International Journal of
Virology

ISSN 1816-4900

Academic
Journals Inc.

www.academicjournals.com

International Journal of Virology 10 (2): 84-93, 2014
ISSN 1816-4900 / DOI: 10.3923/ijv.2014.84.93
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Induction of Selected Deletion in HA Gene of Egyptian HPAI-H5N1 Viruses Using Site Directed Mutagenesis

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ABSTRACT

HA is a critical determinant of the pathogenicity of avian influenza viruses. The HA proteins of highly pathogenic H5 and H7 viruses contain multiple basic amino acids at the cleavage site that are recognized by ubiquitous proteases but low pathogenic virus contain a single arginine residue at the HA cleavage site that are recognized by trypsin-like protease found generally at epithelial cell surfaces. In this study, we made modification of HA gene of six strains representing variant and classic strains circulated in Egypt from 2009 to 2011 by changing the cleavage site to resemble HA cleavage site of LPAI (PQRE----TRGLF). This done by using different types of site directed mutagenesis as inverse PCR, Genetailer® site directed mutagenesis system, PCR-mutagenesis using restriction enzyme and PCR-mutagenesis using blunt end primer to be safely handled under BSL-2 conditions and used for diagnostic and vaccine production purposes. By comparison of the four methods of site directed mutagenesis, we found that all methods were satisfied enough to produce LP virus from HP strain. The PCR mutagenesis using restriction enzyme and The PCR mutagenesis using blunt end primers were more applicable in either research or production use than other methods because they were reliable methods to generate mutations along the entire length of the cDNA, beside they were highly efficient, cost-effective and rapid methods. Also the final PCR products are available for cloning into multiple vectors, thus reducing the time and effort spent on subcloning.

Key words: HPAI, LPAI, H5N1, HA, HACS, SDM, sequencing

INTRODUCTION

Influenza virus type A, which causes disease in both humans and poultry, belongs to the family Orthomyxoviridae. These viruses are enveloped and possess eight segmented genome of single-stranded negative sense RNA, encoded to at least 10 proteins. Influenza a viruses are classified as either Highly Pathogenic Avian Influenza (HPAI) or Low Pathogenic Avian Influenza (LPAI), depending on their ability to cause disease in susceptible birds. Although, some of the H5 and H7 subtypes have been characterized as HPAI, many strains of these subtypes have been shown to be LPAI (Zhou *et al*., 1999). H5 and H7 strains are very dangerous because of their ability to undergo mutation from LPAIV to HPAIV during replication and circulation in poultry (Spackman *et al*., 2002).

HA is a critical determinant of the pathogenicity of avian influenza viruses, with a clear link between HA cleavability and virulence (Horimoto and Kawaoka, 2001). The primary difference

between LPAI and HPAI virus is local versus systemic replication, respectively. The cleavability of the HA protein is one of the major determinants of the tissue tropism of AI viruses. Post-translational proteolytic activation of the precursor HA molecule (HA0) into HA1 and HA2 subunits by host proteases is essential for infectivity and spread of virus (Lee and Saief, 2009). The HA proteins of highly pathogenic H5 and H7 viruses contain multiple basic amino acids at the cleavage site, which are recognized by ubiquitous proteases, furin and PC6. For this reason, these viruses can cause systemic infections in poultry. In cell culture, the HAs of these viruses do not need exogenous proteases to form plaques. In contrast, the HA proteins of avirulent avian and non avian influenza A viruses contain a single arginine residue at the HA cleavage site and are cleaved in only a few organs. These viruses, therefore, produce localized infection of the respiratory and/or intestinal tract that is usually asymptomatic or mild (Palese and Shaw, 2007).

So, the attenuated mutants with altered cleavage sites have been obtained from HPAI viruses by reversed genetic methods. This approach has been successfully applied for the generation of pandemic H5 vaccines (Garten and Klenk, 2008). The knowledge that the pathogenicity of avian influenza viruses is primarily determined by HA cleavability. Conversion of the HA cleavage site sequence of HPAI viruses to that of avian influenza with low pathogenic (LPAI) viruses attenuates virulence but does not affect antigenicity (Horimoto and Kawaoka, 2009) so by using the site directed mutagenesis, the polybasic cleavage site can be removed from the HA of an HP candidate vaccine strain and replaced with a nonvirulent version. This allows production of vaccines under lowered biosafety containment using reverse genetics system as well as possibly enhancing the production of egg-grown virus preparations by allowing increasing egg survival and higher growth titers (Anonymous, 2006).

