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Genetic and Antigenic Diversity of African Swine Fever Virus

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Highlights

- ASFV demonstrates extreme genetic diversity and use multiple mechanisms for acquisition novel phenotype characteristics
- Evolutionary rate of ASFV variable regions may approach similar values to RNA viruses
- More complete ASFV sequences are needed to better understand viral genome plasticity, antigenic diversity, and evolution
- Heamdsorbtion inhibition assay is a reliable tool in defining ASFV antigenic diversity and candidate vaccine efficiency

ABSTRACT

African swine fever virus (ASFV) is the only known DNA arbovirus, and the ability to replicate efficiently in both insect and mammalian cells is encoded in its viral genome. Despite having a relatively low overall genomic mutation rate, ASFV demonstrates genetic diversity in certain genes and complexity in gene content in other genomic regions, indicating that ASFV may exploit multiple mechanisms for diversification and acquire new phenotype characteristics. ASFV antigenic diversity is reflected in the ability to type cross-protective viruses together into serogroups, largely based on antibody-mediated inhibition of hemadsorption. Here we review ASFV genetic signatures of ASFV type specificity, genome variability, and the hemadsorption as a means of defining virus antigenic type, and how these may be used toward defining antigenic and phenotypic diversity that is problematic for development of vaccine solutions to ASF.

INTRODUCTION

African swine fever virus (ASFV) represents the sole member of *Asfarviridae* family (Alonso et al., 2018) and is unique despite sharing structural, genomic, and replicative characteristics with other nucleocytoplasmic large DNA viruses (NCLDV). ASFV productively replicates in ticks (*Ornithdorous genus*) and in pigs of the family *Suidae* and circulates between the two in natural sylvatic cycle of transmission in Africa. Despite several successful attempts still little is known about viral determinants responsible for fitness in different hosts (Burrage et al., 2004; Rowlands et al., 2009). In domestic pigs and wild boar (*Sus scrofa*), ASFV can be highly pathogenic and cause the hemorrhagic, highly lethal ASF disease.

ASFV causes explosive transboundary epidemics of ASF. In recent years, ASF has spread dramatically and now poses a threat to swine production worldwide (Cisek et al., 2016; Jurado et al., 2018; Kyyro et al., 2017; Nurmoja et al., 2017; Sanchez-Cordon et al., 2018; Vergne et al., 2017). Disturbing reports of ASF outbreaks in Europe, and most recently in South-East Asia, appear with increasing frequency (Bao et al., 2019; Garigliany et al., 2019; Le et al., 2019) . There currently is no safe and efficacious vaccine available for control of ASF. While previous studies have shown that protective immunity against ASF is achievable, issues of virulence, immunogenicity, and, importantly, viral phenotypic and antigenic diversity and lack of strain cross-protective immunity continue to confound ASF vaccinology as has been comprehensively reviewed (Arias et al., 2017; Revilla et al., 2018; Rock, 2016).

Key for understanding ASFV diversity, including the viral biology affected by it and evolution driving it, is understanding of ASFV genetic and genomic variation. Several specific genetic targets have been used to assess ASFV genetic diversity. The ASFV major capsid protein (p72) gene (*B646L*) was one of the first genetic targets used for large-scale assessment of ASFV genetic diversity (Bastos et al., 2003). Based on partial *B646L* gene sequencing, Bastos and colleagues identified twenty-two ASF genotypes and established the standard ASFV genotype marker. *B646L* genotype clustering allows relatively fast and easily typing of ASFV strains, and it remains the first method for identification of ASFV origin in case of introduction into the new territories. Unfortunately, p72 genotyping analysis does not always provide adequate typing resolution or ability to discriminate between viruses of different biological phenotype (Malogolovkin et al., 2015a). Increased genotypic resolution has been achieved by additional assessment of p54 (*E183L*), p30 (*CP205L*) and *B602L* genes (Bastos et al., 2004; Lubisi et al., 2007, 2005; Gallardo et al., 2009; Nix et al., 2006). Large-scale

