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The reoccurrence of H5N1 outbreaks necessitates the development of safe and effective influenza vaccine technologies for the prevention and control of avian influenza in Sub-Saharan Africa

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Africa is experiencing reoccurrence of avian influenza outbreaks with huge negative impact on the economy of the continent as a result of high mortality rate and extreme contagiousness of the disease. The epidemiology of highly pathogenic avian influenza (HPAI) in Africa during the 2006-2008 outbreaks was complex and linked to movements of poultry commodities and wild birds. The peculiar risk factors, negative economic impact and the potential of being used as a biological weapon necessitates the development of a comprehensive control programme for the prevention or eradication of the disease. It is the opinion of this paper that development of new influenza vaccine technologies will provide affordable comprehensive control programmes for avian influenza prevention in Africa. To keep pace with the variability of the viruses, there is need for frequent redesign of avian influenza (AI) vaccines to match the circulating subtypes and on this is predicated the necessity of the development of influenza vaccine technology for a country, zone or region. The new vaccine technologies have been shown to have the potentials of giving vaccines with required criteria of purity, safety, efficacy, potency, low cost and short response time. The concept of most new vaccine technologies is biased towards removal of influenza virus from the system of vaccine development and at the same time obtaining more effective, potent and safe influenza vaccines. The new influenza vaccine technologies include gene-based, genomics-based, subunit, plant-based, VLPs and universal vaccine technologies. These technologies have the potential to provide vaccines that will not just be used as intervention strategies to lessen severity of the disease but as preventative vaccination. Also routine vaccination will not just be as a tool of last option in disease endemic areas, but one to prevent the disease.

Key words: Technologies, avian influenza, vaccine, eradication, prevention, comprehensive, control, programme.

INTRODUCTION

During the 2006-2008 outbreaks in Africa, eleven countries reported infection or re-infection of poultry flocks and/or wild and migratory birds (Joannis et al., 2008). As at the beginning of 2013, 13 countries were

reported to have confirmed cases of highly pathogenic avian influenza infection in birds or humans and two of these countries are in Africa: Egypt and South Africa (WHO, 2013). Also as at December 2014, more than fifteen countries reported confirmed cases of avian influenza (AI) outbreaks involving more than five subtypes (H7N2, H5N1, H5N2, H5N6, H5N8 and H7N9) with Egypt as the only African country (WHO, 2014). OIE report of 17th June 2015 showed that six African countries (Burkina Faso, Cote d'Ivoire, Ghana, Nigeria, Libya, Niger, Nigeria) had confirmed cases of H5N1 outbreaks (OIE, 2015). In Nigeria, all the six agro-ecological zones have reported H5N1 outbreak involving up to 18 states of the country, indicative of a more complex reoccurrence in the country. The potential to cause a pandemic is the greatest concern and this calls for a concerted effort for a comprehensive national, regional or continental prevention and control programme. It is evidently clear that AI outbreak is a menace that needs to be given more attention than it receives today in Africa.

The epidemiology of the 2006 - 2008 outbreaks was peculiar due to its complexity involving clades and sublineages (clade 2.2; clade 2.2.1; sublineages I, II and III). The complexity was linked to the identification of multiple introductions through movements of poultry commodities and reassortment events between sub-lineages particularly in Nigeria and Egypt (Fusaro et al., 2010). The evidence of H5N2 virus in apparently healthy wild water fowl in Nigeria (Gaidet et al., 2008) suggests that HPAI could also be introduced to domestic birds in Africa through migratory wild birds. Human cases reported in Egypt, Djibouti and Nigeria raises concern for the possible human health risk of a pandemic as presented by the evolution of genetic properties of A/H5N1 viruses across the African content (Cattoli et al., 2009). The peculiar risk factors in Africa, the negative economic impact and the potential of being used as a biological weapon necessitates the development of safe and effective influenza vaccine technologies for the prevention and control of AI in Africa. Such technologies will afford us the opportunity of having a comprehensive control programme which is necessary for the prevention or eradication of an epidemic or pandemic in a nation, region or even the entire continent.

Presently, many (professional groups, authors) advocate "no vaccination" strategy and even governments' policies in most African countries are also the same. These policies and opinions have immensely discouraged research in the area of influenza vaccine technology in Africa. A critical review of influenza vaccine technologies reveal that the new technologies have more advantages than drawbacks compared to the conventional technologies that gave rise to the current stand. It is the opinion of this paper that development of new influenza vaccine technologies will provide affordable comprehensive control programmes for avian influenza prevention in Africa. Authors still advocate culling as the time-honoured method to control or eradicate AI outbreak and the best way to prevent transmission to humans.