This study aimed to prepare attenuated isolates that representing different groups of HPAI viruses circulated in Egypt from period 2009 to 2011 to be LPAI by application of site directed mutagenesis in HACS using different protocols.

MATERIALS AND METHODS

The AIVs used in this study were obtained from National Laboratory for Quality Control in Poultry Production (NLQP). We selected six strains representing the circulating isolates found in Egypt from different geographic regions and production sectors in period from 2009 to 2011; A/chicken/Egypt/09534S-NLQP/2009(H5N1), A/turkey/Egypt/101474v/2010 and A/chicken/Egypt/10116s/2010(H5N1) of genebank Accession numbers: GU002694, HQ198276, HQ198281 represent to variant group in 2009, 2010 respectively and A/duck/Egypt/1011d/2010, A/chicken/Egypt/1052g/2010 and A/chicken/Egypt/a13/2011(H5N1) of gene bank Accession No. HQ198271HQ198284 represent to classic group in 2010, 2011, respectively.

Using site-directed mutagenesis PCR, the highly pathogenic H5HA cleavage sequence (Proline, Glutamine, Arginin, Glutamic acid, Glycine, Arginin, Arginin, Lysine, Lysine, Arginin/Glycine, Leucine, Phenylalanine) (PQREGRRKKR/GLF) to be mutated to low pathogenic (Proline, Glutamine, Arginin, Glutamic acid, Threonine, Arginin/Glycine, Leucine, Phenylalanine) (PQRETR/GLF) cleavage site by different methods of site directed mutagenesis as inverse PCR, Genetailer^{TM} site directed mutagenesis, PCR mutagenesis using restriction enzyme and PCR mutagenesis using blunt end primers. The H5 HA genes of A/chicken/Egypt/09534S-NLQP/2009, A/turkey/Egypt/101474v/2010 and A/chicken/Egypt/a13/2011(H5N1) were reverse transcribed into complementary DNA using influenza A virus 12 mer primer AGCAAAAGCAGG and AMV reverse transcription system (Promega, Inc., Madison, WI). Full-length H5HA was PCR amplified using

HGGT "5'CTC TTC GAG CAA AAG CAG GGG T3' and Bm-NS 890R primers "5'ATA TCG TCT CGT ATT AGT AGG AAA CAA GGG TGT TTT3' (Hoffmann *et al.*, 2001, SEPRL) and using Pfu DNA polymerase (Ferments, Germany) and then cloning in p-JET1.2 plasmid.

Inverse PCR is the first method of SDM to modify HACS of two strains "A/chicken/Egypt/09534S-NLQP/2009" and "A/turkey/Egypt/101474v/2010" to resemble that of LPAI by PCR amplification of cloned HA using back to back blunt ended primers HA-Inv-F 5'ActAGAGGACTATTTGGAGCTATAGCAG-3' and HA-Inv-R 5'CTCTCgTTGAGGGCTATTTCTG AG 3' and using Platinum Pfx DNA polymerase (Invitrogen, USA). These primers were complementary to sequence before cleavage site at 1020pb and after cleavage site at 1033pb with 'CgT' mutation encode arginin in reverse primer and 'Act' mutation encode threonine 'T' in forward primer and then select the transformed plasmid by DPNI restriction enzyme that destructs the methylated non transformed parental plasmid at GATC sequence then ligated by T4 ligase and transform into competent cells (Weiner and Costa, 1994).

