Review Article

African swine fever virus: A review on its heterogeneity, immunomodulatory property and its extent of virulence

ABSTRACT

African swine fever virus (ASFV) is a causative agent of a lethal haemorrhagic disease in domestic pigs *(Sus scrofa domesticus).* Due to ASFV extensive range of pathogenicity, the World Organisation for Animal Health [Office International des epizooties- (OIE)] classified the infection as being per-acute, acute, sub-acute and chronic. It has a highest mortality rate of 100% at per-acute infection, causing deaths within 4 days. ASF have no vaccine available but can only be contained by strict quarantine and culling procedures. The virus is highly stable with direct transmission through infected swines, and indirectly from soft ticks (*Ornithodoros)*.

ASFV, display a genetic heterogeneity that complexes its mechanism of invading the host immune system. The host macrophage permissive (property to infect macrophage) is due to the dynamic mechanical characteristic of the ASFV virulence proteins. Along with the on-going discovery of a clinical vaccine, evaluation and deciphering of the proper innate and cellular response using wild type ASFV challenges against live attenuated (ASFV) or subunits of ASFV antigen has been the easier strategy apart from Porcine macrophage cell infection in culture. It is due to the invasive and regulatory property possessed by both ASFV essential and non-essential genes that are capable in misregulation and immunomodulation of the immune system that often results in expression of pro and anti-inflammatory cytokines accompanied by haemorrhagic deaths in pigs. Therefore, the need of understanding extent of ASFV virulence machinery, the various immune effectors it evokes and its heterogeneity of infection that contributes to different clinical manifestations are important parameters and steps towards the design of an effective vaccine and therapeutics. This review focus on the roles of major structural and functional ASFV components in host evasion, immunomodulatory potential mechanism along with immunisation experimental evidence for the purpose of diagnosis and prevention.

Keywords: ASFV, Genetic Heterogeneity, Macrophage permissive, Immunomodulation .

1. INTRODUCTION

African Swine Fever (ASF) is an arthropod-borne infection, where its epidemiological data describes it as a sylvatic association of infection between soft ticks (*Ornithodorus moubata)* and its natural persistent host, warthogs *(Phacochoerus africanus)* [1] . However, of all the infected populations, domestic pigs (*Sus scrofus domesticus*) have been greatly affected and devastated by ASFV infection. It's first outbreak was in the year 1907, when it was thereafter described as ASFV on the year 1921 in Kenya [2]. The African nations in the early 20^{th} century had seen a long term susceptibility of domestic pigs to ASFV, where it had been highly endemic. Surprisingly by the onset of 1990's, various parts of Europe, Russia,

the Caribbean and South America have also been threatened with this virus [3]. ASFV first transcontinental drift to Europe was in 1957 was in Portugal with the ASFV genotype I, which alarmed other European nations, however it was strictly controlled and eliminated. By the mid-1990s, a known ASFV Genotype-I had been eliminated across many countries apart from Africa, with the exceptions of an isolated outbreak on 1999 in Portugal and island of Sardinia, where it remained endemic [4]. Since then, mortality rates have decreased and sub- clinical or chronic ASFV infections have become more frequent.
After a few years, ASFV re-emerged and unfurled as a transcontinental genotype, being more than contagious, it was more lethal in causing acute ASF infection, describing it as an ASFV Genotype II in Georgia, 2007 [5]. It was progue to have been introduced during the transport of domestic pigs along with left over infected swills containing survived pathogens, near the port of Poti [6]. The ASFV Genoype-II crossed countries affecting various parts of Russia, Eastern Europe and extending severe outbreaks of swine deaths and by 2013, almost all the southern regions of the Russian Federation (RF) had fallen into the epidemic [7]. The recent major report on ASFV genotype II is pronounced to be a transboundary and 2nd transcontinental drift from Europe to Asia with the virus emergence in China in 2018 [8, 9]. Since then it began to lay out various parts of the North Eastern regions of India [10] and parts of Southeast Asian countries such as Vietnam, Laos and Philippines threatening the pig's population in various continents and sub-continents.

2. MICROEVOLUTION AND GENETIC HETEROGENEITY OF ASFV

ASFV belongs to the genus *Asfivirae* of the family, *Asfaviridae* comprising of a linear ds-DNA, having genomic size of about 170 to 190 k-bp and encoding more than 150 open reading frames (ORFs), varying between different geographical isolates of ASFV [11]. At present, there are 24 different genotypes of ASFV based on the *B646L* gene, a major capsid protein p72 [12, 13]. Genotyping based methods for virus characterisation using distinct genetic markers has been used for differentiating closely related strains and deducing their phylogenetic relationship.

The gold standard in the molecular characterisation of ASFV have been in concordant with the complete nucleotide sequence of B646L or its partial 478 bp variable region located in the C-terminus of the ASFV major capsid p72 gene [14]. Other genetic markers for generating phylogenetic relationship of closely related ASFV genotype isolated from Europe and Africa was the amino-acid tandem repeat sequences (TRSs) located within the CVR (Central Variable Region) of B602L and p54 encoded by the gene E183L [15]. The CP204L encoding p30 protein collected for over 13-years period from Sardinia confirms a remarkable genetic stability is an ideal genetic marker for molecular characterisation [16]. However, the current genotyping procedures cannot differentiate ASFV from the pool of geographical virulent isolates, hence determination of ASFV virulence still rely on the hemadsorption (HAD) a serological test, a characteristics of the functional ASFV CD2v, a haemagglutinin (HA) encoded by the gene EP402R displaying the HAD phenotype, has been precisely used to detect ASFV virulence [17]. The CD2v gene EP402R has also been used for the purpose of molecular characterisation of ASFV. A study during an outbreak in Vietnam placed the ASFV gene EP402R for evaluating genetic relatedness with Eastern European and Chinese strains [18].