However, many reports also show that when the viruses are spread over a wide area and have infected multiple avian species, culling and physical containment alone are not likely to be successful (Marangon et al., 2008; Rao et al., 2009). Hence a comprehensive control programme (to include decreasing susceptibility through vaccination), is an effective defensive barrier for AI prevention in Africa. International organizations such as the World Organization of Animal Health (OIE) and Food and Agricultural Organization (FAO) have recommended that vaccination of poultry be considered for the control of AIV (Park et al., 2006).

Vaccines developed using new technologies have been found to have faster production rate and large scale production methods. They are known to be scalable, safe and have the potential for enhanced efficacy against a broader range of influenza strains (OIE, 2009; Ge et al., 2007).

The main aim of this review was to advocate the need for African scientists to develop or adopt new influenza vaccines technologies and as a wakeup call to face the reality of this highly variable virus which can again come knocking at any time. It is imperative that national, regional or continental AI vaccine technologies be developed as part of a comprehensive control programme as there is no universal vaccine that will protect against all AI viruses. The present day reality is the fact that in the face of a pandemic, the developed countries on which Africa often relies for assistance may not be forth coming when faced with same problem in their countries. Naturally, they will need to control pandemic in their regions or countries before considering assistance to other countries. In such a situation, many may be infected or killed and poultry birds may be greatly lost. This negative impact on the fragile economy of Africa may be avoided by putting up comprehensive control programmes. In this paper, we have highlighted the features and advantages of these new technologies.

AVIAN INFLUENZA VACCINE TECHNOLOGIES

Avian influenza vaccine technologies are the approaches or strategies utilized in the development and subsequent production of avian influenza vaccines. This includes the up-stream and down-stream processes of development

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and manufacture of a successfully tried vaccine candidate. Influenza vaccine technologies can be broadly categorized into conventional and new influenza vaccine technologies. Many of the new technologies have been applied in the preparation of both human and animal influenza vaccines (Swayne, 2006; Hoelscher et al., 2008). These new technologies have been shown to have more advantages as they are able to give vaccines with the required criteria of purity, safety, efficacy and potency at a lower cost compared to the conventional technologies (Rao et al., 2009). Influenza viruses undergo frequent reassortment which results in the emergence of antigenic variants with different surface antigens. To keep pace with the changes, new influenza vaccines must be designed annually to match the circulating viruses and it is a factor in the development of influenza vaccine technology for a country, zone or region. Unarguably, a production facility within a country, zone or region makes the development and production of a new vaccine to match the circulating virus cheaper, affordable and effective. It is in the best interest of Africa to have the new technologies and production facilities within its borders. Response time and production capacity have also given the new technologies an edge over the conventional for use in avian influenza vaccine production. In influenza outbreaks or pandemic, it is of great importance that vaccines become available as soon as possible and enough to cover pandemic or outbreak area(s). The circulation of multiple antigenically distinct subtypes complicates issues as all the outbreak strains have to be taken into consideration when developing vaccines and this is easier and faster done with the newer technologies at a closer proximity.

CONVENTIONAL INFLUENZA VACCINE TECHNOLOGIES

Conventional influenza vaccine technologies rely on influenza virus intermediates during vaccine production cycle. These are the early technologies which give relatively crude or partially purified whole or split virions as vaccines mostly grown in embryonated eggs and based on attenuation or inactivation of the virus. Also included are the cell culture and some subunit based technologies whose drawbacks are more than the advantages. In other to appreciate their drawbacks, some of the conventional technologies and their drawbacks are briefly discussed.

WHOLE-INACTIVATED INFLUENZA VACCINE (WIV)

The classic way of producing whole-inactivated influenza vaccines has been the utilization of seed stocks of the viruses (Swayne, 2009; Kreijtz et al., 2009). The influenza vaccine strains must have high-growth phenotype under laboratory conditions, antigenicity of the epidemic strain and high yield of the viral antigens HA and NA to give complete protection (Stephenson et al., 2004). Vaccine strains are selected by infecting embryonated chicken eggs simultaneously with a selected epidemic strain and laboratory strain with high-growth phenotype for example, A/PR/8/34 strain for humans. The reassortant virus that carries HA and NA of the outbreak strain and the high-growth-phenotype of the laboratory strain is selected as the vaccine strain. Once the vaccine strain is derived, whole inactivated influenza vaccine can be obtained by propagation of the virus in 9 - 11 days embryonated chicken eggs and the allantoic fluid which contains the virus is harvested for vaccine preparation.

Although this technology is still held as the most widely used for both poultry and human influenza vaccines, derivation of a vaccine strain can take a long time. Safety concerns during the development and production of the vaccine in a technologically backward country, zone or region outweigh the advantages of the technology. In our opinion, risk of escape of the virus into the environment in the course of development or production and exposure of the laboratory personnel are the greatest setbacks in developing countries with grave consequences. The other setbacks are common to both developing and developed countries and these include: production in large doses and double vaccination, time of production of a reassortant, high cost of production, scarcity of eggs during outbreaks or pandemic situations, reactogenicity for humans, and difficulty in administration for large poultry (Ellebedy and Webby, 2009; Josefsberg and Buckland, 2012).