molecular epidemiology studies using African ASFV isolates have revealed tremendous variety of ASFV variants across the African continent (Alkhamis et al., 2018; Fasina et al., 2010; Nix et al., 2006). Complementary data of ASFV genotyping and epidemiology data have been used to link ASFV genetic diversity with transmission cycle and number of hosts involved (Lubisi et al., 2005; Quembo et al., 2018; Thoromo et al., 2016). The most complex and contrasting blend of ASFV genotypes has been identified in East African countries where sylvatic cycle transmission takes place (Jori et al., 2013; Quembo et al., 2018). Contemporary studies suggest that additional ASFV genotypes likely exist (Achenbach et al., 2016) and that other genetic markers might be used for intragenotype classification (Gallardo et al., 2014). Recent advances in a whole genome sequencing can provide comprehensive genotyping and data critical for elucidating ASFV biology and diversity. While the number of ASFV genomes available in public databases has increased, genome sequences of additional historical and distinct ASFV isolates are still needed.

In this review, we summarize the outcomes of studies being focused on understanding of ASFV genetic and antigenic diversity, and discuss the questions that we think are most critical for unraveling ASFV diversity.

ASF GENES AND GENOMES

ASFV genomic organization resembles that of others in the NCLDV order. Currently sequenced ASFV genomes are variable in length, ranging in size from 170101 to 193886 base pairs. The ASFV linear genome structure, similar to other NCLDV, contains a relatively conserved, evolutionary stable, and centrally-located ‘core’ of replication-associated genes, whose functions are structural and/or enzymatic and reflect requirements of cytoplasmic replication of a complex DNA virus (Linda K. Dixon et al., 2012; Rodríguez et al., 2015). Many ASFV genes have been named based on homology to poxviruses (Dixon et al., 2013; Galindo and Alonso, 2017), and thorough

gene ontology of ASFV reference strain BA71V and relative to other strains has been presented (Chapman et al., 2008; Rodríguez et al., 2015; Yáñez et al., 1995). Interestingly, functions of many ASFV core genes are still unknown. Notably, within the core region are located “hot-point” variable genes and intergenic regions (e.g. central variable region, or CVR) currently used for ASFV intragenotyping, which complements standard p72 genotyping with additional, high-resolution data (Gallardo et al., 2014; Phologane et al., 2005). Comparative genomic analyses have also identified a range of individual genes, distributed both inside and outside the genomic core, as undergoing positive selection and/or as the most variable gene orthologues in ASFV (i.e. CD2v/EP402R and C-type lectin/EP153R - ultimately representing a source of genetic diversity among ASFV isolates (Chapman et al., 2011; de Villiers et al., 2010). Interestingly, CD2v/EP402R was also found to be essential for virus replication in ticks (Rowlands et al., 2009).

Terminally-located regions of the ASFV linear genome are more variable in size and gene content than the core region, and are referred to as left and right variable regions (LVR and RVR). ASFV terminal genomic regions are predominated by multigene families (MGF) of paralogous genes. Five MGFs (MGF100, MGF110, MGF300, MGF360, MGF505) are named based on their average amino acid length and likely derived by gene duplication and diversification, and have variable compliments in different ASFV strains (Almendral et al., 1990;Chapman et al., 2008; Dixon et al., 2013; González et al., 1990; Pires et al., 1997; Yozawa et al., 1994). Intriguingly, MGFs do not share similarity to other known genes.

