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Full Length Research Paper

The impact of positive selection and mutation in prevalence of representative O/Mya-98 foot and mouth disease strains during 2009-2010

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In this study, we investigated the impact of adaptive positive selection on each protein in Open Reading Frame (ORF) (except for 3D^{pro}) of serotype O Foot-and-Mouth disease (FMDV). The majority of residues under positive selection were located in antigenic sites or close to heparin interacting regions. There were five identical positions between positive selection sites and substitution sites occurred in three FMDV O/Mya-98 strains (O/JX/CHA/2010, O/VN/LC169/2009 and O/HKN/20/2010) prevalent in Southeast Asia during 2009-2010. In addition, no significant recombination signal was detected in ORF of the Eurasian (Asia1, A, C, and O) serotypes including the three strains above, the result indicated no recombination events occurred in the three strains. We inferred selection and amino acid mutation contributed greatly to the prevalence of the three strains during 2009-2010.

Key words: Foot-and-Mouth disease (FMDV), Mya-98, positive selection, mutation, recombination.

INTRODUCTION

Foot-and-mouth disease (FMDV) is a member of *Picornaviridae* family, the genetic diversity results in immunity protection to one serotype does not provide sufficient protection to another. As we know, the main evolutionary forces of FMDV are mutation caused by lack in proof-reading activity of RNA polymerase, homologous recombination, and selection (from immune system of host, vaccination and other environmental conditions (Boerlijst et al., 1996; Domingo et al., 2004; Jenkins et al., 2001; Miralles et al., 1999). Because of the evolution forces, FMDV has a high evolution rate which has been estimated from 10^{-3} to 10^{-5} per replication cycle (Batschelet et al., 1976; Drake and Holland, 1999).

In Southeast Asia, FMDV serotype O has been responsible for most of the reported outbreaks. FMDV serotype O is classified into 11 topotypes, designated as Europe-SouthAmerica (Euro-SA), MiddleEast-South Asia (ME-SA), Southeast Asia (SEA), Cathay (CHY), West Africa (WA), East Africa 1 (EA-1), East Africa 2 (EA-2), East Africa 3 (EA-3), East Africa 4 (EA-4), Indonesia-1 (ISA-1) and Indonesia-2 (ISA-2) (Knowles and Samuel, 2003; Ayelet et al., 2009). During 2009-2010, there were some foot and mouth disease (FMD) outbreaks in Korea, Japan, Mongolia, Vietnam and Hong Kong et al. The disease were mainly caused by FMDV Mya-98 of topotype SEA. For controlling the disease effectively, an understanding of the mechanisms responsible for the maintenance of FMDV and prevalence of certain strains is required. We need improve insight into the evolutionary forces like mutation caused by lack in proof-reading activity of RNA polymerase, homologous recombination, and selection impacting on populations of the virus. In this study, we investigated the recombination events in ORF of the Eurasian (Asia1, A, C, and O) serotypes including O/JX/CHA/2010, O/VN/LC169/2009 and O/HKN/20/2010, and topotype SEA, ME-SA, Cathay, Euro-SA, EA-1, EA-2, ISA-1 were chosen to analyze the

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positive selection in structure proteins and non-structure proteins (except for $3D^{pro}$) of type O.

According to the investigation of substitutions, recombination and positive selection in ORF, although no significant recombination was detected, some important substitutions were found which influence the virus binding to the cell receptor and result in escaping the host immunity system. And some of them experienced positive selection, it indicated error-prone replication and selections contributed greatly to the prevalence of new Mya-98 strains during 2009-2010.

MATERIALS AND METHODS

Virus isolates, primers and sequencing

JX/CHA/2010 was obtained from pig's vesicular fluid, displaying distinct clinical symptoms of FMD. Total RNA was extracted from vesicular fluid using RNeasy Mini kits (QIAGEN) following the manufacturer's instructions. RNA was reverse transcribed into cDNA using oligo (dT) antisence primers (Promega) and MMLV-RT (Promega). The first strand cDNA was then subjected to PCR amplification using the six primers. The PCR products was purified and sequenced (sequenced in Shanghai Sunny Biologic Technology limited company, Shanghai, China).

