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REVIEW

Molecular pathogenesis of H5 highly pathogenic avian influenza: the role of the haemagglutinin cleavage site motif

Jasmina M. Luczo[1,2], John Stambas[2], Peter A. Durr[1], Wojtek P. Michalski[1] and John Bingham[1]*

[1]*Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Geelong, Victoria, Australia*
[2]*School of Medicine, Deakin University, Geelong, Victoria, Australia*

SUMMARY

The emergence of H5N1 highly pathogenic avian influenza has caused a heavy socio-economic burden through culling of poultry to minimise human and livestock infection. Although human infections with H5N1 have to date been limited, concerns for the pandemic potential of this zoonotic virus have been greatly intensified following experimental evidence of aerosol transmission of H5N1 viruses in a mammalian infection model. In this review, we discuss the dominance of the haemagglutinin cleavage site motif as a pathogenicity determinant, the host-pathogen molecular interactions driving cleavage activation, reverse genetics manipulations and identification of residues key to haemagglutinin cleavage site functionality and the mechanisms of cell and tissue damage during H5N1 infection. We specifically focus on the disease in chickens, as it is in this species that high pathogenicity frequently evolves and from which transmission to the human population occurs. With >75% of emerging infectious diseases being of zoonotic origin, it is necessary to understand pathogenesis in the primary host to explain spillover events into the human population. © 2015 The Authors. *Reviews in Medical Virology* published by John Wiley & Sons Ltd.

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INTRODUCTION

Prior to 1997, when the first human case of H5N1 highly pathogenic avian influenza (HPAI) occurred in Hong Kong [1], avian influenza (AI) viruses were thought only to threaten human health by providing a gene pool for re-assorting into the human influenza viral populations [2]. Since then, AI viruses have directly infected humans; the first cases of H5N1 HPAI had a case fatality of 33% [3,4] and this has since risen to almost 55% with subsequent infections [5]. H5N1 HPAI has the potential to cause widespread mortality and morbidity in the human population, particularly if it acquires the ability to sustain human-to-human transmission. Concerns have been heightened by recent reports describing the experimental generation of transmissible H5 and H7 HPAI isolates in a mammalian (ferret) model [6–8]. Recent outbreaks of the novel H7N9 avian influenza virus (AIV) subtype in poultry and humans have intensified attention on the potential impact of AIV on the human population [9]. Furthermore, the recent detection of other neuraminidase combinations with H5, including H5N6 from a fatal human case, demonstrate that it is not only the H5N1 subtype that could threaten human and animal health [10].

The pathogenicity of H5 AI viruses in their avian hosts is highly variable, ranging from asymptomatic to severe acute disease [11–18]. Unlike human

*Correspondence to: J. Bingham, CSIRO, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria, Australia, 3220.
E-mail: John.Bingham@csiro.au

Abbreviations used
AIV, avian influenza virus; HA, haemagglutinin; HACS, haemagglutinin cleavage site; pHACS, polybasic haemagglutinin cleavage site; HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza; NA, neuraminidase; AI, avian influenza; OIE, World Organisation for Animal Health; NP, nucleoprotein; IVPI, intravenous pathogenicity index; SPF, specific-pathogen-free; HV, high virulence; LV, low virulence; SV40, Simian Virus 40; Lys or L, lysine; Arg or R, arginine; Ser or S, serine; Gln or Q, glutamine; Pro or P, proline; Leu or L, leucine; Gly or G, glycine; Phe or F, phenylalanine; Thr or T, threonine; Glu or E, glutamic acid; Asp or D, aspartic acid; Ile or I, isoleucine; B and X, any basic or non-basic amino acid, respectively.

influenza viruses, AI viruses are classified into two pathotypes on the basis of pathogenicity characteristics in chickens and molecular attributes of the haemagglutinin cleavage site (HACS) motif [19]: HPAI viruses possess a polybasic HACS motif (pHACS), whereas low pathogenic avian influenza (LPAI) viruses lack this feature [20–25]. Although the determinants of H5N1 HPAI pathogenicity are multifaceted, the HACS motif plays a predominant role [18,22,26–28]. LPAI viruses replicate locally at epithelial surfaces [29,30] of the respiratory system, kidneys and gastrointestinal tract [26,29–31]. HPAI viruses replicate in multiple tissues, with overwhelming infection within the vascular compartment of chickens [12–15,29,32]; this characteristic is abolished following removal of the pHACS motif by reverse genetics [21,22,33,34]. Therefore, to understand the extraordinarily high pathogenicity of some H5 HPAI viruses and how they evolve from less pathogenic forms, it is necessary to understand the role of the HACS motif.

In this review, we look at the functional evolution of highly pathogenic H5 influenza viruses, focusing on the principal determinant of pathogenicity, the cleavage site motif of the viral haemagglutinin (HA) glycoprotein. We surveyed the experimental studies identifying key residues within the pHACS motif and their influence on the HPAI pathotype. Where we consider the pathogenic effects of HPAI infection, we focus on the disease in chickens, because HPAI is principally a virus of this species; it is in this species that new HPAI variants emerge and it is from chickens that transmission to the human population occurs.

Avian influenza virus

Influenza viruses belong to the *Orthomyxoviridae* family of viruses, which are characterised by their segmented, negative-sense, single-stranded RNA genome [35]. The 13 Kb segmented *Influenza A virus* genome [30,35] allows for re-assortment of viruses within host cells during co-infection [35], resulting in the emergence of novel viruses. Some combinations of genes may result in viruses with pandemic potential [36]. The *Orthomyxoviridae* family consists of six genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, *Isavirus* and *Quaranjavirus* [35,37], of which genus classification is determined by serological cross-reactivity to the nucleoprotein (NP) and matrix proteins [38,39]. Recently, an additional genus, *Influenzavirus D*, has been proposed [40]. *Influenza A virus* species are subtyped according to the antigenic properties of the surface glycoproteins, namely, HA and neuraminidase (NA), with 18 HA and 11 NA antigenic subtypes currently identified [41–47]. Waterfowl play a key role in the perpetuation of avian influenza viruses [38,48,49]. All subtypes in most combinations have been isolated from birds [49–51], with the exception of those recently identified in bats: H17N10 and H18N11 [41,42]. Species within the *Influenzavirus A* genus exhibit zoonotic potential as a result of their ability to infect a wide-range of hosts such as humans, birds, pigs and horses, as opposed to members of the *Influenzavirus B* and *C*, all of which have a limited host range [38].

Haemagglutinin is the surface glycoprotein responsible for both host cell attachment and membrane fusion [52,53]. HA is synthesised as an immature polypeptide chain, HA_0 (Mr 63,000 [35]), encoding HA_1 and HA_2 domains of Mr 40,000 and 23,000, respectively [54]. The immature HA polypeptide is co-translationally translocated across the membrane and into the lumen of the rough endoplasmic reticulum [55] where it undergoes post-translational modifications [55–58] prior to anchoring in the membrane by the C-terminus stop transfer sequence [59]. Following signal sequence cleavage [60], the mature HA_0 monomers (Mr approximately 75,000–80,000 [61–63]) form a homotrimer with approximate Mr of 220,000 [63]. HA_0 exhibits two structurally distinct regions: the membrane-distal globular head comprising anti-parallel β sheets, containing the receptor-binding domain, and the membrane-proximal stem region, which exhibits a distinctive triple stranded coiled-coil of α-helices, containing both the fusion domain and the HACS motif [59]. Proteolytic cleavage of HA_0 into its two subunits, HA_1 and HA_2 (approximate Mr 50,000 and 29,000, respectively [35,61]), is essential for virus infectivity [61].

Haemagglutinin interacts with sialic (neuraminic) acid receptors on the cell surface, and these receptors fall into two major groups according to the linkage configuration of terminal sialic acid moieties to the penultimate galactose within carbohydrate side chains [53]. Avian adapted strains of influenza virus preferentially bind to *N*-acetylneuraminic acid with an α-2,3 linkage, whereas mammalian (including human) adapted strains bind to

N-acetylneuraminic acid with an α-2,6 linkage [64]. Following receptor binding and subsequent endosomal internalisation [65], endosome acidification leads to a conformational change of cleaved HA [66,67], resulting in the hydrophobic fusion domain being inserted into the endosomal membrane and facilitating fusion of the membrane and viral envelope [66,68,69]. The release of viral nucleocapsids into the cytoplasm is followed by dissociation into the ribonucleoprotein and matrix protein components and the subsequent transport of the ribonucleoproteins into the nucleus [70].