SPLIT-VIRION INACTIVATED VACCINE

Split-virion inactivated vaccine is a sterile aqueous suspension of a strain of influenza virus, inactivated and treated so that the integrity of virus particle is disrupted without diminishing the antigenic properties of HA and NA antigens. The process of preparation of split-virion vaccines from vaccine strain selection to sucrose gradient centrifugation and purification can be the same with whole-inactivated vaccines especially when the virus is propagated in embryonated chicken eggs. The optimal conditions for each new strain, however needs to be determined empirically as the yield of HA and NA can vary significantly from strain to strain, year to year, and from one manufacturing process to another (WHO, 2006). Other protocols include further purification by sucrose gradient ultracentrifugation to obtain largely viral HA and NA or filtration to remove bacteria before ultracentrifugation (Jennings et al., 1987; Treanor et al., 2006). Diafiltration is used to remove the detergent. As for whole-inactivated influenza vaccines, HA content is determined by single radial diffusion. The same safety concerns and almost same setbacks with WIV apply to this technology and the further processing steps often

give less immunogenic vaccines as reported by some authors (Hovden et al., 2005; Subbarao and Joseph, 2007).

SUBUNIT INFLUENZA VACCINE

This is a technology that gives influenza vaccines made up of one or more pure or semi-pure antigens (surface glycoproteins: HA, NA, matrix proteins) and can give desirable long lasting protection and immunity against human or avian influenza. The antigen(s) could be egg-based or cell culture propagated influenza viruses (Morein et al., 1983; Mortimer et al., 2012). Generally, preparation using egg-based propagated viruses is the same with split-virion influenza vaccines from propagation to purification of lysates. The additional steps involve dialation, filtration, chromatographic separation and purification of antigens before lyophilization. Quality and quantity of purified antigens are assayed or confirmed using techniques such as HI test, Western Blotting, SDS-PAGE, total protein assay methods such as Lowery method. However, purification may lead to loss of immunogenicity and often necessitates coupling to an immunogenic carrier protein or adjuvants. The final step is formulation which in most cases involves lyophilization. Like the first two technologies safety concerns during development and production are high especially risk of escape of the virus into the environment and exposure of the laboratory personnel. The duration of immunity given by the vaccine is often short and less immunogenic than WIV.

CELL CULTURE BASED INFLUENZA VACCINE

Cell culture based influenza vaccine technology involves influenza virus propagation in vitro in mammalian, insect or avian cells instead of the traditional in ovo propagation. This technology evolved from the use of primary cell lines, followed by their derivatives the diploid cells to continuous cell line (Josefsberg and Buckland, 2012). Cell lines universally used in influenza vaccines production can be grouped into conventional cells(African Green Monkey Kidney-derived Vero cells, Madin-Darby Canine Kidney - MDCK, cell lines of avian origin), and proprietary human cell lines(PER.C6, AGE1.CR®). These cell lines yield influenza virus titers that are sufficiently high enough to be used for commercial production of influenza vaccines (Hess et al., 2012).

Generally, cell lines used for vaccine production must meet an extensive set of regulatory standards: identity (history of derivation, passaging, exposure to animal), stability, purity, ability to support growth of sufficient quantity of virus cells with desired antigenicity, HA and NA titers (Kemble and Greenberg, 2003; Hess et al., 2012). These qualities are established or demonstrated for specific cell lines by carrying out assays such as cell species-specific profiles of isoenzymes, cytogenetic markers, karyotype of cell, DNA finger prints, immunological characterization, life span, purity tests (tests for bacteria, fungi, mycoplasma, mycobacterium) and in vitro tests for adventitious agents (cytopathic effects) and haemadsorpting viruses.

Tissue culture can be used for the production of various types of influenza vaccines such as recombinant protein, life attenuated (LAIV) , inactivated, split-virus and subunit vaccines (Pau et al, 2001; Kister et al., 2007; Hu et al., 2008). Manufacture of live-virus vaccines has the most stringent regulations because downstream processes must not affect the integrity of live virus to maintain potency. To produce influenza vaccine using tissue culture, master and working concentration cell banks are established following regulatory bodies guide lines to meet requirements for continues cell lines used for the manufacture of biological products. Virus seed bank is also generated from the primary seed virus (selected vaccine strain through reverse genetics or wild type isolate) through passages.

Cell culture technology has many attractive advantages such as being more immunogenic and protective because HA and NA of cell culture isolates are known to be structurally identical to clinical isolates and easy supply of cell substrates (Robertson, 1993). Others are large scale production with bioreactors, faster scale up and better characterized vaccine (Ghendon et al., 2005; Steel, 2011). However, apart from safety concerns associated with development and production as for the other conventional technologies, the stringent regulations given by regulatory bodies makes it difficult for developing countries to adopt. It will be a good decision to start the foundation of influenza vaccine development in a country, zone or region with a less sophisticated and safer technology that does not put the very population it seeks to protect at a greater risk.