Phylogenetic analysis and mutation analysis

38 FMDV serotype O reference strains were chosen from National Center for Biotechnology Information (NCBI) included seven Topotype (SEA, ME-SA, Cathay, Euro-SA, East Africa-1, East Africa-2, Indonesia-1), more informations in Table 1. The sequences were aligned with ClustalW algorithm. Based on the aligned VP1 coding sequences, phylogenetic tree was constructed by the Maximum Likelihood algorithm (Strimmer and Haeseler, 1996) with 200 replicates using the Tamura-Nei model. Calculation was performed by the soft Molecular Evolutionary Genetics Analysis (MEGA) 5.0 (downloaded at http://www.megasoftware.net/).

Recombination analysis

To detect possible recombination events in ORF of FMDV serotype A, O, C, Asia 1, we used seven recombination detection methods (RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiSscan, 3Seq) implemented in Recombination Detection Program v.3.44 (RDP3) (Martin et al., 2010) to detect the potential recombination signal in sequence alignments. Bonferroni corrected P values cutoff of 0.01 (Lemey et al., 2009; Lefeuvre et al., 2009), Bootscan was carried out with the window of 200 nucleotides and step sizes of 20 nucleotides and used 200 replicates with a 95% cutoff percentage (Martin et al., 2005; Guimaraes et al., 2010). Other methods had the default settings were used in the analysis. Only potential recombination events detected by four or more methods were considered significant. Additionally, We performed similarity plot analysis in a window size of 200 nucleotides moving in steps of 10 unique recombination events (Lole et al., 1999; Martin et al., 2011).

Positive selection analysis

Recombination events could result in faulty inferences about positive selection, so we carried out recombination detection nucleotides along the alignment to confirm the breakpoints of the method GENECONV implemented in Recombination Detection Program v.3.44 (RDP3) (Martin et al., 2010) to detect possible recombination events among each aligned protein coding sequences of JX/CHA/2010 and reference strains in Table 1 using the default settings. Then, we submitted the aligned protein coding nucleotide sequences of 39 strains and maximum likelihood phylogenetic tree to JCoDA (Steinway et al., 2010). The comparison between M0(one ratio) and M3(discrete) was chosen to test whether ω was variable among sites (Yang, 2007), the comparison between M7 (null,neutral) and M8 (selection) was chosen to test positive selection (Yang et al., 2000). Likelihood ratio test was used as a statistical test to compare the two models (Yang, 1998). Meanwhile, we submitted the amino acid sequences of outer-capsid polypeptides (VP2, VP3 and VP1), $3C^{pro}$ of JX/CHA/2010 to the online homology modelling web site (http://swissmodel.expasy.org/) to got the three-dimensional structure of each peptide, and statistically significant positive selection sites were labeled in the predicted three-dimensional models (Melo and Feytmans, 1998; Benkert et al., 2009; Benkert et al., 2008; Hooft et al., 1996; Laskowski et al., 1993).

RESULTS

Constitute of the full-length genome

The software SeqMan of DNAstar 7.0 softpackage (downloaded at www.dnastar.com) was used to assemble the sequences. From the sequencing result, the length of the full-length genome of JX/CHA/2010 was 8173nt (excluding poly (C) and poly (A)), including 5'UTR (1079 nt), L (603 nt), P1 (2208 nt), P2 (1470 nt), P3 (2724 nt), 3'UTR (93 nt), terminating at TAA stop codon.

Sequence alignments

According to Maximum Likelihood (ML) tree (Figure 1) based on aligned VP1 coding sequences, JX/CHA/2010 was closely linked to O/VN/LC169/2009 and O/HKN/20/2010, it was a Mya-98 strain of SEA topotype prevalent in Southeast Asia recently. The nucleotide identities of the whole genome, ORF, outer-capsid polypeptides, VP1 between JX/CHA/2010 and O/VN/LC169/2009 were 99.2, 99.3, 99.0 and 99.2%, respectively; between JX/CHA/2010 and O/HKN/20/2010 were 98.1, 98.8, 98.3 and 98%, respectively. Compared to 9 reference Mya-98 strains of SEA topotype (Table 1), there were some substitutions exiting in JX/CHA/2010, such as antigenic site I of VP1 G-H loop (A152D), antigenic site II of VP2 E-F loop (S131P), heparin interacting regions (D173G in VP3), H12Y in L^{pro}, T45A in 2B, I99T in 3A, I119V in $3C^{pro}$. Moreover, JX/CHA/2010, O/VN/LC169/2009 and O/HKN/20/2010 had some identical amino acids which were diffenent from the other Mya-98 strains of SEA topotype (Table 1) in the counterpart amino acid sites, for example: amino acids located in antigenic site II of VP2 or near to the region(H79Y, Q133E and K134R), antigenic site IV of VP3 B-B knot (E58D), heparan interacting regions or

Table 1. Reference strains.