The classification of AI into HPAI and LPAI pathotypes is determined by experimental infection of chickens following the World Organisation for Animal Health (OIE) protocols [19], with HPAI viruses having a ≥75% mortality rate within 10 days following intravenous challenge, or an intravenous pathogenicity index (IVPI) >1.2. The IVPI is the mean clinical score (0 = normal, 1 = sick, 2 = severely sick, 3 = dead) of ten 4- to 8-week-old chickens per 24 h over a 10-day observation period. Viruses that do not meet these pathogenicity requirements are to have their HACS motif sequenced whereby the presence of a motif similar to previously characterised HPAI isolates also results in HPAI classification [19]. Of interest are isolates classified as HPAI on the basis of molecular characteristics despite displaying an LPAI phenotype following inoculation of chickens [18,71–74]. All viruses that do not meet the criteria for HPAI classification are classified as LPAI [19]. Of the 18 HA subtypes [41–47], LPAI viruses are present in most subtypes [49–51], whereas HPAI viruses are currently present in the H5 and H7 subtypes only [19,75,76]. HPAI viruses possess a pHACS motif, which facilitates extensive systemic disease, whereas LPAI viruses lack this defining feature [18,20–25,27] and infection is generally restricted to the respiratory system, kidneys and gastrointestinal tract [26,29–31].

Pathogenesis of H5 highly pathogenic avian influenza in chickens

Disease manifestation is a result of a complex interaction between pathogen and host, and one of the important determinants of disease is the tropism of the pathogen, that is, the cell types in which the pathogen replicates. AIV tropism is determined by both viral and host aspects such as: (i) route of entry into the host; (ii) route of spread within the host; and (iii) susceptibility of particular cell types, which is determined by factors such as expression of receptors or activating proteases [77].

Avian influenza viruses are shed in faeces and through secretions of the oral cavity and respiratory tract [11,17,30,31,78–80]. Because of the difficulties in conclusively demonstrating transmission events, it is unclear which route is more important for transmission, but it is likely that both routes of shedding play key roles. LPAI viruses tend to be shed more from the cloacal route [11,81,82] and therefore they rely on faecal–oral transmission. HPAI viruses are generally shed at higher concentrations in respiratory secretions than from the cloacal route [15,17,30,83]. It is unclear how this may lead to aerosol transmission in poultry, as coughing and sneezing, behaviours that may lead to aerosolization of virus are not widely reported. Faecal–oral transmission has been demonstrated between HPAI infected poultry, and despite higher concentrations of virus shed from the respiratory tract, no aerosol transmission was demonstrated [79]. High titres of virus are present within feathers of HPAI infected ducks [84–86], and transmission of AIV in ducks following feather consumption has been demonstrated [84]. Despite currently lacking experimental evidence in chickens, feather consumption may also be a likely mechanism of AIV transmission in this species.

Avian influenza viruses gain entry to new hosts by attachment to mucous membranes via abundant sialic acid receptors (described earlier). Thus, the initial viral replication site depends on the route of entry and the presence of appropriate receptors. LPAI replication is often confined to mucous membranes around the sites of infection (e.g. respiratory tract mucosa), but may also spread to other epithelial surfaces, such as gastrointestinal or kidney epithelia [30]. The presence of a pHACS motif within HA greatly expands AIV tropism in chickens from one dominated by an epithelial cell infection [29,30], limited to the respiratory system, gastrointestinal tract and kidney [26,29–31], to a vascular dominated tropism associated with replication in endothelium and a wide range of other cell types. These viruses therefore have diffuse tropism in chickens, including endothelium, neurons, myocardium, macrophages, connective tissues and, to a lesser extent, various epithelial tissues [12–15,29,32] (Figure 1). Virus replication within the endothelium

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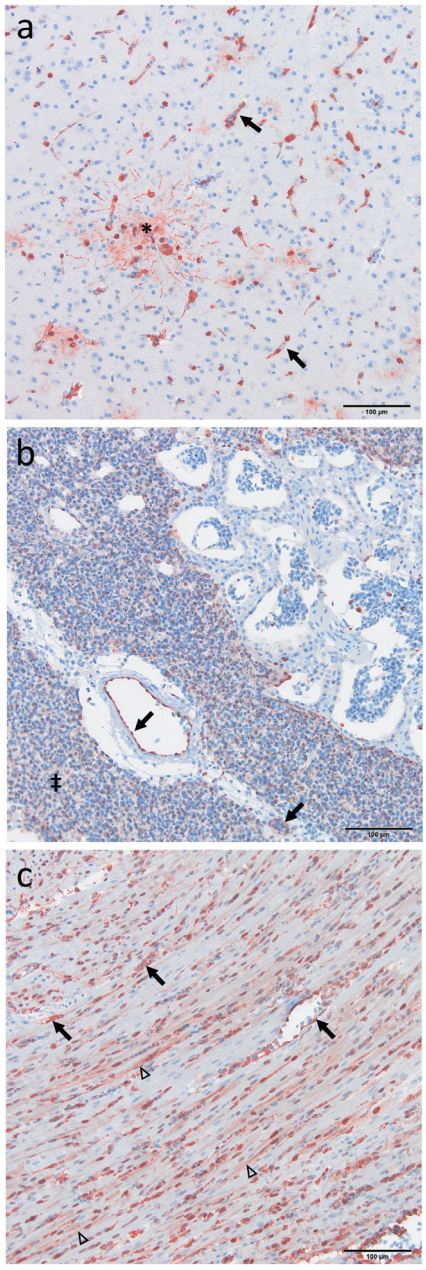

Figure 1. Immunohistochemical detection of *Influenza A* nucleoprotein in the brain (a), lung (b) and heart (c) following experimental infection of chickens with H5N1 HPAI. Systemic dissemination of HPAI enables replication within multiple organs, with a pronounced tropism for the vascular system. Intense antigen is visible within endothelial cells (a–c), in addition to neurons of the brain (a) highly vascularised lung parenchyma (b) and cardiac myocytes within heart tissue (c). Endothelial staining indicated by arrows, neuronal staining indicated by asterisk, vascularised lung parenchyma staining indicated by double dagger and cardiac myocyte staining indicated by open arrowheads. IHC against *Influenza A* NP antigen (brown) counterstained with haematoxylin. Bars represent 100 µm. HPAI, highly pathogenic avian influenza

leads to increased vascular permeability, oedema, haemorrhage, microthrombosis and coagulopathy [12,14,25,29,30], which result in the collapse of the vascular system and death. The mechanism of spread of HPAI viruses beyond the mucous membranes at the initial infection site is unclear; however, as these viruses are strongly vasculotropic, it is likely that the vascular compartment is the main route by which they spread systemically. Multiple studies have demonstrated that the absence of a pHACS motif abrogates the ability of the virus to replicate in vascular tissue and cause severe disease [21,22,33,34,87].

In contrast to pathogenesis in chickens, vascular tropism is not a prominent feature of H5 pathogenesis in mammals [88]. H5 replication in humans is mainly supported in alveolar epithelium and alveolar macrophages, which is reflected by the observed clinical signs [3,89]. Manifestation of human H5N1 infection includes fever, broncho-interstitial pneumonia, acute respiratory distress syndrome with associated hypoxaemia and multiple organ failure [3,90–94]. In the absence of respiratory involvement, infection may also present as a gastrointestinal infection or encephalopathy [95].

In addition to cell and tissue tropism, immunological factors such as hypercytokinemia, or 'cytokine storm', may also contribute to severe disease outcome in mammals. Viral hyperinduction of proinflammatory cytokines (hypercytokinemia) leading to excessive recruitment of inflammatory immune cells has been shown to contribute to the pathogenesis of H5 HPAI infection in humans [3,96–98]. Moreover, pulmonary endothelial cells play a central role in the induction of a cytokine storm [99,100]. Although hypercytokinemia in HPAI infected chickens has been reported [101–104], it is unclear the causal role this plays in disease. Despite considerable evidence supporting the cytokine storm hypothesis, some studies have questioned its contribution to HPAI lethality [105,106].