LIVE ATTENUATED INFLUENZA VACCINE

Live attenuated influenza vaccines (LAIVs) are influenza vaccines made up of modified live viruses which induce protective immunity against challenge influenza virus infection in animals, birds or humans without reversion to virulence. Unlike other types of influenza vaccines, they can be delivered sublingual to birds or by spray or drops intranasal instead of injection, giving them advantage of better protection of vaccinated subjects (Wressnigg et al., 2009; Park et al., 2012). LAIV is derived by genetic reassortment or reverse genetics technology and further modified by adaptation to replicate at lower temperatures (25 - 33°C) to obtain cold-adapted (*ca*), temperature sensitive (*ts*) or attenuated (*att*) virus strain (Snyder et al., 1988; Solorzano et al., 2010). Many authors have reported that LAIVs induce immune response that protects

against heterosubtypic influenza infections, have poor transmissibility, genetic and phenotypic stability (Suguitan et al., 2006; Lanthier et al., 2011). Generally LAIV influenza vaccines development, production and administration has a big setback of the possibility of reversion and re-assortment with a wild type in birds to produce more virulent new strain. Also there is genetic instability potential due to mutations that can affect safety and efficacy. To develop, manufacture or store LAIV influenza vaccines, containment facilities are required to prevent accidental release of virus into the environment. It is evident that LAIV influenza technology is not ideal for a developing country, zone or region due to the above drawbacks.

Convincingly, conventional influenza vaccine technologies quite good as they may be, their development or adoption in a developing country, zone or region could have more disadvantages than advantages. And it can clearly be seen why governments in developing countries especially in Africa are often advised by their technocrats and professionals to opt for "no vaccination" policy and hence little or no attention is paid to avian influenza vaccines development or vaccination. It is however important to notice that most of the safety concerns associated with the conventional technologies also applies to developed countries. And this is evident in the trend of research activities in influenza vaccines development in the 21st century where new approaches and strategies are in the lead to provide vaccines that are safer, cheaper and more effective. Many of these new approaches have shifted away from the direct use of influenza viruses in the development or manufacture of influenza vaccines. These approaches are near ideal for developing countries especially in Africa to lay a solid foundation to tackle eminent reoccurring devastating disease that infects a lot of poultry populations with attendant consequences.

NEW INFLUENZA VACCINE TECHNOLOGIES

Many factors have led to a dramatic increase in the development of influenza vaccine technologies over the years which include lack of sterile immunity by vaccines produced using the conventional technologies, advancement in the fields of immunology and molecular biology, increased AI outbreaks globally since the late 1990's, the 2009 pandemic and the prediction of a likely H5N1 pandemic (Shaw, 2012). The world wide spread of the HPAIV H5N1 and the emergence of other subtypes as highly pathogenic reassortants may also have provided the impetus to find safer technologies for influenza vaccine development and production. New influenza vaccine development processes (concept, discovery, derivation, vector design, construction of recombinants, cell propagation, antigen production) are now broader and differ from the conventional as many are seeking improvement upon or replacement with safer and more efficient processes. Viral or bacterial vector derivation of vaccine cells utilizing gene-based methods together with mutagenesis and reverse genetics(reverse vaccinology) are playing vital roles in the development of new vaccines(Wu et al., 2010; Chen et al., 2010; Peeters et al., 2012).

The direct use of influenza virus is the most critical safety concern in the development, production and utilization of influenza vaccines. Risk of escape of the virus into the environment and exposure of the laboratory personnel have been the greatest concerns in the development, production and administration of avian influenza vaccines in most developing countries. The current concept of withdrawal of direct use of the virus can give advocators the impetus to encourage scientists in these countries to begin aggressive research in the development of influenza vaccines using the new and emerging technologies. It is obvious from passed outbreaks that developing countries hardly have comprehensive control programmes because they depended almost entirely on external help to control avian influenza outbreaks. Unfortunately, it seems so even for a deadly disease like ebola which has killed hundreds across West Africa. In this review, the new influenza vaccine technologies are presented in the light of the fact that they can be applied for human, mammalian and avian influenza vaccines development. New influenza vaccine technologies have been categorized in so many ways by many authors and based on our research we will harmonize them into six categories namely: gene-based, genomics-based, subunit, plant-based, virus-like particles and universal vaccine technologies.