Large MGF-region deletions region have been identified in some laboratory adapted ASFV strains and field isolates. Progressive adaptation of ASFV strain Georgia/2007 to the Vero cell line has led to virus attenuation and acquisition of numerous mutations in different part of virus genome, including long stretch of nucleotide deletions observed in

MGF505 (Krug et al., 2015). Even though the attenuated, cell-passaged Georgia/2007 virus demonstrated an attenuated phenotype, it also has lost immunogenic potential and did not protect pigs against homologous challenge (Krug et al., 2015). Similar patterns of ASFV MGF flexibility have been found in other cell culture adapted ASFV strains (Chapman et al., 2008; Tabarés et al., 1987). In a subsequent study using functional genomics approach, several groups have demonstrated virulence functions associated with MGF360 and MGF505 are one of the virus virulence factors and might be used for future vaccine design(O'Donnell et al., 2016, 2015; Reis et al., 2016). The significance of MGF360/530 region as a host range determinant has been demonstrated by deletion of six MGF360 and two MGF530 genes from ASFV Pr4 which gradually reduced viral replication in infected ticks up to 1,000-fold (Burrage et al., 2004). MGF110 diversity has been noticed in ASFV field isolates and cell culture adapted viruses (Chapman et al, 2008; Almendral et al. 1990; de la Vega et al. 1990), including recently in ASFV isolate Estonia/2014 where 12 out of 14 MGF110 genes were part of a large LVR deletion associated with an attenuation phenotype (Zani et al., 2018).

The continental epidemics derived from the original Georgia 2007 outbreak have provided a view of multi-year, large-scale spread of a clonal ASFV outbreak strain - how it evolves and the diversity generated in defined geographic and temporal scales. Since the first notification of ASFV in Eastern Europe, the number of available ASFV sequences has significantly increased. ASFV gene sequences from Estonia, Poland, Latvia, Lithuania showed high stability of ASFV genome and >99% homology to their parental ASFV/Georgia/2007/wb (FR682468). Nevertheless, multiple single nucleotide changes have been identified in current EU ASFV isolates in different genes and intergenic regions, including some which predict frameshift and truncation of translated proteins (Fraczyk et al., 2016; Gallardo et al., 2018; Śmietanka et al., 2016). Biological significance of these small scale changes remains to be fully understood.

Interestingly, while ASFV genomic GC content is on average approximately 38% for most strains, though areas with relatively low GC content are located in the LVR and RVR and in select central regions (Figure 1). The decreases in GC content may have significant role in virus evolution and possibly being a consequence of the higher biochemical costs of GC base synthesis as it has been shown for other organisms (Šmarda et al., 2014).

Despite of a long history of ASFV research and devastating transboundary potential, the number of publicly available ASFV full genome sequences is limited. GenBank contains 42 complete ASFV genome sequences, and of these only 33 field strains. More complete ASFV sequences are needed to better understand viral genome plasticity, antigenic diversity, and evolution.

The origin of ASFV, as for other viruses, is not clear. Elegant hypotheses as to the natural history of large DNA viruses have been proposed in seminal works by Koonin and Krupovic (Koonin et al., 2015; Krupovic et al., 2019). Data to suggest specific origins of ASFV are limited. Even though ASFV shares common features of genomic architecture with other NCLDV and members of proposed order *Megavirales*, genetic distance and the number of ASFV-specific genes (no orthologues identified) has resulted in ASFV being classified as the single member of family *Asfarviridae* (Andreani et al., 2017). The paucity of characterized ASFV near-neighbors identified in nature makes conjecture on ASFV origins difficult. Recent characterization of environmental giant virus NCLDVs which infect protists indicated potential distant monophyly with ASFV relative to other NCLDVs (Klose et al., 2016), consistent with reports of ASFV-like sequences reported in freshwater environments (Wan et al., 2013). Still other ASFV-like sequences have been also identified in human samples and sewage (Loh et al., 2009) and in marine environments (Ogata et al., 2009), and with potentially more in new metagenomic datasets (Kuhn et al., 2019). Preliminary results of RNA and DNA

sequencing of *Ornithodoros moubata* cell lines have revealed 26 kb ASFV-like sequences in tick genomes (Forth et al., 2017). We anticipate discovery of additional ASFV-like sequences in genomic and metagenomic data from ASFV vectors and wildlife hosts, and addition of more leaves to the *Asfarviridae* phylogenetic tree. It is conceivable that yet-to-be-discovered near-neighbors or novel, divergent variants of ASFV will yield insights toward ASFV evolution and resulting diversity.