Clinically, ASFV virulence has been relying on clinical signs and symptoms. ASFV genetic heterogeneity that contributes towards virulence had also been assessed. The high heterogeneity in ASFV are due to the presence of the multigene families MGFs 100, 110, 300, 360, 505/530 , which reported prominent variation within its genetic locus such as a deletion, addition or an alteration of nucleotides sequences [19]. Another high difference may be explained by the transovarial evolution of ASFV isolates obtained from native ticks and domestic pigs from Portugal and Africa [20]. As a result, such alterations in the ASFV genome led to the emergence of naturally attenuated strains characterised with low virulence. Some reported ASFV non-virulent isolates also corresponded with a mutation or the deletion of the MGFs.

Besides, from the variations in MGF family, which are the usual genetic hot spots of ASFV microevolution, recently in China a new ASFV variant China/GD/2019 was due to certain deletions and mutations on the D1133 gene of China/GD/2018 which resulted with frame mutation [21]. Another aspect in ASFV variations is the number of tandem repeat sequences (TRS's) present in the genic region of p72(VP3) near the C terminal. The inter-genic region between the I73R and I329L genes had also been utilized in comparing other ASFV with Eastern Europe ASFV isolates [22]. Promotion of homologous recombination or unequal chromosomal crossover during DNA replication could also contribute towards genetic heterogeneity in ASFV [23]. Henceforth, since the emergence of transboundary Genotype II in China, there is a need for establishment of additional sub-genetic marker of ASFV Genotype II to determine with higher resolution the origin of a new ASF incursion and to trace the evolution of closely related ASFV isolates, especially with the abundance of pig farms in the region.

3. PATHOLOGICAL SIGNS OF ASFV

ASFV exist clinically from highly lethal pathogenic strains that may kill the entire herd in a pigsty to lesser virulent isolates that cause a milder, asymptomatic African swine fever. ASFV requires strict laboratory diagnosis as per OIE directions, while it shares similar clinical signs like the Common swine fever virus (CSFV). Different clinical manifestations of ASF have been observed in domestic pigs, historically from Spain and Portugal [24]. Disease manifestations may include peracute, acute, subacute and chronic forms **Table1**. In the peracute form, pigs die within 1-3 days characterized by a very rapid clinical course, with high fever (up to 42°C), anorexia, hyperpnoea, and sometimes sudden death without signs of disease.

Table 1. Clinical signs of ASFV

Disease Manifestations	Peracute (highly virulent)	Acute (highly virulent)	Sub-acute (moderately virulent)	Chronic (moderately to low virulent)
Mortality	3-4 dpi (100%)	6-21 dpi (90% -100%)	15-45dpi (30%-70%)	≤30%
Clinical Lesions	No Clinical signs or lesions	Pigs develop cyanotic skin along the ear, snout legs, abdomen and perianal area etc.	Cyanotic skin may appear; similar for acute infection	Multifocal necrosis in the skin and arthritis;
Fever	41^0 C - 42^0 C	41^0 C -42^0 C	41^0 C -42^0 C	41^0 C -42^0 C
Skin	Erythema	Erythema	Erythema	Necroptic areas
Spleen	-	Hyperaemic splenomegaly	Partial hyperaemic splenomegaly or focal infarction	Enlarged with normal colour
Gall Bladder	-	Petechial Haemorrhages	Wall Oedema	-
Heart	-	Haemorrhages in epicardium and endocardium	Haemorrhages in epicardium and endocardium; hydropericardium	hydropericardium Fibrinous pericarditis
Tonsils	-	-	-	Necrotic Foci
Reproductive Alteration	-	Abortion may occur on pregnant sows	Abortion may occur on pregnant sows	Abortion may occur on pregnant sows

Acute cases are characterized by a high fever, anorexia, lethargy, weakness and recumbency. Pigs may also experience diarrhea, constipation or vomiting and/or display signs of abdominal pain. Erythema or

hyperemia, including epistaxis and hemorrhages in the skin and the limb region are usually observed. Severe leukopenia and thrombocytopenia may occur.

Sub-acute African swine fever is similar to acute infections, but with less severe clinical signs. Pigs in the chronic form have nonspecific signs such as an intermittent low fever, appetite loss and depression. Coughing is common, along with diarrhoea and occasional vomiting been frequently reported. Ulcers and reddened or raised necrotic skin foci may appear over body protrusions and other areas subject to trauma.

ASF results in a massive destruction of the lymphoid organs and tissues, including spleen, lymph nodes, thymus, and tonsils. There is a large proportion of B and T lymphocytes and macrophages undergoing cell death in acute ASFV infection [25]**.** ASF is observed with stimulation of pro-inflammatory cytokines [26] and upregulation in the expression of IL-1, TNF-α, and IL-6, abrogates "cytokine storm" [27] which is the responsible for the massive induction of apoptosis in lymphocytes neighbouring the activated/infected monocyte-macrophages in tissues.

4. GENERAL STRUCTURAL ARCHITECTURE OF ASFV

ASFV encodes for at least 150-170 ORF's coding for essential and non-essential genes, with around 38 of 100 ORF's being characterised as having regulatory functions as in nucleotide metabolism, transcription, replication and repair. It is coated with an outer envelope with a morphological feature of being spherical to pleomorphic, exhibiting a slightly icosahedral symmetry. The proteomics analysis identified 68 structural viral proteins with at least 16 structural ASFV proteins involved in virion assembly, morphogenesis and host cell interactions **Figure 1** [28, 29].

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Figure1. Structural Architecture of ASFV: External envelope, capsid, core-shell and inner core.

ASFV major and minor proteins comprises the p72, pp220, pp62, p54, p30, p12, p17, p49, p10, p22 and CD2v **Table:2**. are associated viral component that contributes to primary roles of the virion in interacting with the host [30]. Being non-essential for growth and replication, they have certain roles in productive entry and exhibit a macrophage permissive function.

ASFV consist of four layers of protein shells and an inner genome. Surrounded by the large external envelop is the ASFV major virion capsid protein p72, encoded by the B646L gene [31], and its assembly in virions is assisted by a 45.3 kDa chaperone protein encoded by B602L [32]. Another enveloped viral

Table 2:- Non-Essential ASFV structural proteins components.