GENE/VECTOR-BASED INFLUENZA VACCINE TECHNOLOGY

This is a vaccine technology hitched on the manipulation of influenza virus genes in other organisms that are non-virulent to produce immunogenic antigens in vivo or in vitro to ultimately provide sterile immunity against influenza virus infection. There are many facets to the technology but has the fundamental principle of using influenza virus genes to develop vaccines without necessarily propagating the virus of gene origin. The technology can be used for the development of subunit vaccines, bacterial vector vaccines, viral vector vaccines, recombinant DNA vaccines, conjugate vaccines and plant-based vaccines (Srivastava and Liu, 2003; Kaslow, 2004; Tiwari et al., 2009; DiNapoli et al., 2010). The basic process of vaccine derivation is the same and involves a series of protocols: gene selection, gene isolation or amplification, vector design or selection, construction of clones (for example, recombinant virus or bacteria),

transformation (transfection or transduction), virus recovery, recombinant product isolation and purification and immunogenicity tests among others.

As a source of gene of interest, selection of influenza subtype or reassortant is made based on the epidemiology of the infection in a country or zone or compartment with the knowledge that sterile immunity is best achieved when antigen is a derivative of the circulating virus as recommended by international regulatory bodies such as WHO and OIE. Among the 8 influenza gene segments, HA and NA have proved to be the most immunogenic (Dormitzer et al., 2011). Other genes have also been used in experimental influenza vaccines development alone or in combination with others (monovalent or multivalent vaccines). For instance bivalent antigenically conserved fusion-active subunit of HA (HA_2) and ectodomain of matrix protein (eM2) has been shown to be a broad protective vaccine with a potential to serve as a pandemic vaccine (Stanekova and Vareckova, 2010). Recombinant baculovirus co-expressed HA, NA and M1 proteins have been shown to self-assemble as VLPs and found immunogenic and protective in mouse models (Tao et al., 2009). DNA vaccines encoding nucleoprotein (NP), HA, NA and M1 components have been shown to induce CD8 + CTL responses in mice and ferrets and protected the animal models against challenge with antigenic drift variants of H5N1 (Ulmer, 2002). Influenza A viruses have negative sense, single stranded segmented RNA genomes and to be used for gene synthesis, cDNAs and ORFs are generated by RT-PCR, cloning and restriction enzymes extraction. The ORFs can be modified by silent mutation using primers to obtain mutagenized ORFs and subsequently modified gene products through amino acid alterations. The backbone of gene-based influenza vaccine development is vector design and construction of recombinant clones. Vectors designed may be plasmids, viral or bacterial which are used for recombinant DNA, viral or bacterial vaccines.

Design of licensable vaccine vectors insure high yield, integrity and stability during production as specified by regulatory guide lines, for instance all new vectors require extensive pre-clinical testing to validate safety and performance prior to clinical use (Williams et al., 2009). Today due to advancement in molecular biology and biotechnology, there are many commercially packaged vectors with varieties of expression elements (eukaryotic enhancers, promoters, terminators, polyadenylation signal) suitable for influenza virus genes manipulation and gene-based vaccine development. A few examples are bacteriophage T7 promoter and terminator, RNA Pol I and Pol II cassetes (Hoffmann et al., 2002), polymerase II promoter/terminator cassette from human CMV (Hoffmann et al., 2000) and polI promoter/terminator cassettes from humans and chickens. There are several gene-based influenza vaccine technologies (*in vivo* and *in vitro* expression systems) and we shall discuss some with their advantages and drawbacks.

VIRAL VECTORS INFLUENZA VACCINES

This is a technology based on the use of recombinant viral vectors to develop new influenza vaccines. Viral vectors have been found to express foreign proteins in vitro and to deliver genes in vivo, expressing the protective proteins in vaccinated hosts as replication defective or replicating viruses (Madhan et al., 2010). To avoid biosafety compromise most viral vectors are based on attenuated or replication-defective viruses (Draper and Heeney, 2010).

The technology of using recombinant viral vectors to deliver foreign genes in vivo as vaccines started in 1985 and have been found to induce cell-mediated immunity (CMI) and strong humoral immune response (Perkus et al., 1985; Druper et al., 2008). The upstream processes of viral vectors vaccine development are fast and avoid the handling of live virus of gene origin and the costly biosafety containment. A quick cloning of a new strain can be undertaken and non-specialized production facilities with surge capacity can be used especially during pandemics (Josefsberg and Buckland, 2012). The current technology can remove immunomo-dulatory genes and create a marker for differentiation between infected and vaccinated animals (DIVA) and at the same time ensuring that the protective antigens are expressed. Some authors have reasoned that a few effective veterinary vaccines are available because conventional methodologies may still be the main methods of vaccine development (Brun et al., 2008).

