Structural homology and expression tendency of the natural immune response of the terminal complement components to inoculations in pigs: a review

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ABSTRACT: The transmission of infectious agents from domestic animals to humans is a matter of particular concern at present. Inoculation can enhance the defences of each individual animal but only in the short term. Certainly, it will be of immense benefit if biotechnology and genetic techniques are applied to farm animal breeding and selection programs to improve productivity, performance and health status as well as for the construction of sustainable animal production systems and promotion of animal welfare. In recent years, efforts to drive candidate genes like cytokines, haptoglobin, complement system, C-reactive protein, a 2-macroglobulin, retinol binding protein, transcortin, etc. associated with immune traits have successfully been studied in human and different animal species. Here, we compared the molecular structure and evaluated the expression tendency of the haemolytic complement activity (HCA) of porcine candidate genes encoding the terminal complement components (TCC) C6–9. The results suggested that (1) high homology of complement genes among mammalian species may open new ways in cure/ treatment of disease; (2) Muong Khuong animals (Vietnamese potbelly pig) have a great genetic potential to improve the health status of pigs; and (3) HCA in the classical pathway can be developed further by different activation modes, with the potential improvement of animal health.

Keywords: pigs; terminal complement genes; homology; genetic variation; and haemolytic complement activity

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1. Introduction

Diseases and infections of domestic animal herds are becoming a more and more serious concern. Many new diseases have appeared and old conditions have returned which do not only greatly affect the health and productivity of animals, but also cause directly economic losses and indirectly affect consumer health. In recent years, Bird-flu and blue-ear diseases have rapidly spread on a large scale, especially in some Asian countries (China, Thailand, and Vietnam, etc.). Pathogens may generate variant DNA/RNA structural types/strains within a short time. Therefore, disease prevention using specific vaccines have not been as successful as expected with little or no effects on some diseases.

Mycoplasma pneumonia caused by *Mycoplasma hyopneumoniae* (Mh) is a highly contagious and chronic disease affecting pigs with a significant reduction in the growing performance of animals and estimated economic losses of $ 0.2 to 1 billion per year in the USA (Clark et al. 1991). Mh is spread worldwide and present in almost pig herds (Minion 2002) because antibiotics cannot completely remove the pathogen. Vaccines have been found to reduce the severity of the disease but do not prevent the disease from occurring in infected pigs (Haesebrouck et al. 2004). Unluckily, the immune

response in Mh-infected pigs is slow and ineffective (Minion 2002). Additionally, producers like using antibiotics to treat illnesses and/or to improve the feed-using efficiency, whereas vaccines are used to prevent the spread of disease. All these interventions are to ensure economic objectives. However, the use of antibiotics in the long term may influence the treatment of both animal and human diseases due to the development of medical resistance.

With the development of biotechnology, molecular immunological and genetic methods have been applied to animal breeding and selection programs. These developments have gradually improved the health status and disease resistance of animals leading to higher product quality, a reduction in the transmission of infectious agents or traces of antibiotics, and slight increases in production efficiency. It has been demonstrated that disease reduction by genetic means has certain advantages through cumulative and permanent effects.

The mechanism of complement activation and its role in immune responses for fighting pathogens have been studied in humans and animals. The activation of the complement cascades allows the killing of microbes and the regulation of other immune processes. Many studies have indicated deficiencies in complement components with effects on human/animal health, for instance, bacterial meningitis infection of hosts (Eng 1980; Haeney et al. 1980; Ross and Densen 1984; Inai et al. 1989; Fukumori and Horiuchi 1998; Zhu et al. 2000; Vazquez-Bermudez et al. 2003). These authors have also demonstrated that most deficiencies are associated with polymorphisms present in the molecular structure of at least one complement component. Vissher et al. (2002) drew the important conclusion that there is the possibility for genetic improvement of the immune capacity and response to pathogens. In subsequent years, genetic variation of the C3, C5, MBL1 and MBL2 associated with haemolytic complement activity (HCA) were also investigated (Phatsara et al. 2007; Kumar et al. 2004; Wimmers et al. 2003). Consequently, in this study, we strongly focused on the porcine terminal complement components C6, C7, C8A, C8B, and C9 using the same experimental materials, statistical analytical methods and study objectives.