Virus designation	Isolation source	Topotype	Collection data	Isolation region	Accession no.
O/HLJOC12/03		SEA			DQ119643
O/VN/LC169/2009		SEA	11/2009	Viet Nam:Lao Cai	HM055510
O/HKN/20/2010	Pig	SEA	03/03/10	Hong Kong	HM229661
O/VN/SL22/2006	Cattle	SEA	10/2006	Viet Nam:Son La	GU125647
O/VN/SL01/2006	Buffalo	SEA	10/2006	Viet Nam:Son La	GU125648
O/VN/SL21/2006	Cattle	SEA	10/2006	Viet Nam:Son La	GU125649
O/VN/GL13/2006	Cattle	SEA	04/2006	Viet Nam:Gia Lai	GU125650
O/VN/QB88/2009		SEA	10/2009	Viet Nam	GU582115
O/VN/YB105/2009		SEA	09/2009	Viet Nam	GU582116
O/Tibet/China/1/99		ME-SA			AF506822
O/UKG/35/2001		ME-SA		United Kingdom	AJ539141
O/SKR/2002	Pig	ME-SA	2002	South Korea	AY312588/89
O/SKR/2000		ME-SA		South Korea	AJ539139
O/FRA/1/2001	Bovine	ME-SA		France:Mayenne	AJ633821
O/JPN/2000	Cattle	ME-SA		Japan:Miyazaki	AB079061
O/SAR/19/2000		ME-SA		South Africa	AJ539140
O/TAW/2/99 Bov		ME-SA		Taiwan	AJ539137
O/ PAK/45/2008	Buffalo	ME-SA	2008	Pakistan	GU384683
O/Fujian/CHA/5/99	Swine	ME-SA	05/1999	China:Fujian	HQ009509
O/YUN/TAW/97	Swine	Cathay	04/1997	Taiwan:Yunlin	AF308157
otaiwan97 iso106/112		Cathay	1997	Taiwan	AY593835
O/ES/2001		Cathay			AY686687
O/HKN/2002		Cathay			AY317098
FMDV-O1K		Euro-SA			X00871
o3venezuela iso15		Euro-SA	1971	Venezuela	AY593827
o2brescia iso17		Euro-SA	1947	Italy	AY593826
o1m11 iso57		Euro-SA			AY593822
O/UKG/7/2007	Bovine	Euro-SA	03/08/2007	United Kingdom	EU448371
o1campos94 iso94		Euro-SA		Argentina	AY593819
O/K/52/1992	Ankole cow	EA-1	1992	Kenya: Kiambu	HM625674
o10phil54 iso54		EA-1	1958	Philippines	AY593811
o10phil76 iso76		EA-1	1958	Philippines	AY593812
o5india iso34		EA-1	1962	India	AY593828
O/U/312/2006	Ankole cow	EA-2	31/07/2006	Uganda:Mbarara	HM191257
O/Uganda/2006	Bovine	EA-2	2006	Uganda	EF611987
O/Kumi/Uganda/2002	Bovine	EA-2	07/2002	Uganda	FJ461344
O/Kapchorwa/Uganda/2002		EA-2	10/2002	Uganda	FJ461345
o11indonesia iso52		ISA-1	1962	Indonesia	AY593813

regions nearby(Q133 E, K134R and Y138F in VP2, T174A in VP3). Besides, there were some other substitutions in L^{pro}, capsid proteins, 2C and 3A (Table 2).