Generally, characteristics of good viral vectors for vaccine development should include among other things: efficiency at delivering DNA to target cells, capacity for incorporation of cDNA expression cassettes, low potential for oncogenesis, capability of inducing humoral and T cell-mediated immunity in the absence of adjuvant, elimination of pre-existing immunity and ability to generate antigens with native conformation (Levine and Sztein, 2004). Others are ability to grow to high titers for replication viral vectors, lack of integration into host genome, physical and genetic stability (Rao et al., 2009). Viral vectors for the development of veterinary vaccines have additional characteristics required like ability to distinguish between infected and vaccinated animals (DIVA), time taken to induce protection should be short, low potential for environmental spread of the vaccine virus even in non-targeted species and need for low cost of production (Brun et al., 2008). There are quite a number of DNA viral vectors being used at the experimental and commercial levels for influenza vaccine development and production for example poxvirus, adenovirus, herpes virus, baculovirus, modified vaccinia virus Ankara (MVA) based. RNA viral vectors include recombinant NDV(rNDV) vector, alpha virus vector,

vasicular stomatitis virus vector(Robert-Guroff, 2007; Jin et al, 2008).

TYPICAL VIRAL VECTORS INFLUENZA VACCINE TECHNOLOGIES

Baculovirus expression vector system (BEVS)

This is a recombinant DNA viral vector expression system based on the baculoviruses which have covalently closed circular, double-stranded DNA genome of about 80 - 180 kbp (Ayres et al., 1994). Baculovirus unique characteristic of transducing cells of human, rodent, porcine, bovine, rabbit, fish, and avian origin has made it favorable for viral vaccine development (van Oers, 2006; Hu et al., 2008). Many mammalian cell-active expression elements have been utilized in the construction of BEVS which include promoters from cytomegalovirus (CMV), rous sarcoma virus, Simian virus 40(SV40), chicken beta-actin G(CAG), human ubiquitin C, hepatitis B(HBV), Early to late (ETL), immediate early promoter from White Spot Syndrome Virus(WSSV ie1) (Ghosh et al., 2002; He et al., 2009). BEVS has cloning capacity as large as 38 kb and hence can accommodate large inserts or multiple genes (Chen et al., 2010). Shuttle promoters (ETL, WSSVie1) have been used to enhance the expression of exogenous and de novo antigens to stimulate humoral and cell-mediated immune responses (Fang et al., 2010). BEVS can be used for large scale production of recombinant proteins as it can efficiently express foreign proteins in mammalian cells once the gene is placed under transcriptional control of mammalian cell-active expression elements and mediate efficient gene delivery. Many features have made baculovirus favorable as a viral vector for vaccine development, and these include: inability to replicate in mammalian cells, absence of cytotoxicity, superior biosafety profile when compared to other viral vectors, the potential to mediate innate immunity and acting as an adjuvant by stimulating protective innate immunity against various infectious agents (Elliot, 2012).

BEVS has moved from being a research tool only, to an established manufacturing platform for the production of novel biologic products. It is a technology used for the manufacture of veterinary vaccines: for example, Porcilis Pesti (MSD Animal Health), BAYOVAC CSF E2®/Advasure[a] (BayerAG, Pfizer Animal Health) for classical swine fever; Circuvent® PCV(Merck Animal Health), Ingelvac Circo FLEX®) for Porcine ciruvirus type II. Also human vaccines for human papillomavirus and influenza are produced using the BEVS technology: Cervarix®(GlaxoSmithKline) for human papilloma virus and Flublok®(Protein Sciences Corporation) are typical examples (Felberbaum, 2015). The technology is also used in producing recombinant proteins, developing bioprocesses for producing proteins of interest and as a gene delivery system in vitro and in vivo for gene therapy (Contreras-Gomez et al., 2014). Recombinant baculovirus vector pseudotyped with G glycoprotein of Vesicular Stomatitis virus (VSV-G) has been shown to express influenza virus HA and induced HA-specific antibodies that conferred complete protection against H5N1 challenge in chickens(Wu et al., 2009). Co-expressed with VSV-G in baculovirus, the HA protein could be delivered into host cells to elicit immune response in a long term. Tao et al. (2009) and Behzadian et al. (2013) used BEVS to express recombinant HA, NA and M1 of H5N1 origin which self-assembled to form VLP and conferred complete protection against heterologous H5N1 isolates. These results show that bacullovirus vector based influenza vaccine technology is promising as evidenced by induction of superior immune responses in the pre-clinical studies reported.

Recombinant Newcastle Disease Virus (rNDV) Vector Influenza Vaccine

Reverse genetics (RG) has made it possible to utilize the non-segmented, single stranded negative sense RNA viruses like the Newcastle disease virus (NDV) as the backbone of a viral vector influenza vaccine technology (Römer-Oberdörfer et al., 1999; Peeters et al., 1999; Krishnamurthy et al., 2000; Huang et al., 2001). These authors successfully used both lentogenic vaccine strains (clone 30; LaSota) and mesogenic strains (Beaudette C; NDV/B1) as the backbone for the construction of recombinant NDV vaccine vectors and since then they have been used in the experimental development of bivalent NDV-AIV gene based vaccines. Recombinant NDV vector expressing AIV immunogens has been found to have the potential to simultaneously protect poultry against these highly contagious diseases and at the same time solve the problem of differentiating infected from vaccinated animals (Ramp et al., 2011). Huang et al.,(2003) and DiNaploi et al. (2010) gave elaborate advantages of rNDV vector as a potential ideal influenza vaccine vector which include: low cost of vaccine production and administration particularly due to dual use as a bivalent ND-AI vaccine which can be administered through aerosol sprays, drinking water or eye drops; growth to a very high titer in many cells and eggs; induction of cellular and humoral immune response in vivo; NDV naturally infects via respiratory and alimentary mucosal surfaces, so it can deliver AIV protective antigens; NDV replicates in the cytoplasm of infected cells without a DNA phase, which eliminates the problem of integration of genome into the host cell DNA; NDV does not undergo detectable genetic recombination hence it is stable and safe.