Genetailor™ site directed mutagenesis is second method of SDM to modify HACS of A/chicken/Egypt/a13/2011(H5N1). The cloned HA is methylate by DNA methylase and PCR amplification of cloned HA using back to back overlapped primers Genetailor F 5'GCT CAG AAA TAG CCC TCA AcG AGA GAc tAG AGG ACT ATT TGG A 3' and Genetailor R 5'CTC TCG TTG AGG GCT ATT TCT GAG CCC AGT AGC AAG GAC3' using Platinum Pfx DNA polymerase (Invitrogen, USA). The forward primer contain AcG mutation, the substitution is indicated by small letter, encode arginin "R" and "Act" mutation encode therionine "T" and the reverse primer contain 15-20 nucleotides overlapping region at the 5' end primer. The selection method of cloned transformed gene is depend on McrBC endonuclease that found in DH5α™-T1R *E. coli* cells which digests the methylated template DNA, leaving only unmethylated transformed product.

Site directed mutagenesis by using restriction enzyme is third method of SDM to modify HACS by amplification of HA gene of HACS of "A/duck/Egypt/1011d/2010" strain into two fragments by using primers contain BSMBI restriction site (CGTCTC) BM-H5-1020F 5'ATT ACG TCT CAG AGG ACT ATT TGG AGC TAT AGC AGG-3' and BM-H5-1025R 5'ATT ACG TCT CTC CTC TTg TCT CTC gTT GAG GGC TAT TTC TGA GC-3' and Pfu DNA polymerase (native), the reverse primer contain "TgT" which encoded threonine 'T'and TcG' which encoded arginin 'R' then made digestion by BSMBI restriction enzyme and ligation by T4 ligase then amplification the full length HA gene by Hggt and Bm-NS 890R primers (Liu *et al.*, 2003).

PCR mutagenesis using blunt end primers is fourth method of SDM to modify HACS by amplification of HA gene of A/chicken/Egypt/1052g/2010, A/chicken/Egypt/10116s/2010 (H5N1) strains in two fragments with blunt end phosphorylated primers HA-Inv-F and HA-Inv-R using Pfu DNA polymerase then ligation by T4 ligase and full length amplification of HA gene Hggt and Bm-NS 890R primers (Adereth *et al.*, 2005). The correct mutation in HACS was confirmed by sequencing.

RESULTS

The six strains were positive HA and the titer ranged from 6-10 $\log^2$ HA units and positive by Real Time PCR and the Ct values were ranged from 18 up to 29 (Table 1).

Mutagenesis of the HA gene of selected isolates by different methods: The first method is inverse PCR, The cloned HA of H5N1 strains "A/chicken/Egypt/09534S-NLQP/2009 and

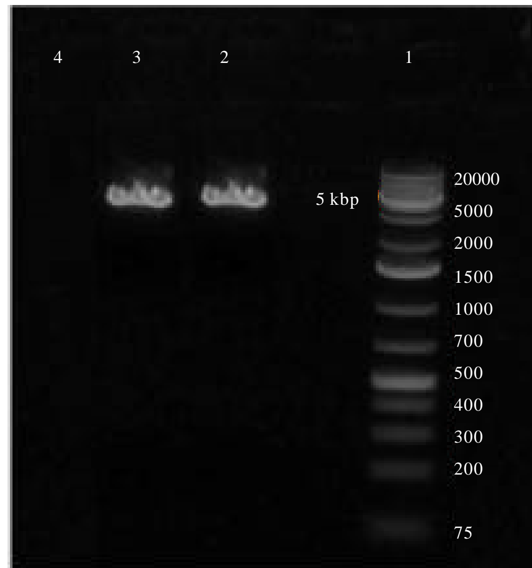

Fig. 1: Positive amplification cloned HA gene (5 kbp) using inverse primers (Lane: 2, 3) for A/chicken/Egypt/09534S-NLQP/2009 and A/turkey/Egypt/101474v/2010, respectively

Table 1: Results of the HA test and real time PCR for selected isolates AIV H5 gene

Isolates ID	HA titer (log^2)	Ct value of H5 real-time RT-PCR
A/chicken/Egypt/09534S-NLQP/2009	6	25.50
A/turkey/Egypt/101474v/2010	10	18.30
A/duck/Egypt/1011d/2010	6	23.21
A/chicken/Egypt/1052g/2010	8	29.34
A/chicken/Egypt/10116s/2010	7	20.08
A/chicken/Egypt/a13/2011	8	22.49

A/turkey/Egypt/101474v/2010" modified by using back to back blunt end primers. The specific DNA bands detectable by Ethedium bromide in the agarose gel appeared at the expected molecular weight 5 k bp (size of cloned HA in P-JET1.2 plasmid) (Fig.1).

