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Genomic Analysis of One Chinese H1N1 Swine Influenza Virus Strain from Healthy Pig Remaining Different Virulence Determinants

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Abstract: The outbreak of Mexico flu pandemic in the Spring of 2009 has arisen the public attention to swine H1N1 influenza virus because H1N1 influenza virus did not bring a large-scale outbreak in human after causing 1918 Spain flu pandemic. Researchers isolated one swine influenza virus strain A/swine/Guangdong/103/2002 (H1N1) from healthy pig in South China and sequenced its full-length genome. BLASTn analysis showed that all segments of the isolate had the above 99% similarities with A/swine/Shanghai/1/2005, A/swine/Shanghai/2/2005 and A/swine/Shanghai/3/2005 from the severe outbreak of respiratory disease pigs. Although, these four virus strains have high similarities they showed very different virulence. The H1N1 virus could replicate in pigs and mousse without causing clinical symptoms according to the animal regressive experiments results. None of the amino acid substitutions were reported to contribute in the pathogenicity of human and avian influenza viruses providing the basis for virulence determinants research by means of reverse genetics.

Key words: Swine influenza virus, H1N1, pathogenesis, determination of virulence, genetics, China

INTRODUCTION

Influenza A virus is a single-stranded negative-sense RNA virus in the family of Orthomyxoviridae causing respiratory disease in avian, human and swine. Although, the infection of influenza A viruses are generally restricted by their host ranges swine is considered to be mixing vessels for gene segments reassortment among avian human and swine influenza viruses thus swine might play a significant role in the emergence of new influenza viruses which can cause human influenza pandemics (Scholtissek, 1990; Centers for Disease Control and Prevention (CDC), 2009a, b).

In swine, four influenza A virus subtypes circulating throughout the world are classical swine H1N1, avian-like H1N2, reassortant H3N2 viruses and various genotype H1N2 viruses (Brown, 2000; Marozin *et al*., 2002; Qi and Lu, 2006; Songserm *et al*., 2006). The classical H1N1 swine influenza virus was first isolated from pigs in 1930 and it was the first influenza virus isolated (Shope, 1931). The first isolated classical H1N1 evolved from the 1918 Spain pandemic and since, 1930 its progenies have been isolated from swine populations in many regions of the world. Until 1979, the classical H1N1 swine viruses were replaced by the avian-like H1N1 viruses (Brown, 2000; Marozin *et al*., 2002). Though H1N1 viruses had been frequently isolated from pigs they did not draw much attention from the public or scientific community until the 2009 Mexico flu pandemic. The H1N1 that caused Mexico flu pandemic was considered to be a quadruple reassortant with two genes from swine influenza viruses normally circulating in Europe and Asia, avian and human influenza viruses (Qi *et al*., 2009).

Most of influenza viruses display different pathogenicity in hosts. Pathogenicity of influenza viruses is dependent on multiple factors. The research about what factors function in the determination of virulence has important implications for public health and agriculture. According to the previous studies many factors can affect the virulence including the quantity of alkalineamino acids

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near the cleavage site of HA protein (Hatta *et al*., 2001) the amino acid residues 97, 108, 126, 138, 212 and 217 of HA (Hulse *et al*., 2004), NA stalk length (Castrucci and Kawaoka, 1993), glycosylation sites in the head (Hulse *et al*., 2004) and the amino acid residues 42, 92 of NS1(Jiao *et al*., 2008; Seo *et al*., 2002) are the most significant.

MATERIALS AND METHODS

Virus isolation and propagation: In this study, researchers isolated and identified one swine H1N1 influenza virus A/swine/Guangdong/103/2002 from a healthy pig in South China. The virus was propagated in 10 days old Specific Pathogenic Free (SPF) chicken embryo. At 48 h post inoculation, allantoic fluid was collected and stored at -80°C.