Protein's name	Gene name	Predicted protein size (kDa)	Protein's function
p11.5	A137R	21.1	Involved in virus attachment
p10	K78R	8.4	Involved in virus attachment
p72	B646L	73.2	Involved in virus attachment
pp220	CP2475L	281.5	Polyprotein precursor of p150, p37, p14, and p34; required for packaging of nucleoprotein core and assembly
p32(p30)	CP204L	23.6	Phosphorylated and antigenic protein, involved in virus entry
p54 (j13L)	E183L	19.9	Binds to LC8 chain of dynein, involved in virus entry; required for recruitment of envelope precursors to the factory
pp62 (p60)	CP530R	60.5	Polyprotein precursor of p35 and p15
CD2v (PEP402R)	EP402R	45.3	Responsible for viral haemadsorption in red blood cells;; viral penetration and entry, CD2 homologue and immunoevasion
p12	P061R	6.6	Involved in virus attachment
p22	KP177R	20.1	Involved in virus attachment
p17	D117L	13.1	Stabilization of capsid components
p49	B438L	49.3	Virus attachment and capsid formation

protein p14.5 encoded by E120R is a late phase viral protein found to have role in transferring virion particles to plasma membrane. The inner core shell is constituted by core shell precursor polyproteins pp-220 and pp-62 encoded by the genes CP2475L and CP530R. The pp-220 is cleaved to yield major proteins p150, p37, p14, p34 and the pp-62 forms p8, p15 and p35, by a virus-encoded SUMO-like protease (S273R). pp 220 is actually a mid- late expressed proteins responsible for the formation of the inner core shell and packaging of viral nucleoprotein supporting early replication; may serve important functions in the assembly of virions and viral infection [34].

p10 encoded by the gene K78R contains highly basic amino acid residues in the primary structure of a polypeptide, predicted as NLS (nuclear localisation signals) was found to be highly accumulated in the host cell nucleus during ASFV infection and adsorption with strong implications in functions aided for transport [35]. The p54 encoded by (E183L) is a 25-kDa polypeptide encoded by the E183L gene that contains a putative transmembrane domain is reported to mediate specific interactions with host cellular receptors and additional implicated roles in microtubule mediated-transport process [36, 37].

The p30 encoded by CP204L is an early expressed highly glycosylated phosphoprotein having property generally associated with virus internalization [38]. p12 encoded by pO61R and the p22 encoded by KP177R are late viral proteins important components required for attachment and virion precursor membranes progression to icosahedral intermediates [39,40]. p17 is another minor capsid protein required in the assembly of the capsid and icosahedral morphogenesis [41].

ASFV CD2v, a polypeptide of 402 amino acid, protein encoded by the gene (EP402R) is a homologue of CD2, a T-lymphocyte surface antigen; has been strongly implicated with hemadsorption phenomenon and it is located at the outer enveloped [42,43]. In addition, ASFV endogenously encodes anti-apoptotic proteins include A179L, a Bcl-2 family member [44]; A224L, an inhibitor of apoptosis proteins (IAP) family member; EP153R, a (C-type lectin); and DP71L which inhibits activation of the stress activated pro-apoptotic apoptotic pathways [45].

5. PRODUCTIVE ENTRY OF ASFV IN MACROPHAGES

ASFV is macrophage trophic, able to survive within the host circulatory monocytes/macrophages and its initial clinical episodes is observed with increase in number and secretory activation of acute phase proteins (increased levels of proinflammatory cytokines) causing clinical infections in ASFV [46]. The ASFV mechanically attach its proteins towards the plasma membrane and provides mechanical

supportive routes for its virion into the host macrophage cytoplasm and infecting it. ASFV possess proteins that acts as molecular antagonist of host cells, inhibits primary anti-viral responses by interfering and inhibition of immunoregulators such as NfkB [47] and by the release of anti-apoptotic signals [48].

Earlier postulates lay that ASFV may have entered through any one of the cytotic mechanisms such as receptor-mediated endocytosis or phagocytosis, macropinocytosis and membrane fusion. Previous studies stated that a favourable physical environment is required by ASFV for binding host-macrophage cell surface. This condition is provided by a low pH of macrophage hydrolytic compartment and is temperature dependent [49]. Macrophage expresses various cell surface receptors during ASFV infection. The major receptors CD163, CD45 and MHC II was predicted as having plausible roles as evidence from in-vitro studies [50]. Transfection experiments in swine macrophages PAM's (Porcine alveolar macrophages), CD163 marker were absent in naïve mononuclear monocytes but its presence and expression in mature tissue macrophages was relevant. However, the role of CD163 in ASFV infection has been controversial, since neither, positive swine cells ($CD163^{+}$) labelled with anti-CD 163 antibody nor the complete Knock out (KO) or gene-edited pigs lacking CD163 on macrophages showed differences in the course of infection with ASFV virulent strains indicating the possibility of other receptors or entry mechanisms that may have been responsible [51]. Similarly, evaluation of Fc-receptors denied its plausible roles, indicating that there was no receptor mediated mechanism that is particularly responsible for ASFV entry[52].

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Figure 2. Entry of ASFV: Adsorption, internalisation and uncoating

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Subsequently, various models in support of ASFV entry **Figure 2** [53] such as the clathrin-mediated dynamin-dependent endocytosis [54], phagocytosis [55] and macropinocytosis [56] were established in unpinning the mechanisms involving ASFV entry. Several viruses utilize macropinocytosis for their entry [57] and also to promote the penetration of viral particles that employs other endocytic mechanisms [58]. Macropinocytosis is a non-specific process, and its active induction is accompanied by actin-dependent membrane protrusion and retraction, ruffling of the plasma membrane and engulfment of large volumes of extracellular fluid that give rise to large endocytic vacuoles called macropinosomes. The process is also accompanied by dextran uptake (a fluid phase marker of macropinocytosis) and stimulation of EGFR which activates the PI3K-Akt, Pak1 and Rac1 signalling.