The second method is Genetailer^{TM} site directed mutagenesis, the cloned HA of H5N1 strain A/chicken/Egypt/a13/2011(H5N1) modified by using back to back overlapped primers. The specific DNA bands detectable by Ethedium bromide in the agarose gel appeared at the expected molecular weight 5 k bp (size of cloned HA in P-JET1.2 plasmid) (Fig. 2).

The third method is site directed mutagenesis by using restriction enzyme to modify HACS of "A/duck/Egypt/1011d/2010" strain by amplification of HA gene into two fragments by using primers contain BSMBI restriction site and The specific DNA bands detectable by Ethedium bromide in the agarose gel (Fig. 3) at the expected molecular weight at 1020 bp forward fragment and 740 bp reverse fragment and then full length amplification of the mutation in HACS gene after digestion by BSMBI restriction enzyme (Fig. 4).

The fourth method is PCR mutagenesis using blunt end primer to modify HACS of A/chicken/Egypt/1052 g/2010, A/chicken/Egypt/10116s/2010(H5N1) strains to resemble that of LPAI by amplification of HA gene in two fragments with blunt end phosphorylated primers and The specific DNA bands detectable by Ethedium bromide in the agarose gel (Fig. 5) at the expected

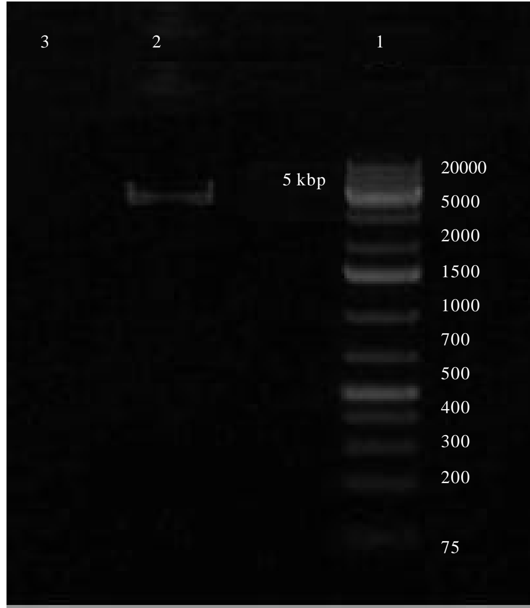

Fig. 2: Positive amplification cloned HA gene (5 kbp) using genetailor primers (Lane: 2, 3) for (H5N1)

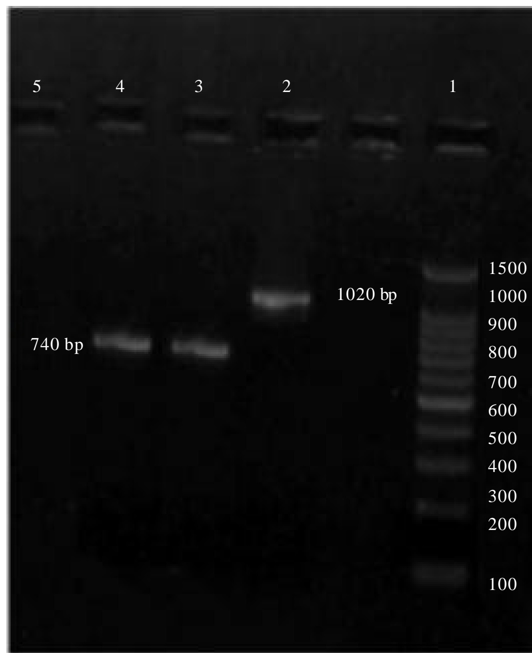

Fig. 3: Positive amplification of 1020 bp forward fragment of A/duck/Egypt/1011d/2010 using Hggt and BM-H5-1025 primers (Lane 2), positive amplification of 740 bp reverse fragment of A/duck/Egypt/1011d/2010 using Bm-NS 890Rand BM-H5-1020F (Lane: 3)