Molecular composition of the haemagglutinin cleavage site motif: a key pathogenicity determinant

For clarity of HACS motif position in proceeding discussions, nomenclature will be based on definitions introduced by Schechter and Berger [107], which designates residues N-terminal of the scissile bond (↓) P1 and those C-terminal as P1';

corresponding residues in the substrate binding domain of the activating enzyme are designated S1 and S1′. The residue numbering increases with distance from the scissile bond (Figure 2). In this review, the HACS motif is flanked N-terminally by PQ/L and C-terminally by GLF sequences (Figure 2, Table 1).

Although evidence exists for the polygenic nature of AI pathogenicity [21,108,109], the molecular composition of the HACS motif is a key determinant of the pathogenic process [18,22,26–28]. Two basic amino acids, lysine (Lys or K) and arginine (Arg or R), play fundamental roles in cleavage site function. The common H5 LPAI HACS motif possesses Arg at P1 and P4: RETR↓ [110], although the Arg is replaced by Lys in some instances (~5% of LPAI motifs possess Lys at either P1 or P4, Table 1). The presence of a pHACS motif is associated with high pathogenicity of AIV, with the consensus motif of the H5 HPAI pHACS being RXR/KR↓ [111,112]. When Lys is present at P4 of the pHACS motif, the minimum consensus is KK/RK/R/XR↓ [113]. The primary and secondary structure of the HACS motif modulates the repertoire of available activating host cell proteases [114], facilitating cleavage by serine (Ser or S) proteases, such as trypsin and trypsin-like proteases in addition to the proprotein convertases.

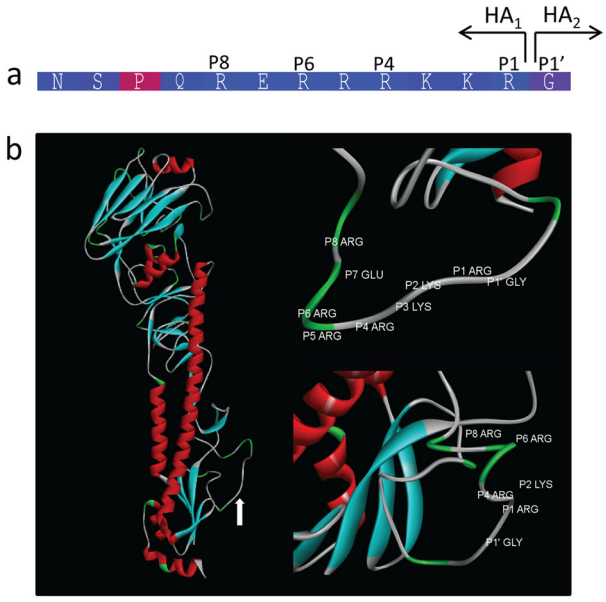

Figure 2. Haemagglutinin cleavage site motif representative of typical Eurasian HPAI isolates. (a) Schematic of pHACS motif typical of Eurasian H5N1 HPAI isolates. In this review, the HACS motif (underlined) is flanked N-terminally by PQ/L and C-terminally by GLF (Table 1). Cleavage of HA_0 into its two subunits, HA_1 and HA_2 occurs between the P1 Arg and the P1′ Gly. Nomenclature of HACS motif position is based on definitions introduced by Schechter and Berger [107], which designates residues N-terminal of the scissile bond P1 and those C-terminal as P1′. (b) Homology structure model of a typical Eurasian HA cleavage site motif, prepared with Swiss Model server [174–176], based on Protein Data Bank ID 1HA0 [114] and manipulated further in Discovery Studio. Left panel shows projection of loop containing pHACS motif (indicated by white arrow) from haemagglutinin monomer. Right top and bottom panels indicate location of P1 to P8 residues within the pHACS motif, side view and distal-to-proximal view of the projected loop, respectively. HA, haemagglutinin; HPAI, highly pathogenic avian influenza; HACS, haemagglutinin cleavage site; Arg, arginine; Gly, glycine; pHACS, polybasic haemagglutinin cleavage site

Activating proteases

Proteolytic cleavage of HA is required for infectivity of influenza viruses [61] and has been demonstrated to occur on the smooth membranes within the trans-Golgi network [54] or at the cell surface [115,116] for highly pathogenic and low pathogenic viruses, respectively. The pHACS motif of highly pathogenic viruses is efficiently cleaved by the ubiquitous proprotein convertases, most notably furin, enabling systemic replication within many tissue and cell types [12–15,29,32]. The HACS motif of LPAI viruses is cleaved by trypsin and trypsin-like proteases [61,117–122]. Hence, LPAI replication tends to be limited to certain cell types, mainly epithelium, resulting in mild infections at mucous membranes and other epithelial tissues [29,30].

Trypsin is a highly specific protease that cleaves exclusively C-terminally to either Lys or Arg [123]. The presence of an aspartic acid (Asp or D) residue (Asp189) at S1, which lies at the base of the substrate binding pocket of trypsin and trypsin-like proteases, dictates substrate specificity, with basic residues preferred at P1 of the substrate. Thus, the presence of Arg (or Lys) within LPAI HACS motif facilitates cleavage by trypsin and trypsin-like proteases with monobasic specificity [20,21,124].

The proprotein convertase, furin, is a ubiquitous transmembrane protease, which cleaves HA_0 [111]. The cleavage site motif of furin substrates was initially identified as paired basic amino acids [125], although the minimum consensus has since been expanded to RXR/KR↓ [126,127]. Furin is active at both the plasma membrane [128] and within the trans-Golgi network [54]; however, evidence of HPAI HA_0 cleavage at the cell surface is currently lacking. The specificity of furin for HA and other

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Table 1. Characteristics of naturally occurring haemagglutinin (HA) cleavage sites from 3140 human and avian isolates of the H5 subtype

Subtype	HA cleavage site motif	Frequency of isolates	Motif length	Number of basic aa in motif	Host
H5N1	PQ_REREGGRRRKR↓GLF	9	11	7	Poultry
H5N1	PQ_GEGRRRKKR↓GLF	1	9	6	Poultry
H5N1	PQ_REGGRRRKR↓GLF	1	9	6	Poultry
H5N1	PQ_IEGGRRRKR↓GLF	1	9	5	Poultry
H5N1	PQ_GEGGRRKKR↓GLF	1	9	5	Poultry
H5N1, H5N2, H5Nx	PQ_RERRRKKR↓GLF	391	8	7	Poultry, wild bird, human
H5N1	PQ_RERRRRKR↓GLF	29	8	7	Poultry, wild bird, human
H5N1, H5N2	PQ_REKRRKKR↓GLF	29	8	7	Poultry, wild bird, human
H5N1	PQ_RERKRKKR↓GLF	5	8	7	Poultry, wild bird, human
H5N1	PQ_GKRRRKKR↓GLF	4	8	7	Poultry
H5N1	PL_RERRRRKR↓GLF	4	8	7	Poultry
H5N1	PQ_RGRRRKKR↓GLF	2	8	7	Poultry, wild bird
H5N5	PL_REKRRRKR↓GLF	2	8	7	Poultry
H5N1	PL_RERRRKKR↓GLF	2	8	7	Poultry
H5Nx	PQ_RDRRRKKR↓GLF	2	8	7	Poultry
H5N1	PQ_RERRRKRR↓GLF	1	8	7	Poultry
H5N1	PQ_RERRKKKR↓GLF	1	8	7	Poultry
H5N1	PL_REKRRKKR↓GLF	1	8	7	Poultry
H5N1	PQ_RERRRKKK↓GLF	1	8	7	Poultry
H5N1	PQ_EKKRRKKR↓GLF	1	8	7	Poultry
H5N1, H5	PQ_GERRRKKR↓GLF	714	8	6	Poultry, wild bird, human
H5N1	PQ_RESRRKKR↓GLF	159	8	6	Poultry, wild bird, human
H5N1	PQ_GEKRRKKR↓GLF	103	8	6	Poultry, human
H5N1	PQ_REGRRKKR↓GLF	62	8	6	Poultry, wild bird, human
H5N1	PQ_REERRKKR↓GLF	41	8	6	Poultry, wild bird, human
H5N1, 'Mixed'	PQ_IERRRRKR↓GLF	17	8	6	Poultry, wild bird
H5N1	PQ_IERRRKKR↓GLF	15	8	6	Poultry, wild bird
H5N1, H5Nx	PQ_GERRRRKR↓GLF	10	8	6	Poultry, wild bird, human
H5N1	PQ_GKSRRKKR↓GLF	9	8	6	Poultry, human
H5N1	PQ_REIRRKKR↓GLF	7	8	6	Poultry
H5N1	PQ_RESRRKRR↓GLF	7	8	6	Poultry
H5N1	PQ_EERRRKKR↓GLF	6	8	6	Poultry, wild bird, human
H5N1	PQ_GERKRKKR↓GLF	3	8	6	Poultry, wild bird
H5N1	PQ_RESRRRKR↓GLF	2	8	6	Poultry
H5N1	PQ_GERRRKRR↓GLF	2	8	6	Poultry