Recombination and positive selection

There was no significant recombination signal detected in JX/CHA/2010, O/VN/LC169/2009 and O/HKN/20/2010 based on the aligned ORF of FMDV serotype A, O, C, Asia 1 according to significant recombination signaldetected by four or more of seven recombination detection methods. Then we used detection method GENECONV to detect the recombination events in the datasets consisted of aligned coding sequences of L^{pro}, VP4, VP2, VP3, VP1, 2AB, 2C, 3A, 3B, $3C^{pro}$, $3D^{pro}$ of JX/CHA/2010 and reference strains in Table 1 using the default settings to avoid false positive selection signals. Result showed except for $3D^{pro}$ coding sequence dataset, no recombination event was found in other datasets. The comparison between M7 (null,neutral) and M8 (selection) for L^{pro}, VP2, VP3, VP1, 3A, $3C^{pro}$ yielded a likelihood test

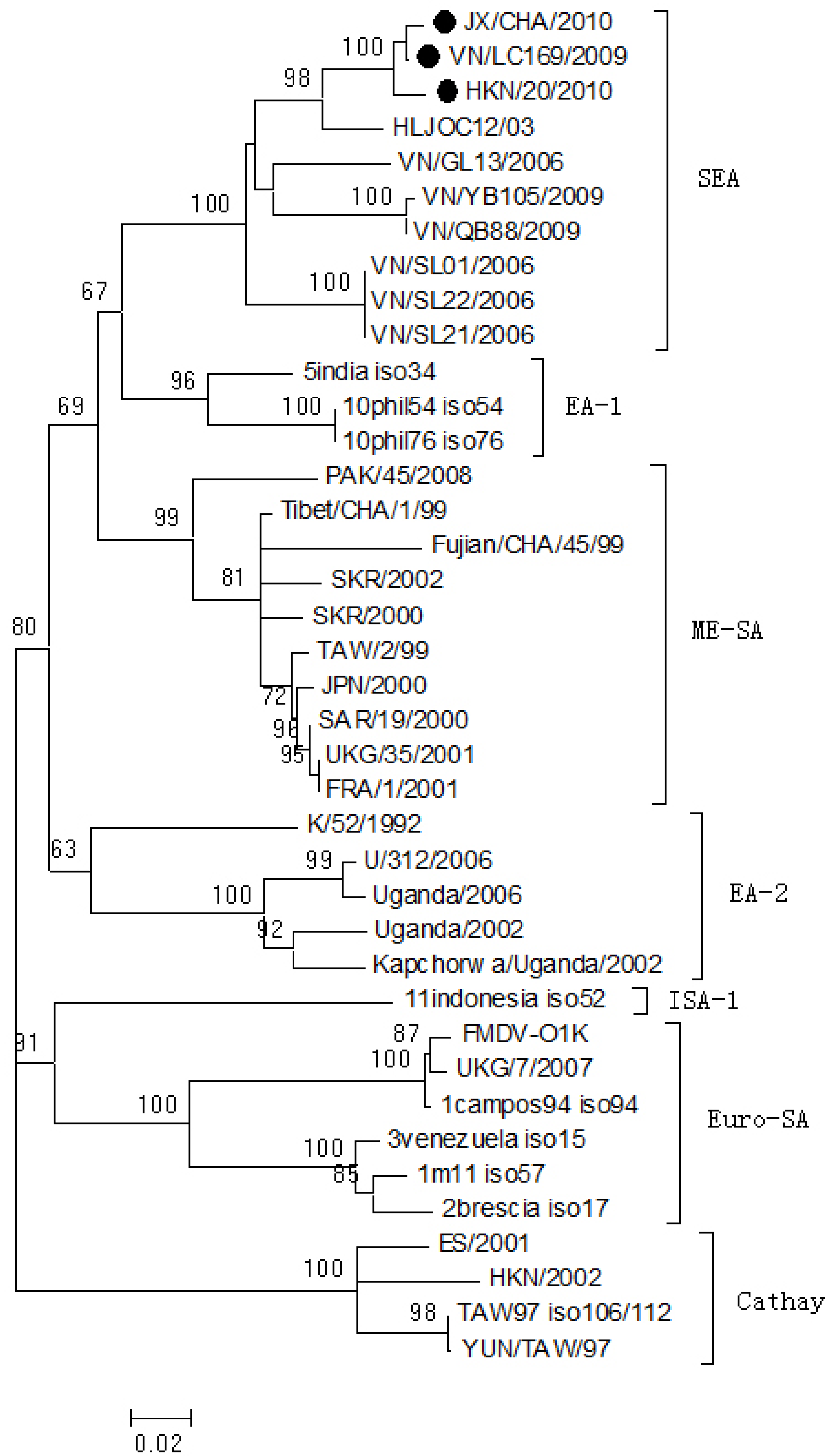

Figure 1. Phylogenetic tree for VP1 coding sequences of serotype O FMDV strains (Table 1) used for positive selection analysis. The tree was generated by Maximum Likelihood analysis with Tamura-Nei model, using MEGA 5.0. Number at nodes indicates the level of bootstrap support (%) of 200 replicates; only values above 60% were given. Three representative strains caused the FMD outbreaks during 2009-2010 are marked with a filled circle.

Table 2. Amino acids substitutions in ORF of JX/CHA/2010 compared to O/VN/LC169/2009 and O/HKN/20/2010 (termed substitutions A). And amino acids were identical in JX/CHA/2010, O/VN/LC169/ 2009 and O/HKN/20/2010 but different from the other Mya-98 strains of topotype SEA (Table 1) in the counterpart amino acid sites (termed substitutions B).

Protein	Substitutions A	Substitutions B	Domain (Bai et al., 2010; Kitson et al., 1990; Crowther et al., 1993; Cooke and Westover, 2008)
L^{pro}		D5G	
	H12Y		
		A19T	
VP2		Y34H	
		H79Y	Close to antigenic site II
	S131P		Antigenic site II, close to heparin interacting region
		Q133E*	Close to antigenic site II and heparin interacting region