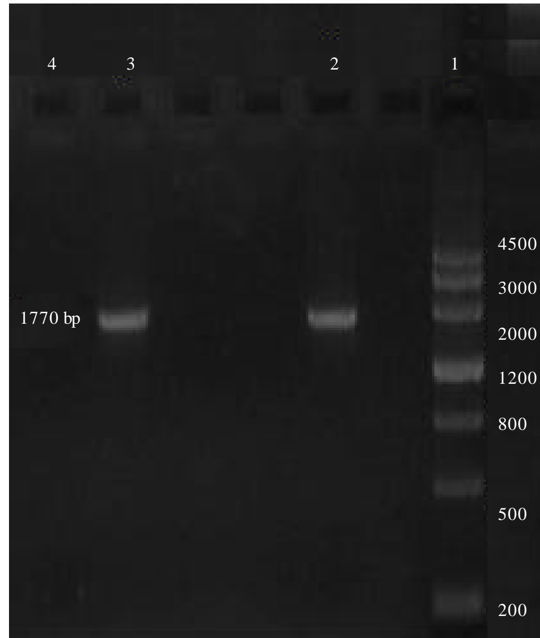

Fig. 4: Positive amplification of HA gene (1770 bp) of A/duck/Egypt/1011d/2010using Hggt and Bm-NS 890R primers (Lane: 2)

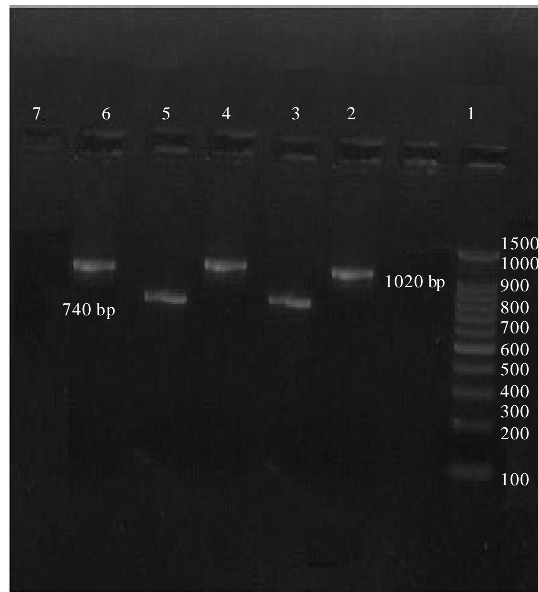

Fig. 5: Positive amplification of 1020 bp fragments using Hggt and HA-Inv-R primers (Lane: 2, 4) for A/chicken/Egypt/1052g/2010 and A/chicken/Egypt/10116s/2010, respectively, Positive amplification of 740 bp fragments using Bm-NS 890R and HA-Inv-F primers (Lane: 3, 5) for A/chicken/Egypt/1052g/2010 and A/chicken/Egypt/10116s/2010, respectively

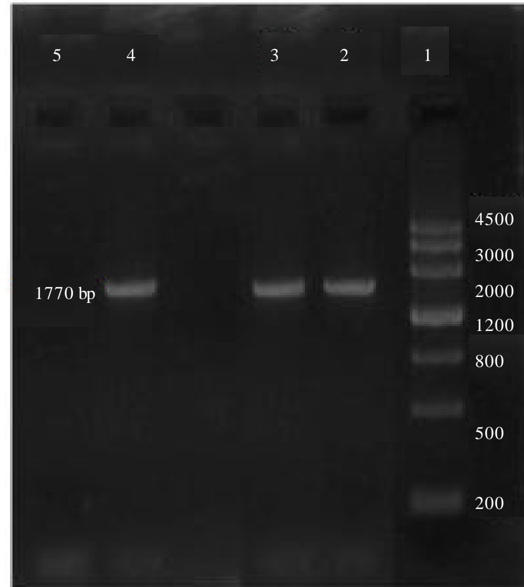

Fig. 6: Positive amplification of HA gene (1770 bp) using Hggt and Bm-NS 890R primers (Lane: 2) for A/chicken/Egypt/1052g/2010 and A/chicken/Egypt/10116s/2010, respectively