In the assessment of actin – dependent macropinocytosis, ASFV Ba71 were used to infect Vero cells pre-treated with macropinocytotic inhibitor EIPA, (inhibitors of Na^+/H^+ ion channels, EGFR, PI3K - Akt, Pak1 and Rac1). A significant decrease in ASFV infection was observed as compared to non –pre-treated controls [59] whereas, in an event to support the above study on actin involvement in macropinocytosis, disrupting actin in vero cells using actin disorganisers (CytoD, jasplakinolide and latrunculin A) ASFV entry seems unaffected [60] suggesting that an alternative route for ASFV other than macropinocytosis.

In order to dissect the specific mechanism of ASFV entry; macropinocytosis, actin and dynamin mediated endocytosis and clathrin mediated endocytosis were all put into a pharmacological assessment using specific inhibitors. Other than active mechanisms for a productive entry that utilize actin mediated endocytosis, a dynamin dependent clathrin-mediated uptake of ASFV was also analysed using flow cytometry. Cells treated with chlorpromazine, and dynasore (dynamin dependent clathrin-mediated endocytosis inhibitors) resulted in reduce uptake of fluorescent extracellular ASFV virions [61].

In addition, the presence of inhibitors of macropinocytosis 5- (N-Ethyl-N-Isopropyl) Amiloride [EIPA]; p21-activated kinase inhibitor III [IPA-3], actin inhibitor (Cytochalasin D); clathrin inhibitor - chlorpromazine (CPZ) and Cholesterol sequestering drugs (nystatin and methyl-β-cyclodextrin (CD), ASFV entry was significantly reduced and thus suggested collective requirements of clathrin, dynamin, and cholesterol in ASFV transport through endosomes in both Vero cells and swine macrophages. In contrast, inhibitors of the Na+/H+ ion channels and actin polymerization inhibition did not significantly reduce infections suggesting ASFV entryway requires both macropinocytosis and dynamin-dependent clathrin mediate endocytic entry pathway for productive entry of ASFV.

6. ROLE OF INNATE IMMUNE SENSORS AND DEFENSE MECHANISMS IN ASFV

ASFV virulent proteins p10, p12, p30, p72, p54 and CD2v are the initial components [62] with higher implications in host macrophages interactions and are the cognitive components that are recognised by the immune system. The p30 is highly implicated for its role involving in activation of macropinocytosis [63]. Besides known ASFV antigens that are recognised by the immune system, uncharacterised ASFV 1215L, CP530R, CP129R, M448R assessed using Gamma Interferon ELispot assay were also found to stimulate IFN response, could have immunomodulative roles [64]. The primary activators of the immune system are the PAMP's (viral nucleic acids DNA/RNA) of the pathogen that signals the immune sensors and immuno-receptors of host cells or vice-versa. The duration and magnitude of the immune response depend on how the virus interacts with host cells. Acute ASFV infections are lethal to cause haemorrhagic fever with the ASFV having a manipulative mechanisms in favouring itself a replicable and surviving environment within host cells.

On ASFV entry within the host, the potential host pattern recognition receptors (PRRs) are cyclic-GMP-AMP synthase (cGAS) that recognize the pathogen associated molecular pattern (PAMPs), dsDNA, of the virus and subsequently generates the cyclic GMP-AMP(c-GAMP) in the presence of ATP and GTP within the cell. cGAMP binds to the stimulator of IFN gene-encoded protein (STING) to recruit TANK-binding kinase 1 (TBK1) to form a complex that activates various transcription factors, such as nuclear factor-kappa B (NF-κB) and IFN regulatory gene 3 (IRF3) [65]. After that, these phosphorylated transcription factors translocates into the nucleus to initiate the transcription of type- I IFN and promote the expression of several IFN-stimulated genes (ISGs), which induce an antiviral protein synthesis and pro-inflammatory factor production through downstream pathways to eliminate the virus. Another possible activated PRR's during ASFV infection are likely to be Toll-like receptor 3 (TLR3) which have been implicated in a point of time gene expression study using PAM's [66] that could sense the viral nucleic acid to activate IRF3 and NF-κB signalling pathway through a TIR-domain-containing adapter-inducing interferon-β (TRIF). TRIF activates tumor necrosis factor (TNF) receptor-associated factors (TRAF) and subsequently TBK resulting in IRF3 activation. The initial type- I IFN induction of IFN-β, may subsequently induce IFN-α through IRF7 phosphorylation in a positive feed forward activation of type I IFN simulation.

Once produced the type-I IFN's triggers signals through the same receptor, the type-I IFN receptor (IFNAR). IFNAR is composed of two subunits (IFNAR1 and IFNAR2) which when bound to type I IFN are endocytosed and activate their associated tyrosine kinases, Tyk2 and Jak1 [67]. The macrophage derived cytokines **(**Type I IFN) is known to be essential for activating the antiviral innate immune response activation of the natural killer (NK) cell, which elicits effector such as IFN-γ response (Type II- IFN).

IFN-γ is a pleiotropic cytokine that modulates both innate and adaptive immune networks; The type- I IFN and antiviral NK cell are tightly interwoven. ASFV modulation of NK cells could be demonstrated by a vaginal HSV-1 infection where type I IFN was required to induce epithelium production of CCL3, CCL4, and CCL5 to recruit NK cells to the vaginal mucosa [68]. During an infection, NK cells have several weapons (IFN-γ, TNF-α/β, CD95/FasL, and TRAIL, as well as cytoplasmic cytotoxic granules containing perforin) under their belt that can induce the manifestation of death receptors on target cells, which in turn activates pro-apoptotic signaling programs. Thus early type-I IFN response is critical for induction of both an antiviral response within infected and target cells, as well as activation of innate immune cells that will ultimately serve to control virus replication and activate the adaptive immune response to both clear the infection and generate memory to create a rapid response against future infections.