While our knowledge of ASFV evolution is incomplete, ASFV is likely to utilize a variety of mechanisms for generating diversity, including single nucleotide mutations, insertion/deletions, gene duplication, and recombination (Dixon et al., 2013). In general, the mutation rate of DNA viruses is lower than RNA viruses and ranges between 10^{-8} – 10^{-6} substitutions per nucleotide per cell infection (Sanjuan et al., 2010), though researchers agree that evolutionary rate for double-stranded DNA is difficult to estimate (Firth et al., 2010). The evolutionary rate for select ASFV gene regions (*B646L*, *CP204L* and *B602L*/CVR) genes have been estimated using Bayesian statistics and may approach those of RNA viruses ($6.9. \times 10^{-4}$ /site/year for *B646L*) and $6{,}6 \times 10^{-4}$ (*CP204L*) (Michaud et al., 2013). Longitudinal study of ASFV isolates from East Africa has demonstrated increase in small-scale, tandem repeat sequence (TRS) number in the *B602L* gene (Lubisi et al., 2007, 2005). In the ASFV strain currently causing Eurasian epidemics, TRSs were noted in intergenic region I73R/I329L among Caucasus, Russian, and European ASFV isolates (Gallardo et al., 2014; Goller et al., 2015; Kolbasov et al., 2017). Many others accumulated mutations were noted in different genes in European and Chinese ASF isolates from 2014-2018 (Bao et al., 2019; Cisek et al., 2016; Gallardo et al., 2018; Garigliany et al., 2019; Ge et al., 2018; Smietanka et al., 2016; Zhou et al., 2018). The biological significance and association between these small-scale nucleotide mutations and ASFV phenotype often are unclear.

In contrast to single nucleotide mutations, variation in gene content can more readily explain profound changes in virus phenotype (Zani et al., 2018). ASFV multigene families (MGF) of paralogous genes are the most flexible genetic components, undergoing dramatic variation in gene content relative to the rest of the genome and demonstrating gene duplication, sequence divergence, gene deletion, and recombination which can greatly affect ASFV genome structure (De La Vega et al., 1990; Yáñez et al., 1995). ASFV MGF gene duplication conceivably is an outcome of the strand slippage mechanism proposed for vaccinia virus (Coulson and Upton, 2011), and may ultimately provide flexibility in viral adaptation to changing hosts or host defenses. ASFV strains harboring multiple copies of MGF have larger genomes (Figure 1) and are often associated with a more virulent phenotype, especially when attenuating loss of MGF occurs through viral passage in *in vitro* cell culture (Chapman et al., 2008; Krug et al., 2015; Rodríguez et al., 2015).

Recombination, described as a major driver of evolutionary change (Barton, 2010), is suspected to occur between strains of ASFV but has yet to be clearly demonstrated. While recombination events have been identified between homologous strains of vaccinia virus during mixed infection *in vitro* (Qin and Evans, 2014), similar has yet to be shown experimentally for ASFV. Phylogenetic reconstructions have indicated ASFV recombination events in MGF, *E183L, B602L, EP153R* and *EP402R* (CD2v) genes (Chapman et al., 2008; Michaud et al., 2013; Nefedeva M. et al., unpublished). How potential recombination contributes to ASFV diversity or confounds its analysis requires further assessment. Together, these evolutionary processes for generating diversity are of great interest as they drives changes in specific genes and encoded antigens and potentially impact vaccination strategies or stability of live-attenuated vaccines.