Table 2: Amino acid sequence of HACS before and after SDM

Isolates ID	Amino acid sequence of HACS before SDM	Amino acid sequence of HACS after SDM
A/chicken/Egypt/09534S-NLQP/2009	PQGEGRRKKRGLF	PQRE----TRGLF
A/turkey/Egypt/101474v/2010	PQGERRRKKRGLF	PQRE----TRGLF
A/duck/Egypt/1011d/2010	PQGERRRKKRGLF	PQRETR----GLF
A/chicken/Egypt/1052g/2010	PQGERKRKKRGLF	PQRE----TRGLF
A/chicken/Egypt/10116s/2010	PQGEGRRKKRGLF	PQRE----TRGLF
A/chicken/Egypt/a13/2011	PQGEKRRKKRGLF	PQRETR----GLF

molecular weight at 1020 bp forward fragment and 740 bp reverse fragment and then full length amplification of the mutation in HACS gene after ligation by T4 ligase (Fig. 6).

The mutation in HACS was confirmed by sequencing as shown in Table 2.

DISCUSSION

HA is a critical determinant of the pathogenicity of avian influenza viruses with a clear link between HA cleavability and virulence (Horimoto and Kawaoka, 2001). The HPAI can be attenuated by induction of mutation in the cleavage site to simulate that of LPAI by site directed mutagenesis. This approach has been successfully applied for the generation of pandemic H5 vaccines by reverse genetic methods (Garten and Klenk, 2008).

The goal of this study was to reduce the pathogencity of different groups of Egyptian highly pathogenic H5N1 virus by conversion the cleavage site to resemble that of LPAI (PQRETRGLF). This modification occur through deletion of four basic amino acids and substitution of glysin (G) to arginin (R) and lysine (K) or Arginin (R) to threonine (T). This allows the virus to be safely handled under BSL-2 conditions and to be used for diagnostic and vaccine production purposes. As reported by Tian *et al.* (2005) that they removed the basic amino acids in GSGD/96 (H5N1) strain by

replacing PQRERRRKKRGLF to RETR/GLF and confirm this attenuation by rescue of virus by reverse genetic, the rescued virus was attenuated and successfully grown in embryonated eggs with high growth titers and the sequencing of HA gene of the virus proved that it was identical to its original plasmids, demonstrating that antigenic changes did not occur during viral replication in the egg.

In this study, the modification of HACS occurred by different methods of site directed mutagenesis as inverse PCR, Genetailer™ site directed mutagenesis, PCR mutagenesis using restriction enzyme and PCR mutagenesis using blunt end primers.

The inverse PCR modify HA gene by using blunt ended primers and DPNI selection mechanism and the mutation are confirmed by sequencing to resemble the HACS of LPAI without appearance of undesirable mutations or parental non transformed plasmid. This result indicated that high fidelity PCR was efficient and DpnI method was suitable for selection mechanism. So, it can be used safely in BSL2 and also can be used as seed for vaccine production as reported by Jadhao and Suarez (2010) that modified the H5 gene of A/duck/Vietnam/Baclieu/09/07 (H5N1) strain by conversion of the HP-H5 cleavage sequence PQREGRRKKR/GLF to low pathogenic PQRETR/GLF cleavage site using site-directed mutagenesis PCR then rescue of the recombinant virus by reverse genetic. Also Horimoto and Kawaoka (2006) that altered the HA cleavage site of the wild-type strain HK213 (RERRRKKR) to the following avirulent-type sequences (RETR), using back-to-back primer pairs, followed by ligation with a PolI-HK213/HA construct containing the HK213 HA gene and used reverse genetics to create recombinant (6:2 reassortant) viruses that comprised genetically altered avirulent-type HA and intact NA genes. The recombinant virus grew well in eggs (9.5 log10 EID 50 mL^{-1}), was highly attenuated, therefore can be used as vaccine against H5 influenza.