		K134R	Antigenic site II, heparin interacting region
		Y138F	Close to heparin interacting region
VP3		E58D	Antigenic site IV
	D173G		Heparin interacting region
		T174A*	Close to heparin interacting region
		D195E	
VP1		Q/P47S	
	A152D		Antigenic site I and V
		E/Q/V198A*	Close to antigenic site I
2B	T45A		
2C		A76T	
		V293I	
3A		L69M	
	I99T		B cell epitope
		C/S/R135G*	
		V/E146G*	
$3C^{pro}$	I119V		

* = positive selection sites as well (Table 3).

ratio statistic of 1.76, 11.32, 9.22, 9.42, 4.20, 18.16, respectively. In other proteins, none positive site were given. According to critical values 5.99, 9.21 at 5, 1%, we knew positive selection sites of VP2, VP3, VP1, $3C^{pro}$ were significant at $p<0.01$, positive selection sites of L^{pro} and 3A were unlikelihood positive selection sites. Most positive selection sites were in the antigenic sites or near to the heparin interacting regions et al. The predicted three-dimensional models of outer-capsid proteins, $3C^{pro}$ of JX/CHA/2010 showed the positive selection sites were in the surface of proteins indicating these sites interacted with host cell extensively. Moreover, positive selection sites of 3A were in the carboxy-terminal which was the most variable region in 3A (Table 3).

DISCUSSION

Based on the substitution analysis and positive selection analysis, we found most substitution and positive selection sites had the same characteristic: locating in epitopes or heparin interacting regions, or near to these regions (Tables 2 and 3). In structural proteins, some important amino acid substitutions were found, for example, positions 131 (key position of antigenic site II), 134 (heparin binding site) in VP2 and position 173 (heparin binding site) in VP3. Although, these amino acid sites did not experience positive selection, regions close to these sites underwent positive selection, for example, positions 133 in VP2 and 174 in VP3. Furthermore, five

Table 3. Positive selection sites under PAML site models (M7vsM8) using bayes empirical bayes analysis.