In a study, ASFV infected Porcine alveolar macrophages (PAMs), on treatment with recombinant IFN, reduced viral titres was observed and an increased regulation and expression of IFN-induced genes (IFIT1, IFITM3, Mx-1, OASL, ISG15, PKR, GBP1, Viperin, BST2, IRF-1, and CXCL10) and MHC molecules which suggested their possible roles in resistance to virus infection [69]. Alternatively, treatment with porcine IFN did not inhibit replication and could not reduce viral titres suggesting that IFN dependent antiviral response is being controlled by multiple factors.

Interestingly an expression analysis on the serum of a virulent Chinese ASFV SY18 infected pigs is observed with upregulation of TNF-α, IFN-α, IL-1β, IL-6, IL-8, IL-12, IL-18, RANTES (regulated upon activation, normal T cell expressed and secreted), and IFN-γ-induced protein 10 (IP-10), but with absence of anti-inflammatory cytokines IL-10 [70]. This abrogative inflammatory response accompanied with peaks of type I Interferon (IFN- α) may have been the regulatory factors that had potentiated secretion of pro-inflammatory cytokines, thus suggesting associated roles of an imbalanced innate immune response with stimulation of pro-inflammatory cytokines. Thus during viral infection IFN response are primeval but its high level induction may accompany an imbalance inflammatory response.

7. IMMUNE ESCAPE MECHANISMS OF ASFV

ASFV is a pathogenic macrophage trophic, possessing mechanism to control and escape the host immune system by either modifying or mimicking, different intracellular signalling intermediates associated with host innate and cellular immune response regulation. Like many haemorrhagic diseases the pathology of ASFV in domestic pigs has been linked to the overexpression of cytokines such as IFN and tumor necrosis factor alpha and alternatively down regulating anti-inflammatory response causing massive cell lymphocytic death [71]. The virus potentially express different sets of protein that differentially inhibits immunoregulation and interferes with the host mechanisms **Figure 3** at different levels such as interferon modulation, inflammation, apoptosis, processing of antigens, and cellular immunity[72].

7.1 *ASFV antigenic proteins in immunomodulation and suppression*

The various host response machinery that ASFV inhibits are Type I interferon (IFN), the NF-κB transport protein importin α2 that are class of karyopherins (nuclear transport proteins), which is a crucial component of the innate response to viral infection [73]. ASFV multi gene family MGF360-12L binding to nuclear transport proteins, importin α2 (KPNA2), importin α4 (KPNA3) and importin α3 (KPNA4), competitively block the interactions of KPNA2, KPNA3 and KPNA4 with p65 subvert cellular innate

Figure 3. ASFV in regulating the host cell immune response mechanism; and apoptosis of infected cells .

immunity impaired the capability of cells to produce IFN-β (Type I IFN), inhibiting NF-κB nuclear translocation and decrease host antiviral responses. The ASFV A528R from MGF 505 inhibits induction of both NF-κB and IRF3 branches of the IFN-I induction signaling pathway [74]. A276R gene from MGF 360 could inhibit IFN-β expression via both the TLR3 and the cytosolic pathways by targeting IRF3, but not IRF7 or NF-κB [75]. ASFV also encodes a gene I329-L gene homologue to a functional viral TLR3 (PRR's) of host and interferes the induction of IFN at the level of TRIF [76]. DP96R inhibits the activation of IFN-β and ISRE promoters by suppressing cGAS/STING and TBK1, but does not inhibit their activation mediated by IRF3-5D [77].

ASFV haemadsorbing CD2v antigenic protein has a structural homologue similar to CD2 a T-cell surface antigen competitively reduce priming of T-cell proliferation through CD58 and deactivating innate immune response by inhibiting nuclear factor-kappa B (NF-κB) which mediates IFN secretion [78]. ASFV F317L impaired NF-κB pathway activation by disruption of NF-κB activity, through interaction with IκB kinase β (IKKβ) and suppressed its phosphorylation, which subsequently reduced phosphorylation and ubiquitination of IκBα and enhanced IκBα stabilization. The accumulation of IκBα then blocked NF-κB activation and inhibited its nuclear translocation, resulting in decreased expression of various proinflammatory cytokines [79].

7.2 *Apoptotic Inhibition*

ASFV inhibits apoptosis favouring survival and replication within its host. The ASFV anti-apoptotic protein machinery, which are encoded within its genome are expressed in its host cytosol during infection such as A179L (a Bcl-2 family member) having BH3 binding domain [80] interferes with apoptotic regulatory machinery; A224L (a member of the family of 'inhibitor of apoptosis proteins', IAP) expression subvert cell apoptosis by reducing caspase 3 regulation [81]; EP153R (a C-type lectin) and DP71L (which inhibits activation of the stress activated pro-apoptotic pathways) has also been described to inhibit apoptosis, interfere with the p53 pathway and caspase 3 activation [82].

7.3 *Inhibition of inflammasome activation*

ASFV have developed complex machinery such as the MGF-505-7R to subvert inflammasome formation by binding to NLRP3 during infection. On a co-immunoprecipitation (Co-IP) and a pull down assay on ASFV infected Porcine alveolar macrophages (PAMs), MGF-505-7R co-precipitated along with NLRP3 showing high physical interaction between the two components. Using high-throughput screening system to identify viral proteins regulating the inflammatory response; they also identified I226L, A151R, NP419L, and QP383R proteins encoded by the virus as potential suppressors of the inflammatory response [83]. Another ASFV protein L83L c binds to IL-1β, a member of the IL-1 family of cytokines. IL-1β is a pro-inflammatory cytokines which is the prime regulator of inflammatory and a mediator of immune responses that contributes to host defense against infection [84].