ANTIGENIC DIVERSITY SEROGROUPS

ASFV antigenic diversity has long been a prominent finding and confounding factor for

disease control. In the seminal studies by Malmquist and Hay, different antigenic virus types were observed (Malmquist, 1963, 1962; Malmquist and Hay, 1960). Key in identifying these different virus types were two features of ASFV: 1) stimulation of type-specific (homologous) acquired immune response in surviving animals that fails to protect against a secondary infection caused by a second (heterologous) viral type, and 2) induction of a hemadsorption (HA) phenotype in infected cells. Using an HA inhibition assay (HAI) assay to assess serological cross-reactivity with different ASFV isolates *in vitro*, Malmquist first identified several ASFV antigenic types (Malmquist and Hay, 1960). After this finding, a key question remains the following: what are the viral determinants which define the antigenic type? More than 20 years after initial findings, Plowright wrote “It is also remarkable that it is still an unexplained phenomenon (HA), poorly characterized and arousing little interest” (Plowright, 1986).

HAI as a tool for defining serotype specificity was used extensively at the Pokrov Institute (currently Federal Research Center for Virology and Microbiology), Russia. An HAI-based ASFV classification was developed to differentiate virus antigenic types (serogroup). Eight ASFV serogroups (SG) were defined and thoroughly characterized; however, more SG may exist. Additional parameters such as HA density (number of erythrocytes per infected cells) and erythrocyte contact maps were used to refine ASFV isolate classification (Makarov et al., 2016; Sereda et al., 1994). The HAI based assay was used for ASFV strains selection and screening for development a potential live-attenuated vaccine candidates (Sereda et al., 1992; Sereda and Balyshev, 2011). Surprisingly, the key significance of HAI classification was discovered even earlier again by Malmquist W.A., who used the HAI assay for ASFV grouping (homologous vs heterologous). According to his results, sera from convalescent pigs could inhibit HA in macrophages infected with homologous ASFV isolate. The key aspects of hemadsorption inhibition are depicted in Figure 2.

Viral determinants responsible for mediating HA were discovered by Rodriguez and colleagues in 1993, when the ASFV-encoded homologue of cellular CD2 (CD2v protein, *EP402R* gene) was described as a major candidate responsible for HA (Rodríguez et al., 1993). In subsequent studies, ASFV C-type lectin like protein (EP153R gene) was also suggested to be an auxiliary HA antigen (Galindo et al., 2000). Comparative genomic work has identified these genes as among the most diverse between discrete strains of ASFV (Chapman et al., 2008; de Villiers et al 2010). Phylogenetic analysis of more than 80 ASFV strains correlated CD2v/C-type lectin genotypic grouping with HAI-based serological grouping, supporting association of CD2v/C-type lectin with protective immune responses and indicating additional potential serogroups which have yet to be characterized serologically (Malogolovkin et al., 2015b). Furthermore, chimeric swap of CD2v/C-type lectin genes between ASFV induces commensurate swap of *in vitro* HAI and *in vivo* cross-protective phenotypes.

Thus after nearly 60 years since Malmquist's discovery, the ASFV CD2v/C-type lectin protein has been correlated with HAI serospecificity, with type-specific protective immunity *in vivo* and cellular immunity *in vitro*, and thus has been proposed as a serotype-specific ASFV antigen (Burmakina et al., 2019, 2016; Malogolovkin et al., 2015b). Still, CD2v and C-type lectin, while important for mediating cross-protective responses *in vivo*, are not sufficient in confiring complete serotype-specific homologous protection, indicating that additional serotype-specific protective antigens remain to be identified.

Indeed, experimental subunit vaccine formulations utilizing or expressing ASFV proteins p30, p54, and CD2v have demonstrated efficacy in inducing partial protection against virulent challenge; however, their roles in contributing to serotype-specific responses is unclear (Argilaguet et al., 2013; Barderas et al., 2001; Gómez-Puertas et al., 1998; Ruiz-Gonzalvo et al., 1996).