Gene	Position	Amino acid	Posterior probability	Post Mean±SE for ω	2Δl	Domain (Ferrer-Orta et al., 2004; Harris et al., 1994; Ohlenschläger et al., 2004; Andino et al., 1993)
L^{pro}	23	S	0.880	1.441±0.305	1.76	
	26	Q	0.684	1.266±0.385		
	84	R	0.620	1.224±0.370		
VP2	74	P	0.858	1.891±0.773	11.32	Antigenic site II
	133	E	0.848	1.877±0.756		Antigenic site II
VP3	174	A	0.924	1.940±0.702	9.22	Close to heparin interacting regions
	219	Q	0.791	1.806±0.805		
VP1	45	K	0.668	1.692±0.778	9.42	Antigenic site III
	96	A	0.989*	1.971±0.589		
	138	G	0.985*	1.970±0.586		Antigenic site I
	140	S	0.932	1.925±0.642		Antigenic site I
	198	A	0.994**	2.013±0.525		Close to antigenic site I
3A	135	G	0.787	1.401±0.408	4.20	B cell epitope
	144	H	0.929	1.547±0.397		
	146	G	0.652	1.266±0.426		
$3C^{pro}$	155	K	0.722	1.796±0.891	18.16	
	200	Q	0.866	2.044±1.041		Close to basic patch

In order to get a accurate positive selection result, the inserted codon for L in L^{pro} of O/HKN/20/2010 was deleted. Amino acid in each positive selection site referred to proteins of O/JX/CHA/2010. The test statistic 2Δl was compared to a χ2 distribution with 2 degrees of freedom, critical values 5.99, 9.21, and 13.82 at 5, 1 and 0.1% significance, respectively.

amino acid sites where substitution B occurred (Table 2) were positive selection sites as well (Tables 2 and 3), whether the five specific amino acids in the three strains were the benefit amino acids or not in the process of FMDV evolution, we need carry out more work.

Analyzing the predicted three-dimensional model of outer-capsid polypeptides, almost all the positive selection sites lay nearly in the same plane as VP1 G-H loop (Figure 2), which facilitated the interaction of positive selection sites with the host cell; $3C^{pro}$ positive selection sites (positions 155 and 200) located in the surface opposite to the active site could generate hydrogen bond with basic patch (Figure 3) which was implicated in RNA binding during viral RNA replication (Harris et al., 1994; Ohlenschlager et al., 2004; Andino et al., 1993). Although the positive selection sites in L^{pro} and 3A were of no statistical significance, the same situation as capsid proteins and $3C^{pro}$ was observed. L^{pro} had three positive selection sites, two sites in the region between two AUGs which was the most variable region in L^{pro} as well. The reason why there were two positive sites maybe had something to do with the region between two AUGs involved in viral pathogenesis (Piccone et al., 2011). Positive selection sites in 3A located in the carboxy-terminus which was the most variable region in 3A (Table 3) as well. Taken together, positive selection sites had extensive interaction with the host and suffered the selection impact from the host. Because the sites that positive selection impacted could play an important role in maintenance of the protein three-dimensional structures and overall antigenic sites of the virus, any substitution at these sites resulting in immune escape cannot be overruled (Mohapatra et al., 2008), additionally, the other uncharacterized amino acid substitution sites and positive selection sites may involve in antigenic drift. As shown in Table 3, different positive selection sites had different ω post mean value; this was interpreted as evidence for different positive selection sites suffered from different selective pressure. In the same way, proteins of FMDV ORF were varied in the strength of selective pressure resulting in the different rates of evolution.

FMDV genome evolves mainly by genetic mutations by error-prone replication, selections (from host immune system, vaccination and so on), recombination (Boerlijst et al., 1996; Domingo et al., 2004; Jenkins et al., 2001; Miralles et al., 1999). Recombination analysis showed the three strains did not experience recombination, however,