2. Structural homology

The architecture of the porcine candidate genes encoding the TCC was identified and analysed. A high similarity was found between human and pig for cDNA (65–86%) and protein (67–83%) sequences. Compared to human protein sequences, the porcine C6, C8A, and C8B are 1, 5, and 20 amino acids longer, respectively. The C9 is 16 amino acids shorter, whereas the C7 and C8G are of the same length. In other words, the C6 is the largest, and the C8G is the smallest. The porcine proteins all show 22–30% similarity to each other except for C8G. In fact, homology was found between the amino termini of the C6 and C7 and the mature form of C8A, C8B and C9. The carboxyl terminus of the C6 and C7 are only extended fragments and therefore they also show high identity to each other. It seems, therefore, that the last molecules of the complement system originate from a common ancestral gene.

In general, the C6, C7, C8A, C8B and C9 are mosaic proteins constructed from smaller cysteine-rich domains like TSP1, LDLa, EGF, CCP, FIMAC and MACPF. The C8G individually is comprised of a lipocalin domain (Figure 1). Interestingly, 58, 45, 17, 18, 3 and 13 of the total of 64, 56, 31, 27, 3, 19 cysteines found in the C6, C7, C8A, C8B, C8G and C9 proteins are located in these functional protein domains, respectively. Each CCP contains four cysteines of which two are at the first and two at the last positions of the sequence. Six cysteines were found in each LDLa segment. In both human and pigs, the amino terminus of C6 has a tandem repetition of TSP1, while the carboxyl termini of C6 and C7 have a tandem of CCP and FIMAC. Each of the five terminal complement mosaic proteins has a MACPF motif in the central area of the sequence. According to Rosado et al. (2007), proteins containing MACPF domains play important roles in vertebrate immunity, embryonic development, and neural-cell migration. The MACPF domain structure is similar to pore-forming cholesterol-dependent cytolysins (CDCs) from gram-positive bacteria. These lytic MACPF proteins may use a CDC-like mechanism to form pores and disrupt cell membranes. The CCP modules mediate specific protein-protein and protein-carbohydrate interactions that are keys to the biological function of the regulators of complement activation and paradoxically, provide binding sites for numerous pathogens (O'Leary et al. 2004). The TSP1 conserved sequence motif has been suggested to play an essential role in mechanisms by which malaria parasites avoid host defences mediated by complement (Goundis and Reid 1988).

In addition to the haemolysis mechanism, cysteine-rich protein domains have another role in bind-