(*Continues*)

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Table 1. (Continued)

Subtype	HA cleavage site motif	Frequency of isolates	Motif length	Number of basic aa in motif	Host
H5N1	PQ_KESRRKKR↓GLF	2	8	6	Poultry
H5N1	PQ_GDRRRKKR↓GLF	1	8	6	Poultry
H5N1	PQ_RVGRRKKR↓GLF	1	8	6	Human
H5N1	PQ_EGRRRKKR↓GLF	1	8	6	Human
H5N1	PQ_RERRREKR↓GLF	1	8	6	Poultry
H5N1	PQ_GEGRRKKR↓GLF	60	8	5	Poultry, wild bird
H5N1, H5N2	PQ_IEGRRRKR↓GLF	13	8	5	Poultry
H5N3	PQ_RETRRQKR↓GLF	3	8	5	Wild bird
H5N1	PQ_REGGRRKR↓GLF	6	8	5	Poultry
H5N1	PQ_REGGRKKR↓GLF	2	8	5	Poultry
H5N1	PQ_EEGRRKKR↓GLF	2	8	5	Poultry
H5N1	PQ_GKNRRKKR↓GLF	1	8	5	Human
H5N1	PL_RKRRRKR↓GLF	1	7	7	Poultry
H5N1, H5N2	PQ_RERRRKR↓GLF	351	7	6	Poultry, wild bird, human
H5N1, H5N2, H5N5, H5N6, H5N8, H5Nx	PL_RERRRKR↓GLF	228	7	6	Poultry, wild bird, human
H5N1	PQ_RERRKKR↓GLF	59	7	6	Poultry, wild bird, human
H5N1, H5N2, H5N5, H5N8	PL_REKRRKR↓GLF	29	7	6	Poultry, wild bird, human
H5N1	PL_RGRRRKR↓GLF	3	7	6	Poultry
H5N1	PQ_REKRKKR↓GLF	3	7	6	Poultry
H5N1	PL_RERRRRR↓GLF	2	7	6	Poultry, human
H5N1	PQ_RGRRRKR↓GLF	1	7	6	Poultry
H5N1	PQ_REKRRKR↓GLF	1	7	6	Poultry
H5N2	PL_RGKRRKR↓GLF	1	7	6	Poultry
H5N1	PQ_GERRRKR↓GLF	1	7	5	Poultry
H5N1, H5N2	PQ_RRRKKR↓GLF	13	6	6	Poultry, wild bird
H5N8	PQ_RKRKKR↓GLF	5	6	6	Poultry
H5N1, H5N2	PQ_RKRKTR↓GLF	6	6	5	Poultry
H5N2, H5N9	PQ_RRKKR↓GLF	5	5	5	Poultry
H5N1, H5N2	PQ_RKKR↓GLF	14	4	4	Poultry
H5N2	PQ_KKKR↓GLF	9	4	4	Poultry
H5N2	PQ_RRKR↓GLF	2	4	4	Poultry
H5N1, H5N2, H5N9	PQ_RKTR↓GLF	12	4	3	Poultry, wild bird
H5N2, H5N3	PQ_REKR↓GLF	9	4	3	Poultry
H5N1-9, H5Nx, 'Mixed'	PQ_RETR↓GLF	589	4	2	Poultry, wild bird, human
H5N1, H5N2, H5N3, H5N7, H5N8, H5N9	PQ_KETR↓GLF	30	4	2	Poultry, wild bird

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H5N2	PQ_KETK↓GLF	6	4	2	Poultry
H5N1, H5N2, H5N7, H5N8	PQ_RGTR↓GLF	5	4	2	Wild bird
H5N2, H5N3	PQ_RDTR↓GLF	2	4	2	Poultry
H5N3	PQ_RATR↓GLF	1	4	2	Poultry
H5N3	PQ_KEAR↓GLF	1	4	2	Poultry
H5N2	PQ_IETR↓GLF	13	4	1	Poultry

*Poultry is defined as domesticated birds such as chicken, turkeys, ducks and geese. This table was constructed by accessing data in the Influenza Research Database (http://www.fludb.org/brc/) and downloading all the amino acid sequences from Segment 4 of Type 'A', with additional filters to limit the data to isolates from avian and human hosts. Laboratory strains were excluded, as were isolates with only partial sequence data for the segment. A quality check was performed and 29 isolates, which indicated poor sequencing, were removed.

substrates is dictated by a group of acidic residues located within the substrate binding domain of the enzyme [126]. Acidic residues within the vicinity of S1, S2 and S4 of the substrate binding domain of furin interact with basic residues present at P1, P2 and P4 within HA and other substrates, and it is these interactions that facilitate protease-substrate binding [129]. A reduction of basic residues within furin substrates may result in presentation of a suboptimal structural geometry of the P1↓P1′ bond to the catalytic site of furin [130], providing further evidence that the presence of basic residues within substrates is preferred. *In silico* studies of the molecular interaction of the furin active site with HA indicated that the presence of a pHACS motif leads to the formation of an increased number of hydrogen bonds, which increase substrate stability [131].

Analysis of binding energies from *in silico* protein docking studies indicated that residues at positions P1, P4 and P6 in the pHACS motif were critical for furin to interact with HA. The interaction at P2 was shown to be less important, although still influential on selectivity (P1 > P6 > P4 > P2) [132]. Additional work suggested that substrate residue requirements at positions P1, P2 or P4 are more stringent than those at P3, P5 and P6 [133]. When comparing the data to the frequency of basic residues at a particular site within the pHACS motif of naturally occurring isolates (Table 1), it would seem that P1 and P4 are the most conserved, with P6 and P2 less conserved among H5 pHACS motifs. The degree of conservation of basic residues P1 and P4 would imply dominance in terms of functional importance for substrate cleavage. Furthermore, mutagenesis of P1 or P4 within another natural furin substrate, anthrax protective antigen, resulted in no cleavage, whilst P2 and P3 had little influence on efficient processing [127].

Diversity of haemagglutinin cleavage site motifs in naturally occurring isolates

To understand the functional and evolutionary significance of the HACS motif sequence, we analysed the sequence patterns and mutation frequency of all full-length HACS motifs reported for H5 human and avian natural isolates. Over 3000 full-length H5 HA protein sequences publicly available on the Influenza Research Database (accessed 23 September 2014) [134] were compiled

in Table 1. A high frequency of sequence patterns gives strong indication that some functional significance can be attributed to them. Conversely, absence or rarity of particular sequence patterns within natural isolates is indicative of low viral fitness. However, the caveat is that highly pathogenic isolates are likely to be more frequently detected by passive surveillance systems and therefore are likely to be over-represented in this database. Additionally, low pathogenic forms are unlikely to cause disease, and for that reason, may not come to the attention of surveillance mechanisms and therefore will be under-represented. We used this method to detect and interpret broad patterns, but we caution against over-interpreting this information. In addition, some of the sequences, particularly those of single frequency, may be erroneous due to sequencing errors, and therefore the common sequences will be more indicative of functionally significant patterns. Geographical clustering of HACS motifs and the frequency of HACS motifs within particular H5 clades are beyond the scope of this review, but would be of great interest.