AIV genes have been successfully expressed in rNDV by insertion between P and M genes (Nakaya et al., 2001; Park et al., 2006; Ge et al., 2007; DiNapoli et al.,

2010), F and HN genes (Veits et al., 2006; Schröer et al., 2009) and between M and F genes (Ramp et al., 2011). A study by Zhao et al. (2015) based on the measurement of the expression of recombinant green flourescence protein (GFP) demonstrated that the non-coding region between P and M genes is the optimal insertion site for foreign gene expression in the rNDV vaccine vector. Recombinant NDV vectors with different AIV gene inserts have been developed and only few have been licensed for use in China and Mexico (Swayne, 2009). In China it has been shown experimentally that, rNDV-based AI vaccines induce greater immune responses than inactivated AI vaccines (Fan et al., 2015) and three of such vectored vaccines have been developed and tested under experimental conditions. Kim et al. (2014) showed that a modified Beaudette C can be used safely as a vaccine vector, with enhanced replication, expression and protective efficacy in avian species. Liu et al. (2015) demonstrated that NDV-vectored H7 and NDV-H5 vaccines are able to induce high HI titer antibodies and completely protected chickens from challenge with the novel H7N9 or H5N1 viruses respectively. Recombinant NDV vector is also a potential human vaccine vector against emerging and re-emerging pathogens such as HPAIV and severe acute respiratory syndrome corona virus (DiNapoli et al., 2007).

Adenovirus vector influenza vaccine

Adenoviruses (Ads) are DNA viruses that have non-enveloped icosahedral linear double -stranded DNA (33-38 kb) genome flanked by two inverted terminal repeats (ITRs), and a packaging signal sequence (ψ) adjacent the left ITR. The genome is divided into early (E1a, E1b, E2a, E2b, E3, E4) and late (L1 - L5) regions and is associated with viral core proteins described as hexon, penton and knobbed fiber proteins (Russell, 2009). The E1 region genes are essential for replication and recombinant adenovirus (rAd) vectors are constructed either by insertion or replacement of viral genes in the principle sites - early regions E1, E3 and E4 (Imler, 1995). Recombinant Ad vectors have been extensively utilized in both human and animal vaccine development since 1977 when the first rAd vector was constructed by Graham and colleagues (Zhang, 2012). Human Ad serotype 5(Ad5) is the best characterized and the most widely used rAd vector and both replication-competent and replication-defective types have been used in experimental vaccine development (Eloit et al., 1990; Zhu et al., 1999). Apart from rAd5 a number of other rAd vectors based on the rare serotype human Ads(Ad11, Ad26, Ad35, Ad48, Ad49, Ad50) and non-human serotypes such as chimpanzee Ad, bovine Ad3, canine Ad2 and porcine Ad3 have been used in rAd vectors construction (Singh et al., 2008; Roy et al., 2007; Patel et al., 2010).

Recombinant Ad vectors have been described as the most widely and heavily exploited viral vectors for vaccine development because the vaccines have been proved to be safe in pre-clinical and clinical studies against a number of infectious diseases (Coughlan et al., 2014). The technology has well established technique for rAd vaccines construction, large scale production in suspension cells (PER.C6) with low cost of production, a quality needed for influenza vaccines production (Kovesdi and Hedley, 2010). Recombinant Ad vectored vaccines can be prepared as suspension solution or lyophilized and stored at fridge temperature of 4°C for at least one year (Evans et al., 2004). They serve as adjuvants because of their potential to induce innate immune response (Hartman et al., 2008). Recombinant Ad vectored vaccines can be administered by various routes because they can infect a wide variety of cell types (Tutykhina et al., 2011). As a result of natural tropism for the respiratory tract, Ad vectored influenza vaccines are well suited for intranasal mass vaccination campaigns and have been found to induce better mucosal and systemic immunity than other recombinant vector systems currently available(Kim et al., 2014). Replication-competent Ad vectored vaccines also have dose-sparing effect due to the replication of the vaccine vector in vivo which mimics natural infection and hence can provide complete protection (Robert-Guroff, 2007).