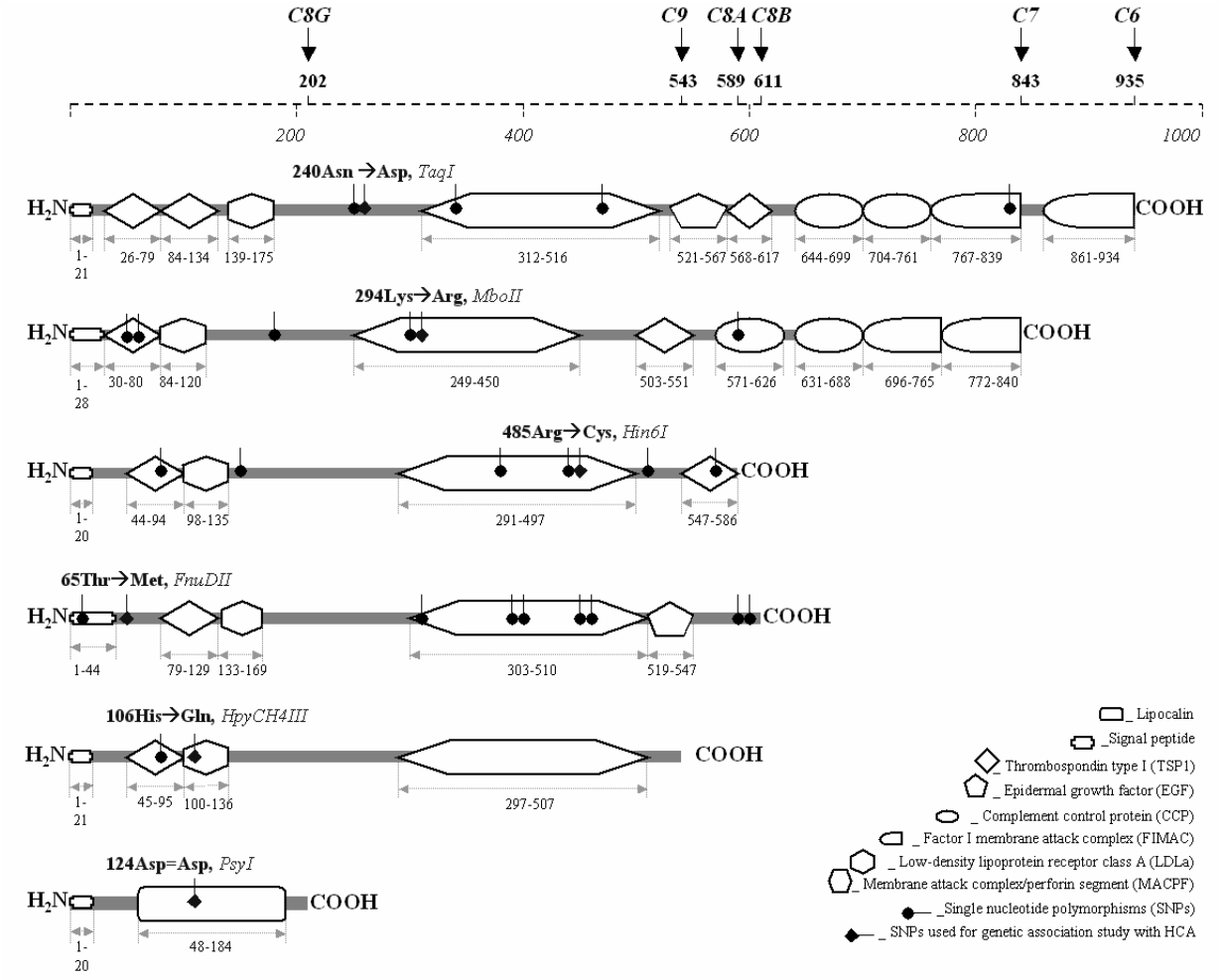

Figure 1. Contribution of cysteine-rich functional protein domains for structural construction of the C6 (DQ333199), C7 (AF162274), C8A (DQ333200), C8B (DQ333201), C8G (DQ333202) and C9 (DQ333198) due to using the SignalP 3.0 (Nielsen et al. 1997) and the SMART (Schultz et al. 1998). The name and length of each protein are discriminated by arrows in the first lines. Similarity among TSP1s, LDLas, EGFs, CCPs, FIMACs or MACPFs in the candidate genes is found in a range of 5–55%, 32–70%, 48%, 14–35%, 13–36% or 26–34%, respectively

ing proteins to each other. The C6 FIMAC module promotes the interaction of C6 with C5 enabling a more efficient bimolecular coupling ultimately leading to the formation of the C5b-6 complex (DiScipio et al. 1999) and binding of C7 FIMAC to the C345C domain in C5 is essential for incorporation of C7 into C5b-6 (Thai and Ogata 2005). The principal binding site for C9 lies within the MACPF domain of C8A (Slade et al. 2006), while the binding specificity between C8B and C8A-G subunits is determined by a cooperative interaction of the asparaginyl-terminal TSP1 module and MACPF (Musingarimi et al. 2002). Site-specific antibodies directed to the LDLa homology unit in C9 cross-reacted with C6 and C7 (Tschopp et al. 1986). The C8G containing a lipocalin module often binds to C8A to form a stable C8A-G complex that depends on the interchain disulfide bonds, although both genes are located on different chromosomes in both pig and human (Monahan and Sodetz 1980; Rao and Sodetz 1984; Dewald et al. 1996; Platteborze et al. 1996). For these reasons, the components of the membrane-attack complex (MAC) are physically formed in an associated arrangement C5b-C6-C7-C8B-C8A.G-C9. The MAC can be regarded as a protein macromolecule constructed from many smaller polypeptide sequences (C5b-9), which merge with each other due to interaction among their cysteine-rich homologous modules to form the membrane association. The

C8A and C8B genes have correspondingly similar roles in MAC-mediated lysis of erythrocytes and bacterial killing, and C8G is not required for the expression of C8 activity (Schreck et al. 1998) and for complement-mediated killing of gram-negative bacteria (Parker and Sodetz 2002). The pore-like characteristic appearance of the MAC is attributed to the association of as many as 12–18 C9 molecules that self-polymerise to form a circular structure in the membrane (Tschopp 1984). The individual components are hydrophilic proteins; however, when combined they form an amphipathic complex that is capable of binding to and disrupting local membrane organisation (McCloskey et al. 1989). The increase in membrane permeability leads to osmotic lysis of simple cells such as erythrocytes or the initiation of a variety of intracellular signalling events in the case of nucleated cells (Mold 1998). In bacteria, the MAC disrupts the outer membrane, thereby increasing permeability and inducing lethal changes in the inner membrane (Esser 1994). Regulation of the membrane-attack pathway is essential to protect host cells from damage at sites of complement activation (Morgan 1999). In most tissues, ubiquitously expressed complement regulatory proteins prevent autologous destruction, protecting host cells from the powerful cytolytic activity of activated complement (Scolding et al. 1998).