8. EFFORTS AND IMPACTS ON IMMUNISATION EXPERIMENTS

With plausible efforts, ASFV immunisation experiments proved to be effective while they were capable of generating both the innate and humoral response. Partial and complete protection was attained when precise combinations of viral antigens were formulated. The attenuated forms such as naturally attenuated ASFV vaccine (NAV) and modified live attenuated ASFV(M-LAV-produced via genetic manipulation in cell cultures), the bacculovirus expressed ASFV antigenic protein as subunits vaccine, the physically or chemically inactivated ASFV vaccine, the disarmed viral-vector based vaccine have been extensively studied and tested **Table2**. Immunisation experiments on ASFV vaccines had corresponded with different range of protection and responses along with inconsistencies in results. The efficiencies of these vaccine were determined at various protective levels such as its capability of eliciting antigen specific neutralising antibodies, T-cell response, cross protectivity and persistence of virus (viremia) on a challenged with both heterologous and homologous strains of the ASFV. It was demonstrated that neutralising antibodies (Nabs) alone, without Antibody Dependent Cell Cytotoxicity (ADCC) are not sufficient for protection [85]. Most of successful viral vaccines that pass clinical trials are those that exhibited efficacy (multi-valency, long term immunological memory, ease of administration, thermal stability) and safety. Earlier the search for ASFV vaccines was primarily aimed at achieving primary antibody response using potent ASFV structural proteins or antigen. Few studies observed induction of appropriate cellular response and also achieved a higher cross protection but had to face adverse effects of immunological reactions.

Surprisingly, in the event of ASFV infections the discovery of naturally attenuated strains prompted their use in immunisation experiments. The first studied naturally attenuated ASFV were that of the ASFV OUR/T88/3 and ASFV OUR/T88/1 Genotype I strain, which resulted in cross-protection along with cellular immune response that protected the pigs on a challenge with a non-homologous ASFV Benin 97/1 [86]. Another cofound naturally attenuated strain NH/P68 (genotype I) had the capability to achieve 100% protection against a heterologous challenge with the virulent strain L60 (genotype I) and most importantly induce protective immune response such as antibodies specific to ASFV antigens and induction of NK cells [87]. This ASFV NH/P68 Genotype I was later demonstrated for its capability of inducing ASFV specific cytotoxic T- cells (CTL's) in Porcine alveolar macrophages (PAM's) with enhanced NK activity against a virulent ASFV L60 which were relevant to primeval activity and immune response against ASFV infection [88].

Inactivated viral vaccines comprises of the whole virion or part of it, generally devoid of its genetic material through chemical or physical treatment or by combining both. A wide range of well-established inactivating agents have been described to successfully inactivate viruses for vaccine purposes. Inactivated viral vaccines are known to be less protective in that they possess low immunogenicity, induce weak response and less long lived, therefore they are enriched with adjuvants. An inactivated vaccine of ASFV Armenia 08 when administrated with adjuvants (Polygen or emulsigen) did produced ASFV specific antibodies but they could not resist post challenge experiments [89].

Table 3. Immunisation Experiments on Pigs.

Vaccine Type	ASFV Strain/Viral Antigen	Vaccine Design	Survivability/Protection	Challenged ASFV Strain	Ref.
Attenuated Live ASFV Vaccine	ASFV Georgia 2007/1	ΔMGF -360 and ΔMGF -505	Pigs survived post challenged	ASFV Georgia 2007/1	[102]
	ASFV Benin 97/1	ΔMGF -360 and ΔMGF -505	Significant number of Pigs survived	Parental Benin 97/1	[91]
	ASFV Georgia 2007/1	Recombinant; ΔI177L	Pigs survived protected / significant antibody responses	Parental ASFV-Georgia 2007/1	[105]
		Recombinant; Δ DP96R and ΔB119L	Pigs survived/protected/significant antibody responses	Parental ASFV-Georgia 2007/1	[104]
	ASFV OUR/T88/3 (Naturally Attenuated)	Δ- DP71L and Δ -DP96R	Significant number of Pigs survived	Sub-lethal OUR T88/1	[103, 86]
		Unaltered native state	Pigs survived/	ASFV Benin 97/1	
	ASFV Pr4	Δ-9GL	Pigs Survived	Parental virus Pr4	[85]
	ASFV NH/P68 (Naturally Attenuated)	Unaltered native state	Pigs survived with high levels of protection	Heterologous ASFV/ L60	[87]
	ASFV Ba71	Δ-CD2v (ΔEP402R)	Pigs survived/Cross Protection	ASFV -E75	[92]
			Pigs Survived / Protected	ASFV - Georgia 07	
			Partial Protection	ASFV Ba71	
Whole ASFV-Virion Inactivated Vaccine	ASFV Armenia 08	Freeze-dried inactivated ASFV Armenia 08	No protection/ pigs with lethal deaths	ASFV Armenia 08	[89]
Subunit-Antigen based ASFV Vaccine	ASFV (E75CV)	Baculovirus-expressed proteins CD2v	Pigs survived post challenged/ No Antibodies	ASFV E75	[95]
	ASFV (E75)	Baculovirus-expressed Proteins (p54 + p30)	Partial Protection	ASFV E75;	[101]
	ASFV (E75) 5	Baculovirus-expressed chimeric protein (p54,p30)	Protected/ Neutralizing Antibody	ASFV E75;	[95]
	ASFV (Pr4)	Baculovirus-expressed proteins (p54 + p30 + p72 + p22)	Pigs died of viremia at 4 d.p.i.	ASFV Pr4	[85]
Viral-vectored ASFV vaccine (Fusion)	ASFV Georgia 2007/1	Adenovirus tailored synthetic (p30+p54+pp62+ p72 genes)	Pigs did not survived	ASFV Georgia 2007/1	[99]
	ASFV Georgia 2007/1	Adenovirus tailored synthetic (A151R+ B119L+ B602L+ EP402RΔPRR+, B438L +K205R-A104R)	Pigs survived/ Protected/ antibody and IFN-γ^{+}	ASFV Georgia 2007/1	[100]
	ASFV E75	BacMam tailored synthetic (sHA/p54/p30 fusion (E75)	Partial Protection	E75; 2x sub-lethal challenge 102	[97]
DNA Vaccine	ASFV E75	p-CMV (sHA/p54/p30)	Pigs dying 6-8 days (p.i.)/Absence of neutralizing Ab/no protection	ASFV E75 Virulent	[98]
		p-CMV (sHA/p54/p30/ Ubs)	Insignificant number of Pigs Survived/ T-cell	ASFV E75 Virulent	[98]

In another study, a modification in the delivery approach was aimed at achieving protective response through the use of strong adjuvants, intradermal administration in addition to the usual intramuscular administration, the increase in dosage, yet the inactivated administration did not achieve protection [90].