HAI mediated by antibodies from recovered animals remains a valuable tool for study of antigenic diversity and protective potential of ASFV vaccines. In several studies, Ruiz-Gonzalvo and colleagues demonstrated that the inhibition potential of sera from ASFV recovered pigs depended on the serum activity, virus dose and virus type (homologous or heterologous) (Rodríguez et al., 1994; Ruiz Gonzalvo et al., 1986a, 1986b). Notably, results from HAI classification and infection-inhibition assays are independent of results from "classic" virus neutralization, indicating a novel mechanism of affecting protection *in vivo*. Here we will not address the controversial role of ASFV neutralizing antibodies, an elegant review on antibody-mediated neutralization of ASFV has been published by Escribano with colleagues (Escribano et al., 2013).

ASFV antigenic diversity is a key component which hampers universal ASFV vaccine development. Homologous protection has been demonstrated for multiple ASFV strains and isolates. However, heterologous ASFV challenge still presents a challenge for immune responses generated against a single virus serotype, confounding broad cross-protective immunity (King et al., 2011; Rock, 2016; Sanchez-Cordon et al., 2017). Recent study has indicated cross-protective heterologous potential for ASFV live attenuated vaccines (ASFV strain BA71 lacking CD2v) against ASFV Georgia/2007 (Monteagudo et al., 2017). Another example of cross-protection was demonstrated using naturally attenuated ASFV NH/P68 and ASFV Armenia/07 as a challenge virus (Gallardo et al., 2018)

Contemporary advances in proteomics research have demonstrated the complexity and flexibility of ASFV proteome (Alejo et al., 2018) and has led to discovery of several novel ASFV proteins (Kessler et al., 2018). The ASFV proteome is a dynamic entity tightly regulated by virus and cell machinery, and thus may substantially vary depending on virus strain and the host cells in which they replicated. Proteomics is also now indicating diverse effects of live-attenuated ASFV strains on the host proteome relative

to a virulent counterpart. Herera-Uribe with coauthors have discovered that inflammatory and immunological pathways are severely altered by live-attenuated ASFV E75CV1, but not by virulent E75 (Herrera-Uribe et al., 2018). More recent collaborative work has indicated the particular alteration of cGAS-STING pathway in infected macrophages by virulent ASFV Armenia/07, but not attenuated ASFV NH/P68 (Garcia-Belmonte et al., 2019). Thus, ASFV genomic and/or antigenic diversity also affects diversity in host response – further study of ASFV-host interactome hold promise for correlating viral genotypic differences with phenotypic diversity.

Anti-ASFV immunity is a type-specific and likely correlates with the level of serogroup-specific antibodies. A preliminary result of concern with regards to ASFV diversity is induction of more severe, or exacerbated, disease in immune animals after subsequent infection by a heterologous ASFV strain. A similar phenomenon has been recognized for flaviviruses, when previous immunity may induce more severe disease pathogenesis through a mechanism of antibody-dependent enhancement (ADE) (Khandia et al., 2018; Li et al., 2018). While pre-existing ASFV immunity may shape the T-cell and antibody responses and the constraints are poorly understood so far, ADE is primarily caused by cross-reactive antibodies with low neutralization capacity. Non-neutralizing antibodies bind to virus surface antigens and expose its Fc-fragment to the Fcγ receptor bearing cells (mostly dendritic cells, NK cell and macrophages) (Nimmerjahn and Lux, 2014). The antibody-virus complex is an easy target for macrophages, where the virus efficiently replicates after entry. Whether the ADE mechanism takes place in ASFV pathogenesis is largely unknown, but it may prove to be of importance in the future when considering how to address the issue of ASFV antigenic diversity.