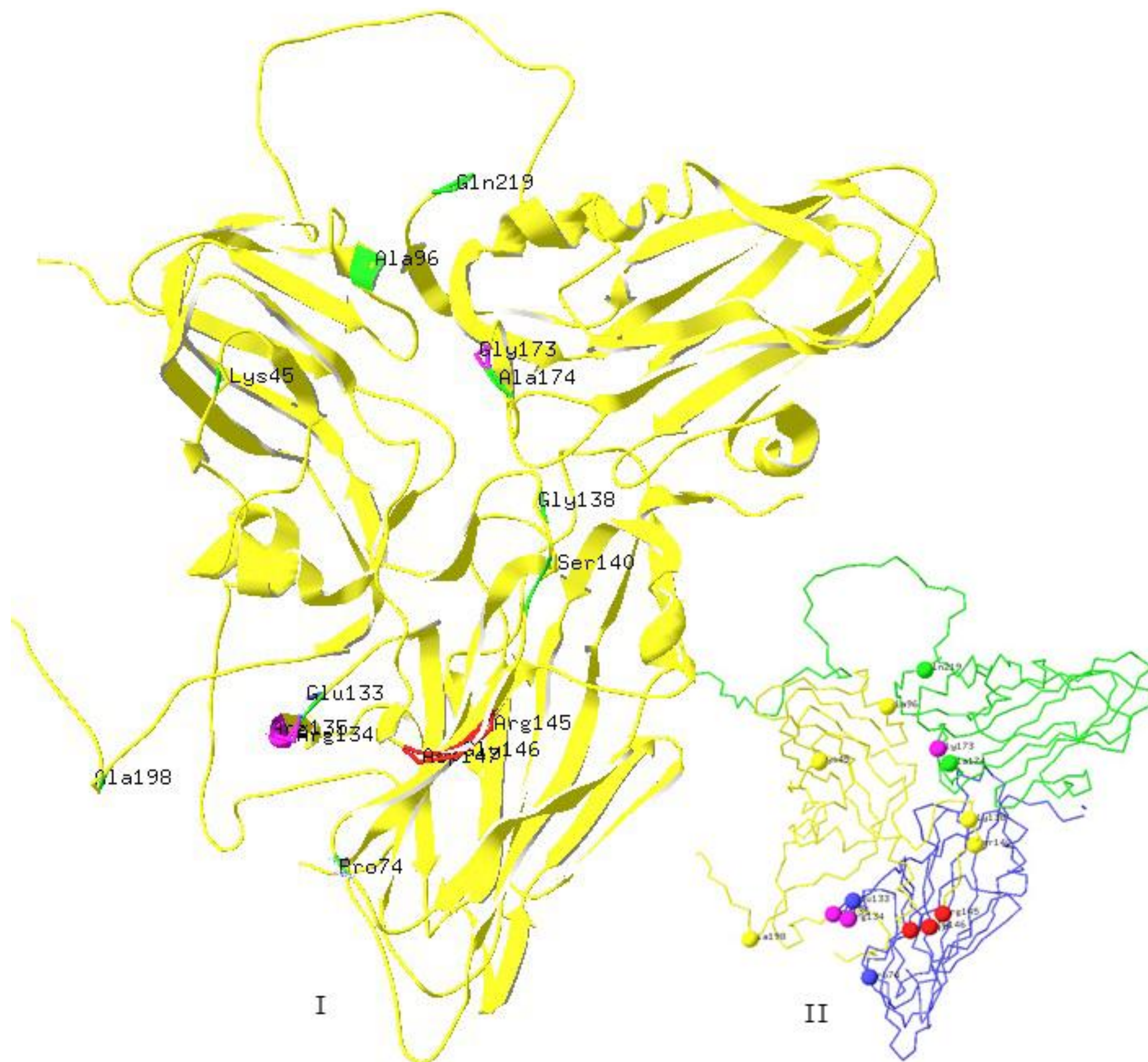

Figure 2. The predicted three-dimensional model of outer-capsid polypeptides of O/JX/CHA/ 2010. In map I, red regions stand for integrin interacting regions (positions R145, G146, D147 (RGD) in VP1 G-H loop) (Kitson et al., 1990; Crowther et al., 1993), pink regions stand for heparin interacting regions (positions R134, R135 in VP2, and G173 in VP3) (Bai et al., 2010; Jackson et al., 1996; Jackson et al., 2003), green regions stand for positive selection sites (positions K45, A96, G138, S140, A198 in VP1; P74, E133 in VP2; A174, Q219 in VP3). Map II is the line view of I. In map II, yellow stick stands for VP1, blue stick stands for VP2, green stick stands for VP3, red balls stand for RGD region, pink balls stand for positions R134, R135 in VP2, and G173 in VP3, yellow balls stand for positive selection sites in VP1, blue balls stand for positive selection sites in VP2, green balls stand for positive selection sites in VP3.

in the antigenic sites, heparin interacting regions and the regions nearby, we found many amino acid substitutions and some of them located in positive selection sites, it indicated the prevalence of new Mya-98 strains was primarily due to the immune escape and changes in capacity of virus binding to receptors on cells surface. We need to put the huge manpower and resources in the research of assessing selection impact of FMD