Genetically modified live attenuated ASFVs vaccine were prepared by specific deletion of ASFV virulence gene the multiple members of multigene families (MGF), genes involved in virus replication, anti-apoptotic genes and morphogenesis. Promising results were achieved using genetically modified Benin 97/1 strain with deletion of Multi gene Family - ΔMGF 360 and MGF 505 [91] with early detection of antibodies post immunisation (pi) , impart protection in pigs with detected serum levels of IFN- γ and IL-10 (anti-inflammatory cytokine) against a homologous challenged. In another study, a dose dependent protection was observed using live attenuated ASFV Ba71 strain which resulted with secretion of Type II- IFN (IFN-γ) and T-cell modulation in response to recall antigen on a homologous and heterologous ASFV challenge [92]. Another gene pA137R interacts with TANK-binding kinase 1 (TBK1) repressed type I IFN production and attenuated ASFV (ASFV-ΔA137R) inoculated animals were protected when challenged with the virulent parental strain ASFV-G. Also, on an immunization with ASFV-ΔA137R there were lack of evidence of the virus replication [93].

Subunit-vaccines may incorporate a defined pathogen component such as structural, non-structural or unassigned proteins as antigens to elicit protective immune responses [94].Subunit ASFV vaccine makes use of virulent structural proteins p30, p54, p72 and CD2v that display antigenic epitopes are a pre-requisite requirement to generate ASFV specific antibodies in vaccine. DNA vaccine platforms are viral, bacterial, or plasmid-based vectors that can be incorporated with a either a single or more than a single viral antigen (mono or multi- antigenic designed). The first pioneering study with ASFV subunit to demonstrate protection against ASFV challenge was the baculovirus-expressed ASFV CD2v, a hemagglutinin (HA). Pigs vaccinated thrice with recombinant CD2v proteins, then challenged with the virulent ASFV genotype- I E75 strain, resulted in CD2v-specific antibodies, with one pig resulting in virus-neutralizing activity [95]. Few pigs were protected from lethal challenge, although animals did developed viremia and subsequently died.

In order to strengthen immunisation a chimeric preparation of ASFV antigens were preferred over the use of single antigenic protein as subunits. The first studied chimeras of Bacculovirus expressed ASFV antigen (p54/p30) developed neutralizing antibodies with sterile immunity checked after 55 day post- inoculation on a challenged with virulent ASFV strain E75 as compared to that of controls[96]. Another expression vector design is the BacMam which comprises the Bacculovirus driven by Cytomegalovirus promoter (pCMV) is an enhancing model and versatile system for the expression of genes in mammalian cells. The fusion protein consisted of the ASFV Hemagglutinin (sHA), p54 and p30 in tandem and were used to immunise live pigs that co- responded with large number of virus-specific IFN γ-secreting T-cells in blood at 17 dpi on a homologous sub lethal challenge [97] highlighted the importance of the cellular responses in protection.

DNA vaccines are transforming the system in vaccines with the capacity of tailoring wide range of viral genes and inducing wider immunological response. In ASFV a plasmid DNA cloned with three ASFV antigens (sHA/p54/p30) were immunised in pigs corresponded only with antibodies against the p30 and p54, induced IFN γ but did not protected the pigs. In slight modification to the above experiment Ubiquitin tagging was strategized to improve MHC- Class I antigen presentation bearing the plasmid and ASFV (Ubs/sHA/p30/p54) corresponded as predicted with high levels of cytotoxic T-cell response, but lacked B-cell priming of antibodies post challenged however proportion of immunized-pigs survived lethal challenge with ASFV (E75) [98]. This study demonstrated a new prospective of DNA vaccine in ASFV, with a platform in newer approach for vaccine development.

Although the multivalent properties of subunits vaccine were capable to elicit antibodies upon subsequent challenged, they were not sufficient to recall collective immunological response necessary for protection. Moreover, there has not been any conclusive data where an ultimate successful vaccine in ASFV been developed.

The on-going trend in ASFV vaccine stands reliable on disarmed (deletion of virulent genes) viral vector based vaccine preparations that incorporates combinations of ASFV antigens. Adenoviral vectored vaccine (Ad-ASFV) carrying cocktail of antigens (p32, p54, pp62, p72) was found to induce strong antibody and IFN-γ+ cell response with the first to show antigen-specific CTL's response in commercial swine stimulating both specific and non-specific anti-ASFV immunological response [99]. Additionally, a refined approached, a broad spectrum, design is a disarmed Adenovirus (E1 deletion) vector carrying both structural ASFV antigens (Georgia 2007/1 isolate) and non-structural proteins such as pA151R, , pB119L, B602L, EP402R, B438L, K205R -A104R that resulted in a strong ASFV antigen-specific IgG responses and also ASFV-specific IFN-γ-secreting cells that were recalled strongly upon boosting [99]. Most importantly in relevant to the prevailing ASFV, this fusion induced antibodies that recognized viral proteins from ASFV Georgia 2007/1.

Overall, the subunit vaccines were very much success in imparting recognition and inducing response. Complementing the antibodies generated from infected swine cell culture models, Bacculovirus expressed ASFV subunits antigen are also capable of inducing antibody specific for ASFV p30 and 54 in live pigs [101]. The limit of virulence, whether acute or chronic causing ASFV, are important clinical properties and attributes towards choice for ASFV to be used in live attenuated vaccines. The efficiency and range of protection appears that the moderate naturally occurred attenuated isolates ASFV OUR/T88/3 had better results in immunization experiments than its weakened attenuated form, the deletion of Δ- DP71L and Δ -DP96R of ASFV OUR/T88 in achieving protection, since the latter native form could resist heterologous challenge with ASFV Benin 97/1 [102].