RNA extraction and RT-PCR: Viral RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The first strand cDNA was synthesized using Superscript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, CA) and random primers. The reverse products were amplified by segment-specific primers. The primer sequences are shown in Table 1.

Genetic sequencing: The PCR products were purified and then cloned into the pMD19-T vector (TaKaRa Biotechnology, Dalian, China). Three clones were chosen and sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The nucleotide sequences of A/swine/Guangdong/103/2002 are available in GenBank accession numbers GQ422382-GQ422389.

Animal regression experiment and PCR detection: The pathogenicity of A/swine/Guangdong/103/2002 was detected in influenza viruses free mousse by animal regression experiment. Viruses affected mousse through nasal cavity.

Mouse were divided into two groups separately, control group and experiment group. After inoculation, physiological indices were recorded every 24 h. Meanwhile, samples from lungs, spleen, kidney and bowel were detected by PCR. The primer sequences are HA-F: 5'-AGCCTTAACGGGATAGCTCCCCTAC-3', HA-R: 5'-CTTTGTTATCCACAGCAAATTCCGA-3'.

RESULTS AND DISCUSSION

The BLASTn anlysis: The BLASTn analysis of eight gene segments of A/swine/Guangdong/103/2002 revealed all gene segments had >99% similarities with A/swine/Shanghai/1/2005, A/swine/Shanghai/2/2005 and A/swine/Shanghai/3/2005 (A/swine/Shanghai/1, 2, 3/2005) which were isolated from the lung sample of dead pigs (Qi *et al*., 2009). It is fascinating that influenza virus strains have much similarities regarding gene segments but diverse in pathogenicity in pigs. In this research, we compare protein sequences of the strain with 3 strains isolated from Shanghai. The different amino acids residues are shown in Table 2.

There are 18 different amino acid residues between A/swine/Guangdong/103/2002 and A/swine/Shanghai/1/2005. PB2, NS1 and NS2 amino acid sequences are 100% identical and the comparison of PB1 protein showed one different residue. PA, HA, NP, NA, M1 and M2 showed 2, 5, 6, 2, 2 and 1 different residues, respectively. About 14 amino acid residues are different between

Table 1: H1N1 segment-specific primer sequence

Genes	Primer sequences (5'-3')	Length of PCR product (bp)	Temperature (°C)
PB1	Upper: AGC G/A AAAGCAGGCAAACCATTTG	2341	56
	Lower: AGTAGAAACA AGGCATTTTTTCATG		
PB2	Upper: AGC G/A AAAGCAGGTCAAATATATTC	2341	56
	Lower: AGTAGAAACAAGGTCGTTTTTAAAC		
PA	Upper: AGC G/A AAAGCAGGTACTGATCC	2233	56
	Lower: AGTAGAAACAAGGTACTTTTTTGGAC		
HA	Upper: AGCA A/T AAGCAGGGGAA AATCAAATC	1777	55
	Lower: AGTAGAAACAAGGGTGTTTTTCCATAC		
NA	Upper: AGC A AAAGCAGGAGTTT AAAATGAATC	1463	55
	Lower: AGTAGAAACAAGGAGTTTTTTTTCAACG		
NP	Upper: AGCA AAAGCAGGGTA A/G ATAATCACTC	1565	55
	Lower: AGTAGAAACAAGGGTATTTTTCATTAATTG		
M	Upper: AGCA AAAGCAGGTAGATATTG AAAG	1027	55
	Lower: AGTAGAAACAAGGTAGTTTTTTACTC		
NS	Upper: AGC AAAAGCAGGGTGACAAA A/T AC	890	55
	Lower: AGTAGAAACAAGGGTGTTTTTTAGTAC		

/: Means degenerate base

Table 2: The different amino acid residues between A/swine/Guangdong/103/2002 with A/swine/Shanghai/1, 2, 3/2005