Glutamine (Gln or Q) and proline (Pro or P) residues commonly flank the HACS motif N-terminally, although leucine (Leu or L) may replace Gln in some instances (Table 1). C-terminally, the HACS motif is commonly flanked by glycine (Gly or G), Leu and phenylalanine (Phe or F); these amino acids constitute the first three residues of the HA_2 fusion domain. Insertions into the HACS motif shift the Gln and Pro N-terminally to accommodate the additional residues.

Low pathogenic avian influenza HACS motifs are dominated by the presence of Arg residues at both P1 and P4 (RXXR↓) (92%, Table 1). The P1 residue within HACS is most frequently Arg, which is a minimum requirement of the activating proteases [24,123]. P2 and P3 are predominately threonine (Thr or T) (~99%) and glutamic acid (Glu or E) (~98%), respectively.

The HPAI pHACS motif frequently contains six basic residues, from P1 to P6, with the presence of another basic amino acid at P8 (Figure 2, Table 1). The P4 residue within naturally occurring AI viruses is preferentially Arg for both LPAI and HPAI viruses (Table 1), suggesting its presence is important for optimal interaction between HA and the host cell activating proteases. P6 displays a high level of polymorphism (49%), indicating that the presence of a non-basic amino acid is not detrimental to HA processing. The P8 position within the pHACS motif is dominated by basic residues, although this position is also polymorphic (40%).

A higher frequency of a particular HACS motif within naturally occurring isolates suggests that the motif may occupy a state of optimal fitness and correspond with optimally fit pathotypes. Data presented in Table 1 can be divided into three categories of cleavage site motifs: (i) short motifs (≤2 basic residues) similar to those in LPAI isolates; (ii) mid-length motifs (3–4 basic residues) that may indicate transition species, and includes isolates classified molecularly as HPAI; and (iii) extended cleavage site motifs (≥5 basic residues), which are inclusive of HPAI isolates.

Short cleavage motifs represent ~21% of the 3140 HA sequences analysed (Table 1), indicating that these HACS motifs occupy an ecological fitness peak. All short HACS motifs possess a basic residue at P1, 98% of short HACS possess a basic residue at P4 and 98% possess basic residues both at P1 and P4, suggesting that despite the monobasic specificity of trypsin, the presence of a basic residue at P4 in addition to P1 is favoured. The consensus motif of the short HACS motifs is BXTB↓G, with B and X being basic and non-basic residues, respectively (98%, Table 1). Of the short HACS motifs, viruses harbouring RETR↓G sequences dominate (91% of shorter sequences). Selection pressure must be placed on the motif, in addition to accumulation of genetic changes elsewhere in the genome, to facilitate switching from a LPAI to HPAI pathotype. Although incorporation of a pHACS motif is sufficient for pathotype switching in the majority of cases, some isolates may lack an optimal pathogenetic backbone for pathotype switching to occur, such as those isolates that possess a pHACS motif despite being low pathogenic in chickens [18,71–74]. This is further supported by experimental evidence suggesting that incorporation of a pHACS motif into *any* isolate is itself insufficient to transform from an LPAI into an HPAI pathotype [21,109], without the accumulation of mutations elsewhere in the genome [23,135,136].

The number of isolates harbouring mid-length HACS motifs is relatively small (1.5%, Table 1) in comparison to isolates with either short or extended HACS motifs. Viruses encoding mid-length HACS motifs are considered highly pathogenic if they possess a HACS motif similar to that previously observed in HPAI isolates, regardless of their

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pathogenicity in chickens [19,137]. The mid-length HACS motifs, RKTR↓G and RKKR↓G are the most frequent, constituting 56% (26% and 30%, respectively) of the mid-length HACS sequences. Mid-length HACS motifs may be representative of viral species in a transition phase during pathotype switching. For example, mutation of P3 within a shorter cleavage site motif (PQRETR↓G) to form a mid-sized cleavage site motif (PQRKTR↓G) potentially results in an unstable intermediate HACS motif that readily incorporates additional residues into cleavage site motif to gain stability. This seems to have occurred during the 1994–1995 HPAI outbreak in Mexico where a series of extension events within the cleavage site motif was reported; PQRETR↓G to PQ*RK*RKTR↓G and finally PQ*RKRK*RKTR↓G [138]. Coupled with the relatively low number of natural isolates harbouring mid-length HACS motifs, this suggests that a mid-length HACS motif is an unstable intermediate, driving duplications within the cleavage site motif. Experimental evidence suggests that extension of the cleavage motif confers increased sensitivity to furin [133].

Isolates harbouring extended pHACS motifs constitute the majority of HACS sequences in Table 1 (78%). These sequences correspond to highly pathogenic isolates and the high prevalence may be mainly due to surveillance over-representation, as HPAI viruses have been a major cause of poultry disease in recent years. The presence of basic residues dominates positions P1 to P6. H5 HPAI viruses commonly encode the extended cleavage site motifs, PQGERRRKKR↓G and PQRERRRKKR↓G (29% and 16%, respectively). Of the extended size cleavage motifs, a relatively large number of isolates harbour an acidic residue (e.g. Glu) at P6 (29%), such as PLRERRRKR↓G (9% of extended sequences) and PQRERRRKR↓G (14% of extended sequences). Having an acidic residue at P6 seems counterintuitive, as the residue would impart repulsive forces when bound to activating proteases. On the other hand, this cleavage site motif may be indicative of isolates that are nearing the completion of their cleavage site motif extension via a common mechanism; incorporation of another basic residue would push the suboptimal acidic residue from P6 to P7 position, thus increasing substrate-protease interactions. Furthermore, this may also explain the polymorphism seen at P6 in pHACS motifs (49%, Table 1). The pressure to insert another residue at P6 to displace the acidic residue to P7 results in incorporation of a replacement residue, with the prerequisite being that the residue is not acidic.

A prominent feature of naturally occurring isolates in Table 1 is the significant diversity within HACS motifs originating from domestic poultry isolates (99.6% of sequences). This suggests that the variation may be a host adaptation, with the mutation and evolution of H5N1 viruses progressing upon transmission to poultry. Additionally, the majority of diversity within the HACS motifs is present within the H5N1 subtype (98%). Furthermore, a comparison of the consensus LP HACS motif and the consensus furin substrate motif (BXTB↓G and RXR/KR↓G, respectively) reveals that, on an optimal genetic backbone, only one mutation within the LP HACS motif may be required to initiate pathotype switching.

Mechanisms of natural sequence change

Highly pathogenic viruses arise from low pathogenic precursors through the stepwise accumulation of mutations [138] and positive selection of a low-level virus quasispecies [136,139]. Because of an inherently high mutation rate during replication of *Influenzavirus A*, as a consequence of a lack of RNA polymerase proofreading ability [140], mutations can occur with every replication cycle. The nucleotide substitution (error) rate of HA is $\sim 10.15 \times 10^{-3}$ substitutions/site/year [136], and this property, in addition to it also being malleable to insertions [141], highlights the plasticity of HA for incorporation of mutations.

Polymerase slippage, due to RNA secondary structure, has been put forward as a mechanism for the extension of the HACS motif [138,142]. Duplication of purine rich regions located within the HACS motif, adjacent to a region of high secondary structure, was implicated in an increase of pathogenicity during the 1994–1995 Mexico HPAI outbreak [138]. Additional basic residues within the HACS motif have also been linked with increased pathogenicity during the 1976 Australian outbreak of H7N7 HPAI [143]. Not only was the length of the cleavage site loop extended by the addition of the three amino acids, the residue present at P4 was now a basic residue, which has previously been implicated as critical in the cleavage activation of AI [20]. Recombination of HA with

host 28S ribosomal RNA has also been implicated in the extension of the cleavage site [144]. Interestingly, a palindrome sequence at the cusp of the positive sense viral 5′ sequence and the 3′ ribosomal RNA insert sequence was identified, leading to the hypothesis that this may be a priming site for recombination events [144]. A recombination event with host 28S ribosomal RNA was identified during the 2012 HPAI outbreak in Mexico, in addition to palindromes in the vicinity of insertions, strengthening the earlier suggestion that a yet to be identified mechanism facilitates RNA recombination events [145]. Recombination events between viral genes have also been identified during HPAI outbreaks [146–148].