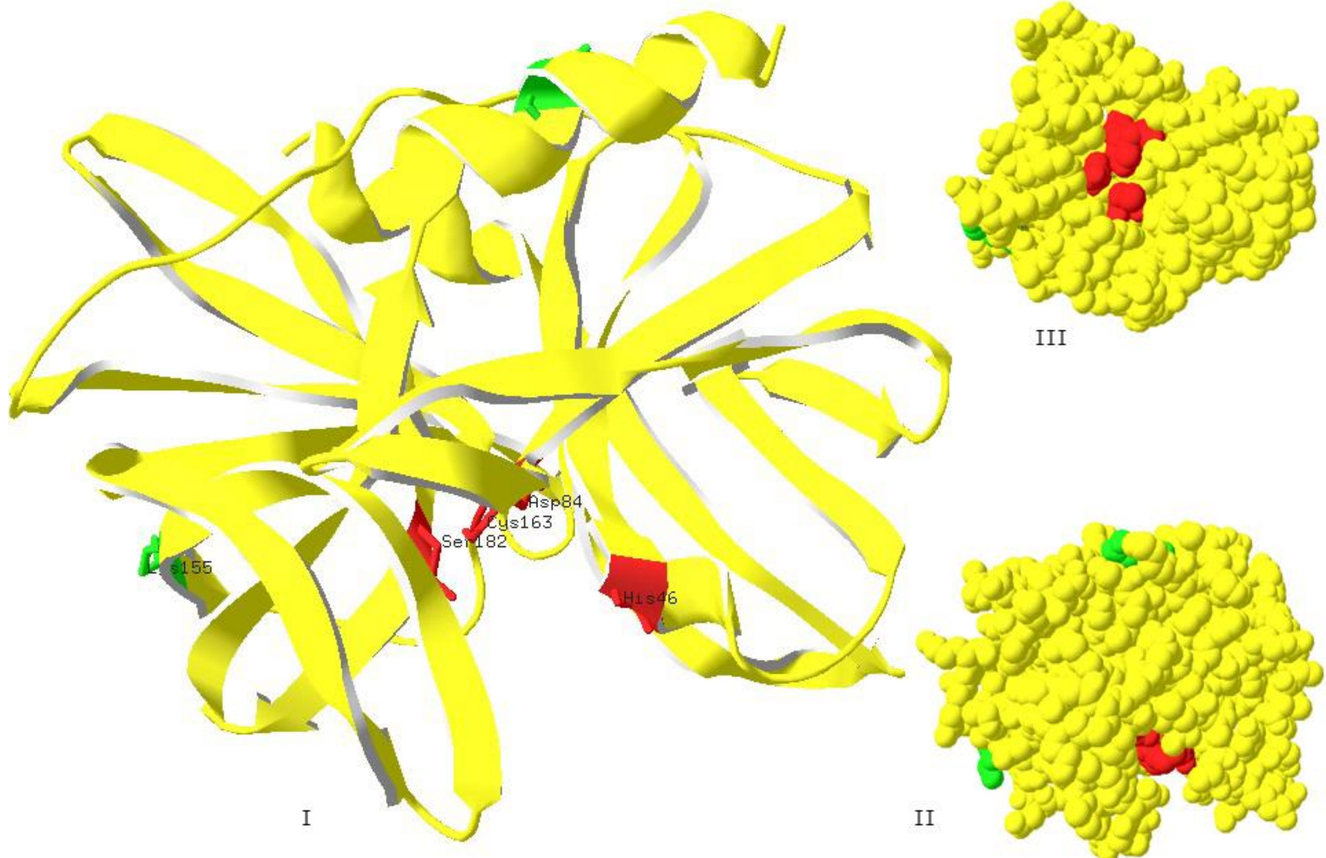

Figure 3. The predicted three-dimensional model of $3C^{pro}$ of O/JX/CHA/2010. In map I, red regions stand for the active sites (positions H46, D84, C163, S182 (Birtley et al., 2005)), green regions stand for positive selection sites (positions K155, Q200). Map I is the ribbon view of map II. Map III is the view from the bottom of map II. In map II and III, red regions stand for active sites, green region stand for positive selection sites.

controlling policy such as vaccination policy and predicting FMDV evolutionary trends to control the disease efficiently.

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