Protein	Amino acids sites	A/swine/ Guangdong/ 103/2002	A/swine Shanghai/ 1/2005	A/swine/ Shanghai/ 2/2005	A/swine/ Shanghai /3/2005
PB1	6	T	A	-	-
PA	105	Y	-	H	H
	378	K	-	R	R
	428	I	-	G	G
	455	A	S	-	-
	470	L	P	-	-
	519	N	-	D	D
	645	V	-	I	I
HA	7	F	V	-	-
	14	T	A	-	-
	16	N	K	-	-
	241	T	-	-	A
	550	I	V	-	-
	565	C	Y	-	-
NP	81	E	-	K	K
	101	D	N	N	N
	162	R	-	K	K
	166	L	-	Q	Q
	190	V	-	A	A
	201	I	V	-	-
	306	I	-	I	I
	307	L	P	-	-
	341	L	I	-	-
	398	Q	K	-	-
	471	A	T	-	-
NA	3	P	T	T	-
	188	V	M	M	-
M1	10	del	P	P	P
	34	I	T	T	-
M2	30	D	-	-	N
	35	K	R	R	-
	47	K	-	-	T
NS1	14	M	-	-	K
NS2	171	D	-	-	E

del means deletion: -: Means amino acid as the same as A/swine/Guangdong/103/2002

A/swine/Guangdong/103/2002 and A/swine/Shanghai/2/2005 which locate on protein PA, NP, NA, M1 and M2. Researchers also, discovered 11 amino acid residues changed in A/swine/Guangdong/103/2002 and A/swine/Shanghai/3/2005. These residues are HA 241, NP 81, 101, 162, 166 and 196, M1 10, M2 30 and 47, NS1 14 and NS2 171. Researhers notice that M1 protein has one missing amino acid at position 10 compared to the 3 strains.

Result of animal regression experiment: The PCR detection results (Fig. 1) showed that H1N1 could replicate in mouse but this strain did not cause clinical symptoms. Researchers consider that A/swine/Guangdong/103/ 2002 could infect mouse but this virus has no pathogenic. This result keeps identical with the source of the virus which isolated from healthy pig.

Although, pigs are considered as influenza virus genes mixing vessel, swine influenza viruses did not getenough attention until 2009 Mexico flu happened.

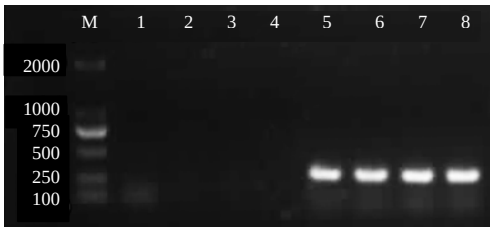

Fig. 1: Polymerase Chain Reaction (PCR) analysis of samples from animal regressive experiments. MW: DL2000 DNA Marker; Lane 1-4: PCR results of samples from control group, Lane 1-4 are lungs, spleen, kidney and bowel of mouse, respectively; Lane 5-8: PCR results of samples from experiment group, Lane 5-8 are lungs, spleen, kidney and bowel of mouse, respectively.

Most studies about determination of influenza virus pathogenicity focused on the human influenza viruses and avian influenza viruses.

CONCLUSION

According to results of comparing protein sequences of A/swine/Guangdong/103/2002 to A/swine/Shanghai/1, 2, 3/2005, researchers found some amino acid substitutions. But none of them were reported to contribute in the pathogenicity of influenza viruses. Considering the total different pathogenicity of A/swine/Guangdong/103/2002 and A/swine/Shanghai/1, 2, 3/2005 maybe two reasons can explain. Firstly, determinations of pathogenicity of human and avian influenza viruses which revealed by researches are not suit for swine influenza viruses or these factors do not play important role in the pathogenicity of swine influenza viruses. Secondly, there are many factors are not discovered responsible for pathogenicity. We can get information about which amino acids are related to the pathogenicity from this study and reverse genetics can be used as a good tool to confirm it.

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