3. Chromosomal localisation

Probably at least a part due to homology in structure and function, the C6, C7, and C9 proteins have all been assigned to chromosome 16q1.4. Meanwhile, the C8A and C8B were physically mapped to chromosome 6q3.1 to q3.5 (Khoa 2010). The C8G was assigned to chromosome 1q2.13 (Khoa 2010), the location of several members of the lipocalin family, LCN1 for example. The lipocalin protein also plays an important role in the innate immune system and the acute phase response to infection (Flo et al. 2004). The location of either the C6-C7-C9 or C8A-C8B of other animal species (cattle, human, mouse, etc.) is also found to be close and in the same chromosome regions as in pigs.

4. Genetic variation

Screening the cDNA sequence of C6, C7, C8A, C8B, C8G, and C9 revealed 5, 6, 7, 9, 1 and 2 single nucleotide polymorphisms (SNPs) between European breeds (Hampshire, Duroc, German Landrace, Pietrain, Berlin Miniarture Pig) and the Vietnamese potbelly pig breed (Muong Khuong), in which 2, 3, 2, 8, 0, and 1 of each them have amino acid substitutions, respectively. Of note, there are three different alleles at nucleotide 935 of the C8B (A>G>T). All SNPs belong to the coding region of the candidate genes (Khoa 2010). Some of them are located in cysteine-rich functional protein domains such as TSP1, LDLa, MACPF, CCP, FIMAC, or lipocalin (Figure 1). These may play important roles in the structural formation and function of the protein domains and therefore may affect the activity of the complement components in cell lysis.

5. Association

In humans, the genes encoding the proteins of the MAC of the terminal lytic pathway (C5b-9) are required for complement bactericidal activity (Schreiber et al. 1979; Esser 1994). Here only the tendency of expression of C6–9 genotypes on HCA in the classical (CP) and alternative (AP) pathways is described. In the CP, HCA tended to increase on Day 4 after inoculations and then came back close to the innate value before vaccination on Day 10. The highest haemolytic value (from 74.13 ± 5.48 for C7 to 81.50 ± 4.87 for C6) was always reached on day 4 after immunisation with ADV vaccine.

Genetic association of C7, C8A, or C9 genotypes with CH50 was different at 95% significance level. Interaction between genotypes of the C8B and time points of inoculation was also found to be significantly different. There was no significant difference for C6 and C8G. Probably, the C6 and C8G have other roles in formation of the MAC. Actually, it has been suggested that the C8G may not contribute to lysis but rather enhances MAC activity (Schreck et al. 1998, 2000; Figure 2).

In the AP, HCA always decreased on Day 4 and came back to starting values on Day 10 after vaccination with Mh. After that, this activity showed a linear incremental increase throughout the experiment and reached the highest values either before the PRRSV vaccination for the C6 (75.51 ± 4.82), C8A (74.03 ± 3.20), C8B (73.00 ± 3.38), and C9 (72.18 ± 3.22) or after for the C7 (72.35 ± 6.07) and C8G (71.68 ± 3.28). The later time points always tended to have higher values than the early ones. Difference in genotypes of all candidate genes with HCA was