Selective mutagenesis of the H5 haemagglutinin polybasic cleavage site motif and associated pathogenicity

It is now widely reported that removal of the pHACS motif by reverse genetics renders HPAI viruses low pathogenic in both *in vitro* systems and *in vivo* studies in avian and mammalian animal models [20,22,25,33,34,87,104,149–158]. Reverse genetics have been used as a powerful tool in determining the molecular 'signature' of the HPAI pHACS motif critical for pathogenicity. Studies investigating amino acid residues within the HACS motif important to the cleavage activation and pathogenicity of H5 HPAI are summarised in Table 2; these studies include *in vitro* and *in vivo* approaches utilising simian virus 40 (SV40) and vaccinia virus expression systems, biochemical assays and reverse genetics generated viruses. Unless characterised according to the OIE guidelines, experimentally determined pathotypes described in this review will be referred to as either as high virulence (HV) or low virulence (LV).

Naturally occurring H5 isolates are dominated by Arg at P1, with the exception of a few with Lys at this position (Table 1). The presence of a P1 Lys in 0.2% of naturally occurring AI isolates (predominately harbouring short HACS motifs, Table 1) suggests that Lys is somewhat tolerated *in vivo*, although not favoured. Mutation of the P1 Arg to Lys using SV40 and vaccinia virus recombinant systems abolished pHACS cleavage *in vitro* [24,159–161]. To date, reports of *in vivo* studies investigating mutations to non-basic residues at P1 are lacking. Moreover, there are no naturally occurring isolates with a non-basic residue at P1, indicating that a mutation away from a basic residue at P1 is detrimental.

There is a substantial body of work characterizing the consequences of a basic to a non-basic mutation at P2. All studies have incorporated a mutation to Thr, presumably because this residue is commonly found at P2 in naturally occurring isolates (21%, Table 1). *In vitro* studies suggested that mutations of P2 (Lys to Thr) reduced HA cleavage [20,24,162]. Subsequent reverse genetics studies reported that a virus with Thr at P2 displayed an LV phenotype following intranasal or oronasal inoculation of 4-day-old specific-pathogen-free (SPF) chickens; intramuscular inoculation yielded variable pathogenicity results [22,162]. Despite experimental evidence supporting reduced HA cleavability and an LV phenotype following a P2 mutation to a non-basic residue, a naturally occurring isolate from the 1991 HPAI outbreak in England, which harboured Thr at P2, was not shown to be detrimentally affected [163].

Mutations at P3 from Lys to a non-basic residue, such as Glu, have not affected cleavability of HA *in vitro* [20,24], suggesting that this position is not critical for HA interactions with activating proteases. Within naturally occurring isolates, a basic residue at P3 is not present within short HACS motifs, although is present in 80% of mid-length pHACS motifs and 99.9% of extended pHACS motifs (Table 1), suggesting that P3 undergoes selection pressure. Mutation of the P3 residue may not detrimentally affect extended pHACS motif cleavage, although its mutation (or insertion at this site) may support the transition from an LP HACS motif to an HPAI extended pHACS motif.

Experiments investigating the presence of non-basic residues at P4 within H5 pHACS have shown that an Arg to Thr mutation prevented HA cleavage *in vitro* [20,24,160]; no reported *in vivo* data are currently available. No naturally occurring isolates with mid-length or extended pHACS motifs possess a non-basic residue at P4, suggesting its critical importance for HA processing by furin.

P6 of the H5 extended pHACS motif has been shown to display high polymorphism [164] (49% of isolates, Table 1), suggesting that mutations at this position are tolerated. Indeed, experimental mutations at P6 have no effect on cleavage *in vitro* [22,24,159] and were also shown not to affect

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Table 2. H5 haemagglutinin cleavage site (HACS) motif: reverse genetics viruses, reassortants, peptide sequences and haemagglutinin (HA) glycoprotein sequences tested experimentally

Subtype	HACS motif†	LP/HP††	HA cleavage†††	Method	Reference	Naturally occurring
H5N8	**Q_RKRKKR↓G**		+	*In vitro* cleavage of SV40-HA	Kawaoka & Webster 1988 [20]	Y
	Q_RK<u>T</u>QKR↓G		-	recombinant virus.		N
	Q_RK<u>T</u>KKR↓G		-			N
	Q_RKR<u>Q</u>KR↓G		+			N
	Q_RKRK<u>T</u>R↓G		±			Y
	Q_<u>T</u>ERKKR↓G		±			N
	Q_ΔΔRKKR↓G		-			Y
	Q_ΔΔR<u>Q</u>KR↓G		-			N
	Q_ΔΔRK<u>T</u>R↓G		-			Y
	Q_ΔΔR<u>E</u>TR↓G		-			Y
H5N8	**Q_RRRKKR↓G**		+	*In vitro* cleavage of SV40-HA	Kawaoka & Webster 1989 [165]	Y
	Q_ΔΔRKKR↓G		-	recombinant virus. ◊Indicates		Y
	Q_ΔΔRKKR↓G		+◊	no carbohydrate present at		Y
H5N1	**Q_RKKR↓G**		+◊	residue 11.		Y
	Q_RKKR↓G		-			Y
	Q_*RR*RKKR↓G		+			Y
H5N8	**PQ_RKRKKR↓G**		+	*In vitro* cleavage of SV40-HA	Walker & Kawaoka 1993 [159]	Y
	PQ_<u>T</u>ERKKR↓G		±	recombinant virus. Extension		N
	PQ_RKRKKR↓<u>A</u>		+	of Kawaoka & Webster 1988		N
	<u>Y</u>Q_RKRKKR↓G		+	study.		N
	<u>G</u>Q_RKRKKR↓G		+			N
	PQ_RKRKK<u>K</u>↓G		-			N
	PQ_<u>T</u>KRKKR↓G		+			N
	PQ_ΔKRKKR↓G		±			N
	PQ_ΔΔRKKR↓G		-			Y
H5N8	**PQ_RKRKKR↓G**		+	*In vitro* cleavage of vaccinia-HA	Walker *et al.* 1994 [24]	Y
	PQ_RKRKK<u>K</u>↓G		-	recombinant virus. Extension of	Also refer to Horimoto	N
	PQ_RKRK<u>T</u>R↓G		±	Walker & Kawaoka 1993 study.	*et al.* 1994 [160]	Y
	PQ_RKR<u>Q</u>KR↓G		+			N
	PQ_RK<u>T</u>KKR↓G		-			N
	PQ_<u>T</u>KRKKR↓G		+			N
	PQ_<u>T</u>ERKKR↓G		±			N
	PQ_<u>TET</u>KKR↓G		-			N

(*Continues*)

Table 2. (Continued)