The Genetailer™ site directed mutagenesis used mainly in the single mutation with high efficient as used in previous study in Watanabe *et al.* (2011) that detected the effect of amino acid substitution of HA gene in binding to human receptor and its effect on pathogencity of avian influenza, the induction of the single mutation in Glutamine (Q) to Histidine (H) and Serine (S) to Proline (P) at position 192, 235 in HA gene markedly increased viral binding to human receptor α 2,6 SA and double mutation in Serine (S) to Asparigine (N) at position 120 and Isoleucine (I) Threonine (T) at position 120, 151 in sublineage B of avian influenza in Egypt increased α 2,6 SA binding. It was used also in studying the effect of replacing amino acid Asparigine (N) with Alanitionne (A) at position 27, 28, 40, 286, 304, 498, or 557 in N-linked glycosylation sites of HA gene on the efficiencies of processing in the posttranslational modification of viral glycoprotein in the Golgi apparatus (Ueda *et al.*, 2008).

In this study, we used the Genetailor™ site directed mutagenesis in deletion of four basic amino acids and substitution of other two amino acids in HACS and the mutation are confirmed by sequencing from PQGEKRRKKRGLF to PQRE----TRGLF resemble to HACS of LPAI without appearance of undesirable mutations or parental non transformed plasmid. This result indicated that high fidelity PCR was efficient, the McrBC endonuclease selection mechanism was suitable and the Genetailor™ site directed mutagenesis could be used effectively in the induction of multiple mutations. As previously done by Ueda *et al.* (2010) that they modified the HA genes of strain Cw/Kyoto by replacing RRKKR with TR at the cleavage site by Genetailer™ site directed mutagenesis then rescued the virus by reverse genetic to comparison the cellular apoptosis of highly and low pathogenic avian influenza.

In this study, the site directed mutagenesis by using restriction enzyme and PCR mutagenesis using blunt end primer was efficient and rapid method due to the HA gene modified by

amplification of HA gene in two fragment flanking the HACS by primers containing BSMBI restriction site or blunt end primers respectively without need the cloning or selection steps so it used directly in further application without subcloning steps and the mutation are confirmed by sequencing without appearance of undesirable mutations. This was similar to Liu *et al.* (2006) that amplify the HA gene segment of A/Goose/HLJ/QFY/04 by RT-PCR in two fragments then digested and ligated into pHW2000-BsmBI by three-fragment ligation reaction to delete the multibasic amino-acid cleavage site from PQRERRRKKRGLF to PQRETRGLF and Webby *et al.* (2004) used PCR-based mutagenesis using BsmbI restriction enzyme for conversion the HACS of strain A/Hong Kong/213/03(H5N1) from PQRERRRKKRGL to PQIETRGL to resemble that of the avirulent strain A/teal/Hong Kong/W312/97(H6N1) then cloned into pHW2000 plasmid.

By comparison the four mutagenesis methods, we found that all methods were satisfied enough to produce LP virus from HP strain. However, the Genetailer™ site directed mutagenesis system and inverse PCR were expensive and slow. They keep the modified HA plasmid for long period due to mutagenesis has been occurred only in cloned HA, so it will be very easy to propagate and preserve the plasmids in *E. coli* competent cell. They need subcloning step to transfer the modified HA from a parent vector to a destination vector in order to further study. This work also indicated that the Genetailer™ site directed mutagenesis system is faster and cheaper than inverse PCR.

The PCR mutagenesis using blunt end primers and restriction enzyme were very rapid and cheap because they can be used directly without the need for further subcloning steps, but they need further steps to keep the mutated gene as transfomed plasmid. The PCR mutagenesis using blunt end primers was faster and cheaper than that using restriction enzyme method.

So, the modified HA gene induced by four methods can be used safely in the BSL2 and not need BSL3 facility for handling and it used as seed for vaccine production from Egyptian strains of HPAI as DNA vaccine or reverse genetic vaccine and it could be used in diagnostic purpose like HI antigen and ELISA kits. This work also indicate that importance to establish protocols for HPAI attenuation that would potentially allow the virus to be safely handled under BSL-2 conditions and use further in different purposes like in diagnostics and in vaccine production and comparison of different types of site directed mutagenesis protocols used in the lab for their effectiveness and suitability.

ABBREVIATIONS

HPAI = Highly pathogenic avian influenza
LPAI = Low pathogogenic avian influenza
HA = Heamaglutinine gene
HACS = Heamaglutinine cleavage site
SDM = Site directed mutagenesis
PCR = Polymerase chain reaction
HI = Heagglutination inhibition
ELISA = Enzyme linked immune sorbent assay

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