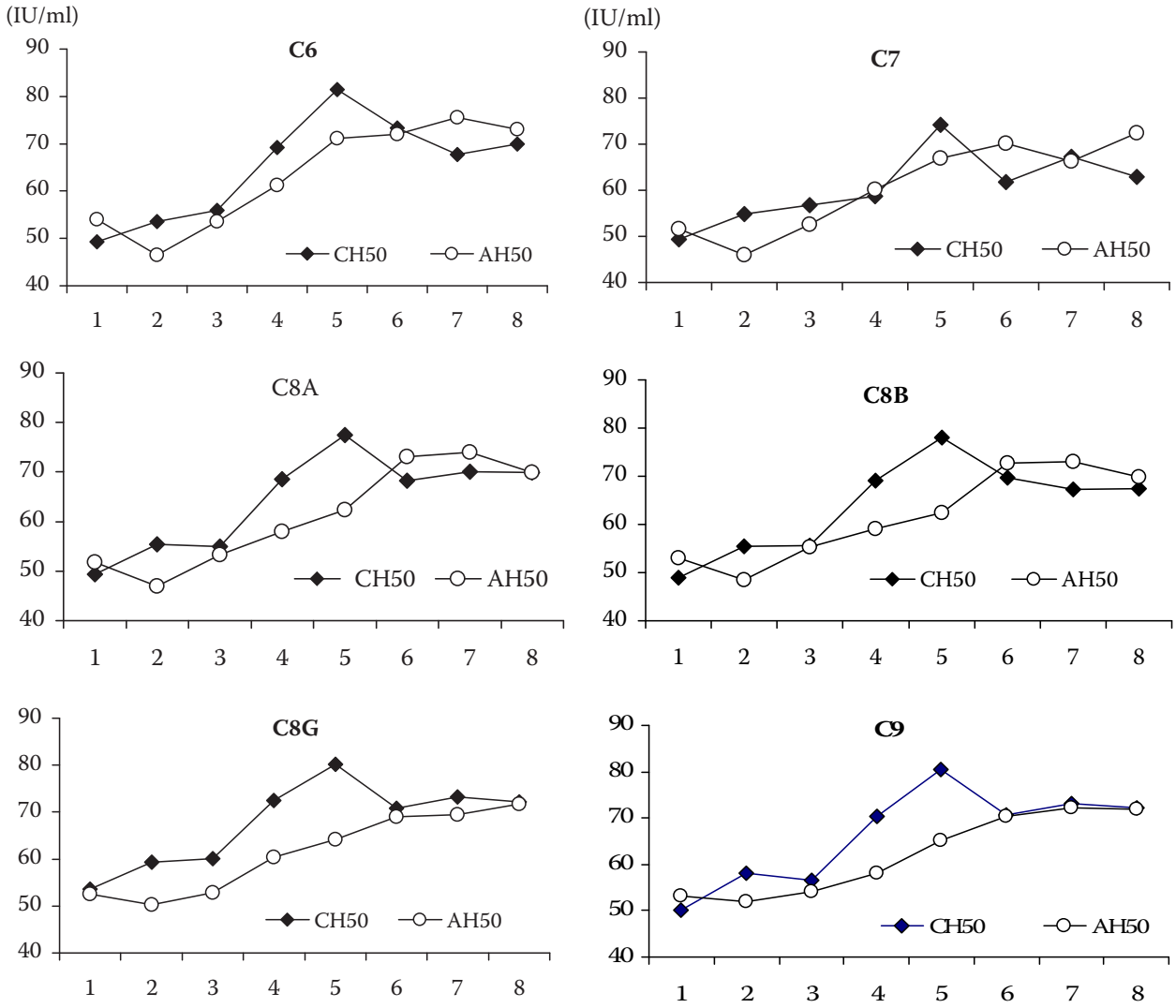

Figure 2. Expression tendency of hemolytic complement activity in the classical (CH50) and alternative (AH50) pathway at eight time points of inoculation (Khoa 2010)

not significant. However, in the interaction of genotypes and time points, a significant difference was found for C8A, C8B, and C9 ($P < 0.05$) (Figure 2).

Initiation of HCA before inoculation with Mh via the CP seemed to be weaker than the AP. But after that it increased faster and became stronger, especially on Day 4 after ADV vaccination. Perhaps the HCA of the CP must wait for the activation of several factors binding to the C1q, whereas the HCA of the AP can start with the little available free C3b proteins circulating in blood stream. The linear increasing tendency of the HCA in the AP was similar among the candidate genes, but with many fluctuations in the CP over the experiment, particularly after each inoculation. However, a highly significant difference was found between time points of inoculation in both pathways ($P < 0.001$). Since the CP is known as the major pathway of the HCA and is supported by lectin and AP, many additional factors, which contribute to complement activation of the CP, have been recognised. For example, the structure of the bacterial membrane, especially protein contents of lipopolysaccharide (LPS)-like lipid A (Morrison and Kline 1977), O-antigen (Merino et al. 1998; Eisenschenk et al. 1999), porin (Loos and Clas 1987; Alberti et al. 1996), kind of vaccine (Bredt et al. 1977), age (Yonemasu et al. 1978; Tyler et al. 1988; Durandy 2003), C-reactive protein (Jiang et al. 1991; Mold et al. 1999), adiponectin protein (Peake et al. 2008), and sex (Olaho-Mukani et al. 1995), acute phase response (Wimmers et al. 2003) are strong evidences for this.