Subtype	HACS motif†	LP/HP††	HA cleavage†††	Method	Reference	Naturally occurring
	PQ_TESPKR↓G		-			N
	PQ_RKTQKR↓G		-			N
	PQ_ΔKRKKR↓G		±			N
	PQ_ΔΔRKKR↓G		-			Y
	PQ_ΔΔRKTR↓G		-			Y
	PQ_ΔΔREKR↓G		-			Y
	PQ_ΔΔRQKR↓G		-			N
	PQ_ΔΔKKKR↓G		-			Y
	PQ_ΔΔIEKR↓G		-			Y
H5N9	**PQ_RRRKKR↓G**	LV^	+	Intranasal/oral inoculation of 4-day-old SPF chicks with 10^6 plaque forming units PFU. ^HV phenotype following intramuscular inoculation.	Horimoto & Kawaoka 1994 [22]	Y
	PQ_RRRKTR↓G	LV^	±			N
	PQ_TTRKKR↓G	LV^	+			N
	PQ_TRRKKR↓G	LV^	+			N
	PQ_ΔΔRKKR↓G	LV	-			Y
	PQ_ΔΔRETR↓G	LV	-			Y
H5N9	**Q_RRRKKR↓G**	LV^	+	Intranasal inoculation of 4-day-old SPF chicks. Dosage not reported. ^HV phenotype following intramuscular inoculation.	Horimoto & Kawaoka 1997 [162]	Y
	Q_*RR*RRRKKR↓G	LV	+			N
	Q_RRRKTR↓G	LV	±			N
H5N1	PQ_RETR↓G	LP[∂]		IVPI determined in 4-week-old White Leghorn chickens. LV also following intranasal/intratracheal inoculation of Plymouth Rock chickens. Virus rescued on cold adapted backbone. [∂]HA from HK/156/97. [Σ]HA from HK/483/97	Li *et al.* 1999 [156]	Y
	PQ_RETR↓G	LP[Σ]				Y
H5N1	**PQ_RERRRKKR↓G**	HP	+	IVPI in 4-week-old White Rock chickens. 10^6 EID_{50} in 6- to 8-week-old BALB/c mice. LP rescued on H1N1 backbone.	Subbarao *et al.* 2003 [149]	Y
	PQ_RETR↓G	LP	-			Y

(*Continues*)

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Table 2. (Continued)

Subtype	HACS motif†	LP/HP††	HA cleavage†††	Method	Reference	Naturally occurring
H5N2	**PQ_RKKR↓G**	LP	-	IVPI determined in 4-week-old SPF White Leghorn chickens. Parent classified HPAI molecularly though LPAI. in chickens	Lee *et al.* 2005 [73]	Y
	PQ_*R*RKKR↓G	LP	-			Y
	PQ_*RK*RKKR↓G	LP	+			Y
H5N1	PQ_RESR↓G	LP		IVPI determined in 6-week-old chickens. Viruses rescued on H1N1 backbone.	Lu *et al.* 2005 [168]	N
H5N2	PQ_RESR↓G	LP				N
H5N1	RETR↓G	LP		IVPI determined in 4-week-old SPF chickens. LP virus rescued on H1N1 backbone.	Tian *et al.* 2005 [157]	Y
H5N1	**PQ_RERRRKKR↓G**	HP*		IVPI determined in 4- to 6-week-old SPF chickens. 6-week-old BALB/c mice inoculated with 10^5 EID_{50}. *LP in mice.	Lu *et al.* 2006 [87]	Y
	PQ_RESR↓G	LP				N
H5N2	PQ_RESR↓G	LP				N
H5N1	**PQ_RERRRKKR↓G**	HV		Intravenous inoculation of 6-week-old SPF chickens with 10^7 EID_{50}. Viruses rescued on H3N1 backbone.	Muramoto *et al.* 2006 [25]	Y
	PQ_RETR↓G	LV				Y
H5N1	PQ_RERRRKKR↓G		+	Biochemical peptide cleavage assay. Cleavage conducted in the presence of furin and heparin at pH 7.5.	Pasquato & Seidah 2008 [169]	Y
H5N2	PQ_RKRKRKTR↓G		+			Y§§
H5N1	PQ_RERRRKR↓G		+			Y
H5N1	PL_RERRRKR↓G		+			Y
H5N2	PQ_RETR↓G		-			Y
H5N1	RRRKKR↓G		Furin -37% PC2 - 8% PC4 - 31% PC5/6 - 33% PC7 - 26% PACE4 - 40%	Biochemical multiplexed peptide cleavage assay assessing cleavage by various proprotein convertases.	Remacle *et al.* 2008 [133]	Y
H5N1	**PL_RERRRKR↓G**	HV		IVPI determined in 6-week-old mallard ducks.	Tang *et al.* 2008 [170]	Y
	PQ_RERRRKR↓G	LV				Y
	PL_RERRRK*K*R↓G	LV				Y
	PQ_RERRRK*K*R↓G	LV				Y

(*Continues*)

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Table 2. (Continued)

Subtype	HACS motif†	LP/HP††	HA cleavage†††	Method	Reference	Naturally occurring
H5N1	**PQ_RERRRKKR↓G**	LV				Y
	PL_RERRRKKR↓G	LV				Y
	PQ_RERRRKR↓G	LV				Y
	PL_RERRRKR↓G	LV				Y
H3N8	**PE_KQKR↓G**	LV		Inserted H5 pHACS into LV H3 HA.	Stech *et al.* 2009 [109]	Y§§
	PQ_R*RRKK*R↓G	LV		Oculonasal inoculation of 4-week-old		Y
	PQ_RER*RRKK*R↓G	LV		SPF chickens with 1.7 x 10^6 PFU.		Y
H5N1	**PQ_RERRRKKR↓G**	HP	+	IVPI determined in 4-week-old White	Suguitan Jr. *et al.* 2009 [150]	Y
	PQ_RETR↓G	LP	-	Plymouth Rock chickens. Intranasal		Y
	PQ_RERRRKKR↓G	LP§▫	+	inoculation with 10^6 $TCID_{50}$ mimicked		Y
	PQ_RETR↓G	LP§	-	IVPI results. Mouse LD_{50} determined in		Y
	PQ_RERRRKKR↓G	LP§¤	+	6-to 8-week-old BALB/c mice. §H2N2		Y
	PQ_RETR↓G	LP§¤	-	backbone. ¤Cold adapted. ▫HV in mice.		Y
H5N1	**PQ_KETR↓G**	LV	-	Inserted H5 pHACS into LV H5 HA.	Bogs *et al.* 2010 [21]	Y
	PQ_RER*RRKK*R↓G	LV	+	Oculonasal inoculation of 2-week-old		Y
	PQ_GER*RRKK*R↓G	LV	+	SPF chickens with 10^5 PFU.		Y
	PQ_RER*RRKK*R↓G	HVº	+	ºReassortant virus.		Y
H5N1	**PQ_RERRRKKR↓G**	HV		Intranasal inoculation of 16-32-week-old	Abt *et al.* 2011 [171]	Y
	PQ_RETR↓G	LV		ferrets. LV rescued on H1N1 backbone.		Y
H5N1	**PQ_GERRRKKR↓G**	HV	+	Oculo-nasal inoculation of 2-week-old	Gohrbandt *et al.* 2011 [34]	Y
	PQ_GETR↓G	LV	-	SPF chickens with 10^6 $TCID_{50}$.		Y§§
	PQ_GER↓G	LV	-			N
H5N1	**PQ_RERRRKKR↓G**	HV		Oculo-nasal-oral inoculation of 4-week-	Karpala *et al.* 2011 [104]	Y
	PQ_RETR↓G	LV		old broiler chickens with either 2 or 10 EID_{50}.		Y
H5N1	**PQ_RERRRKKR↓G**		+	Intranasal inoculation of ferrets with 10^6	Schrauwen *et al.* 2011 [151]	Y
	PQ_IETR↓G		-	$TCID_{50}$ (age not reported). Inserted H5		Y
H3N2	**PE_KQTR↓G**	LV	-	pHACS into LV H3 HA.		Y
	PE_KQR*RRKK*R↓G	LV	+			N

(*Continues*)

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Table 2. (Continued)