CONCLUSIONS

ASFV is a unique virus within *Asfarviridae* family. Almost a century since the first report of African swine fever by Montgomery in 1921, and the disease is threatening the world swine population more than ever before. ASFV diversity and complexity are the key factors which hinder vaccine development. Understanding the diversity among ASFV genomes from different hosts will help to reveal new aspects of ASFV evolution and determinants conferring antigenic and phenotypic variation – additional sequencing of historical and distinct ASFV strains genome sequences should aid in these efforts. We and others have made considerable strides in defining ASFV antigenic groups. ASFV CD2v based hemadsorbtion and comparative genomics has provided a robust means of identifying genetic signatures associated with viral antigenic phenotypic and cross-protective immunity. Identification of additional serotype-specific protective antigens is likely necessary to design of specific and efficacious vaccine formulations.

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Figure captions

Figure 1 Genomic characteristic of ASFV genomes. A) Relative GC content (%) of ASFV/Georgia/2007/wb (FR682468). Low GC content areas marked in blue, high GC content areas marked in red. The genes in low GC content areas in the central part of the genome are listed. B) Comparative analysis of ASFV genomes which are publicly available from the GenBank. The graph shows the relationship of ASFV genome length with its GC content.

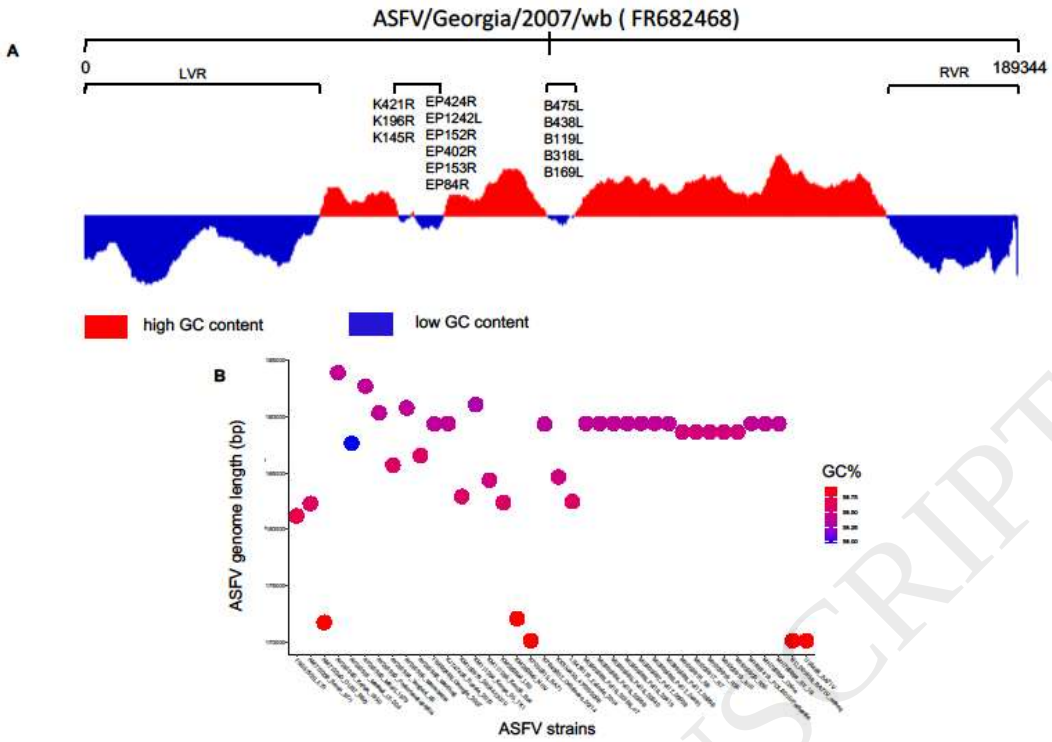

Figure 2 The principle of ASFV hemadsorption (HA) and hemadsorption inhibition (HAI). The macrophage infected with ASFV is surrounded by red blood cells (hemadsorption). The serum from recovered pigs contains antibodies which may inhibit hemadsorption (HAI) caused by homologous ASFV strains (same serogroup). The macrophage infected by heterologous ASFV strain (different serogroups) will demonstrate “classical” HA picture regardless the presence of antibodies against heterologous ASFV serogroup.