The complement system is increasingly activated due to aging as well as accumulation after various vac-

cinations. The activities of the TCC (C6–9) depend on the activity of other components acting on higher levels of the cascade. Especially the formation of the C3 convertase and subsequently the C5 convertase are keys to the final overall HCA. The release of the C5b from the C5 that binds to the C6–9 to form the MAC is the cause of interaction of different protein sequences in the three pathways of complement activation. The HCA in the AP partly depends on the C3b formed after the complement activation of the CP. Generation of the C3-convertase has strong direct effects on the formation of the MAC. HCA in subsequent time points was higher than in early ones over the course of the experiment due to both antigen-specific and antigen-nonspecific immune responses and responsiveness that accumulated after each vaccination. According to Loos and Brunner (1979), the increase in complement components shortly after infection with *Mycoplasma* may represent an early unspecific defence mechanism of the host before the specific immune response becomes effective. Of course, complement activation also depends on other signals of the immune system like interleukins. Vaccinations are known to affect the acute phase response through cytokines like IL1, IL6 and TGFB1 that are released during the immune response and in particular trigger the expression of C3 (Castell et al. 1989; Mackiewicz et al. 1990; Gonzalez-Ramon et al. 2000).

Unlike other complement components with specific functions, C6–9 assembly has the same function in cell lysis because they are all members of the MAC macromolecule. This means that reciprocal influence among the TCC on HCA is indispensable. Catalytic activity of previous components may affect not only the subsequent others but the activity of the whole MAC complex as well. Determining the genetic variation of the TCC C6–9 on HCA in the CP and AP is complicated. It is quite indirect to use CH50 and AH50 for evaluating genetic effects of the terminal complement genes on HCA because direct products at the end of the complement activation from three pathways (classical, lectin, and alternative) are C5-convertases. However, measurement of the CH50 and AH50 has been the best screening test for deficiencies in the complement components of the CP, AP or terminal pathway so far. Besides the genotypes of candidate genes, a number of other factors that affect complement activity were evident in the experiment. These factors have been taken into account when analysing the use of adequate statistical models. Some of these factors may contribute to the fact that the association of the candidate genes was shown for the CH50 but not for the AH50. Therefore, HCA in the CP is stronger than in the AP.

6. Further prospects

Genetic variation had been demonstrated in response to pathogens or immune system challenges (Mallard et al. 1998; Wilkie and Mallard 1999; Henryon et al. 2002) and particularly in the response of pigs to infection with PRRSV (Petry et al. 2005). This study has identified valuable genetic variations between European (German Landrace and Pietrain) and Vietnamese (Muong Khuong) porcine breeds. The genotypes which performed the strong HCA also came from the Muong Khuong. These genotypes, which might have been selected through many generations of the raising of Muong Khuong pigs in bad conditions (no vaccination, insufficient feed and nutrient imbalance, poor hygiene, ...), can be considered as candidates for enhancing the natural resistance in pigs in the future. This may represent one of the common good characteristics of local Asian breeds because the Meishan breed also showed variations of complement activities higher than European breeds (Duroc, Landrace and Large White), as a result of natural selection of the host against infectious environmental conditions (Komatsu et al. 1998).

It is recommended that further studies should focus on the genetic association of candidate genes and immune traits to select valuable genotypes, which may then be assembled on multi-SNP-GeneChips and used to select the best individuals for nuclear herds in larger populations of different porcine breeds. The high similarity in molecular structure of the candidate genes among other species has opened a new way to treat animals and humans with deficiencies of complement components. Complement proteins can be extracted, purified, produced, and used as commercial medicines/DNA therapeutic products for disease control.

The results also illustrate that the performance of the AH50 gradually increases in a linear fashion, whereas that of the CH50 always varies depending on the vaccine program for all genes. Thus, the HCA in the CP may be improved via various activation routes to enhance the defence system of the host. If possible, ADV should be inoculated at earlier weeks of age to make a basic foundation for

boosting HCA along the vaccine program. Age is one of the important factors with regard to breeding and selection for resistance to diseases.

In summary, the obtained results provide the means for further understanding the role of the TCC in the natural immune response of the host against pathogens. It also highlights C6, C7, C8, and C9 as extremely valuable candidate genes in efforts to genetically improve general animal health, a goal of all breeding programs for food animals.

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