Subtype	HACS motif†	LP/HP††	HA cleavage†††	Method	Reference	Naturally occurring
H5N1	**PQ_RETR↓G**	LP	-	IVPI determined in 4-week-old Boris Brown chickens. Intranasal inoculation with $10^{6.5}$ EID_{50} mimicked IVPI results. Viruses rescued on a H5N1 LP backbone. »Acquired pathogenic phenotype. following multiple *in vivo* passages.	Soda *et al.* 2011 [135]	Y
	PQ_RE<u>R</u>*RKK*R↓G	LP»	-			Y
	P<u>R</u>_R<u>KK</u>R↓G	LP»	+			N
H5N1	**PQ_RERRRKKR↓G**	HV≠		Oculo-nasal-oral inoculation of 4-week-old SPF chickens and 5-week-old Pekin ducks with $10^{5.2\text{-}6.1}$ EID_{50}. ≠LV in ducks.	Schat *et al.* 2012 [33]	Y
	PQ_RE<u>T</u>R↓G	LV				Y
H5N1	**PQ_RERRRKKR↓G**	HV	+	Intranasal inoculation of adult ferrets with 10^6 $TCID_{50}$.	Schrauwen *et al.* 2012 [152]	Y
	PQ_<u>I</u>E<u>T</u>R↓G	LV	-			Y
H5N1	**PQ_RERRRKKR↓G**	HV≡		Intranasal inoculation of 6- to 8-week-old BALB/c mice and 6-month old ferrets with 125 $TCID_{50}$ and 10^7 $TCID_{50}$, respectively. Intratracheal and intranasal inoculation of African green monkeys with 2 x 10^6 $TCID_{50}$. ≡LV in African green monkeys.	Suguitan Jr. *et al.* 2012 [153]	Y
	PQ_RE<u>T</u>R↓G	LV				Y
H5N1	PQ_RRRKKR↓G	HV≈	+	Oculonasal inoculation of 10-week-old SPF chickens with 10^6 PFU. ≈LV on H9N2 LP backbone.	Veits *et al.* 2012 [172]	Y
H5N2	PQ_GERRRKKR↓G	HV≈	+			Y
H5N1	**PQ_RERRRKKR↓G**		+	*In vitro* assessment of replication in human pulmonary endothelial cells.	Zeng *et al.* 2012 [154]	Y
	PQ_RETR↓G		±			Y
H5N1	**PQ_REGRRKKR↓G**	HP*	+	IVPI determined in 6-week-old SPF chickens. Intranasal inoculation of 6-week-old BALB/c mice with 50 µl 10^6 EID_{50}. *LV in mice.	Zhang *et al.* 2012 [164]	Y
	PQ_RE<u>I</u>RRKKR↓G	HP*	+			Y
	PQ_RE<u>R</u>RRKKR↓G	HP*	+			Y
	PQ_RE<u>S</u>RRKKR↓G	HP	+			Y
	PQ_RERRKKR↓G	HP*	+			Y

(*Continues*)

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Table 2. (Continued)

Subtype	HACS motif†	LP/HP††	HA cleavage†††	Method	Reference	Naturally occurring
H5N1	**PQ_RERRRKKR↓G**	HV		Median lethal dose determined in 6-week-old BALB/c mice.	Tchitchek *et al.* 2013 [158]	Y
	PQ_RETR↓G	LV				Y
H5N1	**PQ_RERRRKKR↓G**	HV		Intranasal-intraocular-intratracheal inoculation of 6-week-old White Leghorn chickens with 3-5.6 times their median chicken lethal dose.	Yoon *et al.* 2013 [173]	Y
	PQ_GERRRKKR↓G	HV				Y
H5N1	PQ_RETR↓G		-₴	*In vitro* cleavage in MDCK cells. HA and NA from indicated isolates, H1N1 backbone. ₴HA from duck/Anhui/1/2005 ¢HA from duck/Guangdong/s1322/2010	Wen *et al.* 2015 [155]	Y
	PQ_RETR↓G		-¢			Y

†Parental sequence bold. Δ indicates deletion, italicised indicates insertion and underlined indicates mutation. ↓ indicates scissile bond cleaved by activating protease. HACS motif defined as between PQ_ and↓ G, N- and C-terminally, respectively.

††LP/HP according to OIE definition. LV/HV indicates pathogenicity not determined according to OIE definition (LV <75% mortality, HV ≥75% mortality).

†††Cleavage in the absence of exogenous trypsin.

§§HACS motif reported to be naturally occurring, although is not represented in Table 1.

Table 2 was generated by conducting a literature search of H5 HACS motif manipulations. AIV subtype, HACS motif and associated virulence and/or HA cleavability, brief information about the study undertaken in addition to whether the HACS motif is represented in naturally occurring isolates is summarised.

LP, low pathogenic; HP, highly pathogenic; HV, high virulence; LV, low virulence; IVPI, intravenous pathogenicity index; SPF, specific-pathogen-free; pHACS, polybasic haemagglutinin cleavage site; OIE, World Organisation for Animal Health; SV40, Simian Virus 40.

pathogenicity following intramuscular inoculation. A slight reduction in pathogenicity was however noted following oronasal inoculation of SPF chicks [22]. A recent study by Zhang *et al.* [164] reported that mutagenesis of P6 from Gly to Isoleucine (Ile or I), Arg or Ser did not affect pathogenicity following experimental inoculation of chickens; all mutated viruses displayed an HPAI phenotype. Interestingly, the presence of Gly or Ile at P6 resulted in a low pathogenic phenotype in a mouse model. This clearly reflects the need for careful selection of appropriate animal models in assessing pathogenicity of generated mutants.

The length of the pHACS motif has also been implicated in AI pathogenicity. Deletion of residues at P5 and P6 (resulting in a sequence shift) has been shown to abolish cleavage of HA *in vitro* [20,22,24,159,160]. Steric hindrance resulting from a carbohydrate present at asparagine 11 within the HA stalk was identified as modulating cleavage; mutation of the N-linked glycosylation site eliminated steric hindrance imposed by the carbohydrate, allowing subsequent HA cleavage [72,165,166]. Deletion of basic amino acids at P5 and P6 resulted in an LV phenotype, despite molecular HPAI classification, following intranasal/oral or intramuscular inoculation of chickens (0% mortality) [22]; mutation of both P5 and P6 to non-basic residues maintained an LV phenotype following intranasal/oral inoculation (40% mortality), although displayed a HV phenotype following intramuscular inoculation (80% mortality) [22].

Despite multiple studies investigating individual residues within the HACS motif critical to HA cleavage and/or pathogenicity, the majority of conclusions are drawn from studies conducted *in vitro*. Revisiting these specific mutations, assessing their effect on pathogenicity in chickens and sequence analysis of re-isolated viruses would be of considerable interest. Reverse genetic manipulations of the H5 HACS motif have encompassed mutations that are both naturally occurring and those that are not (Table 2). As a result, conclusions drawn from HACS motifs represented in naturally occurring isolates bear more biological relevance than those motifs that are not naturally occurring. Combining experimental approaches with biologically feasible mutations will assure that results and subsequent conclusions are representative of relevant protease-substrate interactions following H5 infection.

Based on both the experimental data and the frequencies of HACS motif present in naturally occurring isolates, two positions within the HACS motif, P1 and P4, are critical for HA cleavage. All naturally occurring AIV isolates in Table 1 harbour a basic residue at P1, particularly Arg, suggesting its critical role for HA_0 cleavage. Additionally, all naturally occurring AIV isolates harbouring mid-length or extended pHACS motifs harbour a basic residue at P4 (Table 1); mutation of P4 from a basic to a non-basic residue abolishes cleavage. The extremely low tolerance for non-basic residues at these positions confirms their functional importance for HA_0 cleavage.

CONCLUSION

H5 HPAI continues to threaten the human population through direct transmission from avian to human hosts. Despite the current inability of the virus to sustain human-to-human transmission, the genesis of laboratory-generated transmissible HPAI viruses [6–8] has alarmed scientists and policy makers alike [167]. With the HACS motif playing a critical role in the pathogenicity and pathogenesis of HPAI infection, it is imperative to understand the functional evolution and molecular components that contribute to the overall disease severity. The advent of *Influenza A virus* reverse genetics has facilitated identification of residues key to the HPAI pathotype, with several residues —particularly P1 and P4—proving critical to HA-protease interactions. Additionally, despite the advances in knowledge of molecular determinants of pathogenicity, gaps remain in our knowledge of the evolution of LPAI viruses into HPAI. To date, there is currently no information in the literature describing the mechanism for conversion of LPAI viruses to HPAI viruses. Time-course monitoring of viral quasispecies following experimental infection of chickens may help to elucidate the sequence of events required to initiate the incorporation of a pHACS motif and AI pathotype switching. Coupled with reverse genetics techniques, further studies could survey and target the molecular events leading to pHACS motif incorporation and subsequent pathotype switching. Knowledge of these events may allow us to better predict and respond to the emergence of HPAI viruses.

CONFLICT OF INTEREST

The authors have no competing interest.

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