With the on-going ASF epidemic, the ASFV Georgia Genotype II is considered to be the most versatile in the history of ASFV epidemic. Therefore considering the facts and reports, in concordant with the epidemic, the virulent ASFV Georgia itself finds position as the most favourable candidate in live attenuated vaccines. The ASFV MGF families 360 and 505 are a group of genes sharing partial sequence and structural identities that have profound roles in virus cell tropism, IFN modulation and also may be required for efficient virus replication in macrophages. The ASFV Georgia attenuated forms through deletion of Δ-MGF360 and Δ-MGF 505 replicated efficiently in primary swine macrophage cell cultures as efficient as the parental virus demonstrating the role of MGF genes acting as independent determinants of ASFV virulence. Surprisingly, ASFV Georgia Δ-MGF immunized pigs, on a challenge with highly virulent parental ASFV Georgia clearly had no signs and symptoms of infection, although a proportion of these animals consisted very less residual titers of the challenged ASFV Georgia. In fact, ASFV-Georgia Δ-MGF was reported as to be the first live attenuated preparation to resist challenge against an epidemic causing ASFV-Georgia [103]. Another ASFV Georgia live attenuated vaccine was a double-gene-deletion recombinant with attenuated deletions on ASFV Georgia- UK protein encoded by the DP96R (TBK inhibitor) and the 9GL, encoded by B119L (viral processing and assembly). The ASFV Georgia double deletion Δ-9GL/-DP96R was found to protect immunized pigs against the wild type ASFV Georgia 2007 within less than 2 weeks post vaccination [104]. The presence of protection correlates with the appearance of serum anti-ASFV specific antibodies, but lacked circulating ASFV specific gamma interferon (IFN-γ) producing cells.

On an analysis of gene expression in porcine alveolar macrophages (PAM's) infected with virulent ASFV, revealed that the genes B646L (p72), CP204 (p30) and I177L were highly upregulated. ASFV I177L a non-putative ASFV gene was shown to be highly expressed in PAM's at early stage as 18 hpi, thus predicted its possibility to have viral early roles in invasion. Most of live recombinants attenuated vaccines that have been created unto date were most deprived of more than one ASFV protein virulent genes. As predicted, attenuation was done on ASFV Georgia gene I177L. This single gene completely attenuated ASFV Georgia Δ I177L vaccine and on immunization experiments resulted with sterile immunity against the parental ASFV when administered intra muscularly [105] showed no virus shedding, low viremia titers, and developed a strong virus-specific antibody response; importantly, pigs were protected when challenged with the virulent parental strain ASFV-Georgia. Similar results were found when this vaccine was administered through oronasal routes [106] with same levels of anti ASFV specific antibody response. Also in another transcriptomic sequencing analysis a single gene attenuated deletion (ASFV-ΔA137R) from the wild type HLJ/2018 strain induced higher type I interferon (IFN) production in primary porcine alveolar macrophages (PAMs) than did ASFV-WT. The ASFV-ΔA137R had promising results as a vaccine candidate where the A137R protein is known to negatively regulate the cGAS-STING-mediated IFN-β signaling pathway through targeting TANK-binding kinase 1 (TBK1) for autophagy-mediated lysosomal degradation [107]. Accordingly, if certain mining of gene and proteins for their application in ASFV vaccine, whether as attenuated deletion or a DNA vaccine or as cocktails antigen subunits, their underlying mechanism to ASFV protection has to be certain and is the pen ultimate goal for their use as a clinical vaccine.

9. CONCLUSION

ASFV is a major global threat to the pig industry with the infection when manifested, do not have a mechanism for cure and mostly require culling procedures. There are no precise clinical signs when spotting ASFV and therefore requires a strict laboratory diagnosis. Transmission and surveillance has been less programmed since its emergence. The microevolution accompanied during ASFV epidemic is relevant for its heterogeneity in virulence and is a major concern. Mostly, the reports on ASFV transmission were those that were being transmitted directly through infected pigs and transmission vectors such as ticks. There must be inclusive need for screening probable vehicles which includes common insects such as housefly (*Stombyx calcitrans, Musca domestica)* fomites and pigsty during infection for prevention purposes. Mono-clonal antibodies have promising roles and may neutralize ASFV avoiding further cascades of transmission.

The recent progress in ASFV characterization of structural genes is the key to the development of a novel vaccine. Cell culture models had created a platform in identification and functional characterization of only about 30% known essential genes of ASFV. The ASFV essential genes are mostly involved in its multiplication, survival and shedding. Late viral genes associated with apoptosis, nuclear localization, hyper stimulation of immunological response and modulating host normal regulatory process are potential targets in combating ASFV. There are certain development in the disovery of biomarkers in ASFV, but its slow progress delays many therapeutical shifts.
Antivirals apart from the natural IFNs are known conventional antivirals (nucleoside analogues, protease inhibitors, polymerase, topoisomerase inhibitors) used in treating viral infections. Previously, viral proteins or antigens were major potential anti-viral targets or vaccine components, it was only recent, that antivirals developed were allowed to target host-cellular machinery.

Molecular aptamers, small interfering RNA and CRISPR/Cas9 etc. are potential biotechnological tools in the diagnosis of ASFV. Another recent progress in viral diagnosis and treatment is the applications of CAR-T cells (Chimeric Antigen Receptors) in HIV and HCV infections which is of a

tremendous progress in the field of immunotherapy reaching clinical trials, had promising results. Thus developing CAR-T cells against emerging viral infections such as ASFV and similar diseases that prevails amongst animal infection may be an alternative approach which may prove beneficial especially preventing livestocks from infections which tends to develop resistance with simultaneous use of antibiotics and antivirals. Moreover, ASFV immunization experiments had not involve the use of combinatorial treatment such as the use of vaccines along with antivirals which could prove a different outcomes in disease progress and interventions.

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