However despite the substantial progress in molecular engineering of rAd vectors, they have proven difficult to construct and produce (Seregin et al., 2009; Dharmapuri et al., 2009). It has been observed that when cell lines like HEK293 are used during homologous recombination, replication-competent adenoviruses (RCA) can be generated within the rAd vectors, giving rise to undesired Ad infection(Zhang, 2012). Also high doses are needed to elicit immunity in the case of replication-defective forms (Robert-Guroff, 2007). As at few years ago adenovirus vector based human influenza vaccines preparations are many and at various stages of trials e.g. preparations by Vaxart San Francisco, USA and GenPhar, NY, USA were at preclinical stage, while preparations by PaxVax San Diego CA, USA and Vaxin Birmingham, AL, USA were at phase I stage of trials (Stephenson et al., 2010; Zhang et al., 2011). Vemula et al. (2013) tested an Ad vectored multivalent AI vaccine and observed high levels of humoral and cellular immune responses which conferred protection against challenge with H5, H7 and H9 subtypes. Kallel and Kamen (2015) described the general principles of large scale production of adenovirus vectors for vaccines with manufacturing methods that rely on well-established cell culture technologies.

Fowl pox virus vectored influenza vaccine

Fowl pox virus (FPV), a member of the *Avipoxvirus* genus

is limited to avian species and made up of double stranded DNA genome of up to 300 kb. It has been used as a live virus vector for the delivery of antigens both to its natural avian hosts as well as to mammalian hosts (Bublot et al., 2010). The non-essential regions of the genome such as thymidine kinase gene locus are used as insertion sites and foreign proteins expressed under the control of strong promoters like vaccinia virus H6 promoter (for example, ATI-p7.5 x 20), $P_{E/L}$ promoter (Fuchs et al., 2009). Sites for insertion of foreign DNA on FPV vector are the TK gene locus, terminal inverted repeat regions and the site homologous to the vaccinia virus TK. The poxvirus promoter is necessary to ensure expression of inserted vaccine antigen. The fowl pox recombinant viruses trade named VFP89 and VFP2211 have been used in the development of commercial AIV inactivated vaccines like TROVAC® - AIV H5(Merial Select, Inc. Gainesville, GA) and GALLIMUNE® H5N9 (Merial Italia, SPA Noventa Italy) (Bublot et al., 2010). Chicken embryo fibroblasts cells, chicken embryo skin cells, human 143B cells, murine L929 cells have been used in transfection and propagation of fowlpox virus influenza vaccines (Leong et al., 1994). Fowlpox virus vector can accommodate over 25 kb foreign DNA and hence can be used for the development of multivalent influenza vaccines (Boyle et al., 2004). When used as a live virus vector vaccine, it is not likely to pose a danger as it has no potential of insertional mutagenesis because it replicates in the cytoplasm of infected cells and does not integrate into the host genome. Replicating FPV vaccine can induce a long-lasting immunity after a single dose because it is capable of inducing humoral and cellular immunity depending on the promoter controlling the expression of immunogen (Skinner et al., 2005). However, replication-deficient FPV influenza vaccine or the inactivated form requires high titer or immunogen concentration, adjuvant and booster injection to achieve sufficient protection (Qian et al., 2012). Also toxic side effect and inhibitory action on weight gain of chickens and immune function has been observed (Mingxiao et al., 2006).

BACTERIAL VECTORS INFLUENZA VACCINES

Bacterial vectors have been used as live vaccine delivery systems (heterologous or homologous expression) or for the production of recombinant immunogenic proteins which can be isolated and purified as subunit influenza vaccines (Srivastava and Liu, 2003). The disprove of influenza vaccine dogma that recombinant HA molecule has to be produced in a vertebrate cell substrate for proper folding, glycosylation and secretion has given an impetus to the utilization of bacterial vectors in influenza vaccines development(Shaw, 2012). For instance it has been shown that knocking out the various N-linked glycosylation sites on HA had no measurable impact on immunogenicity in mice (Bright et al., 2003). Good as it seems, authors have their concerns in the use of bacterial vectors especially as live bacterial vectors. The expression of foreign antigens in live bacterial vectors can be complicated and faulty. There can be improper protein folding, lack of post translational modification, degradation of foreign proteins by bacterial proteases, inclusion of toxic protein sequences, lack of proper secretion, ineffective presentation to the immune system. And these can lead to the induction of non-protective immune response or the complete abolition of immune response (Kotton and Hohmann, 2004). At the level of construction, the author should note that choice of promoter, localization within the vector, codon optimization and plasmid copy number can all affect foreign antigen expression and careful consideration of these concerns can lead to the development of the best bacterial vaccine delivery system. Some bacterial vectors being used in the development of influenza vaccines include, *Salmonella*, *Listeria monocytogenes*, *Shigella*, *Lactococcus* and *E. coli* among others (Hargis et al., 2008; van den Berg et al., 2008).

Salmonella vectors are widely considered as delivery systems for heterologous antigens because of their ability to invade nonphagocytic cells such as those in the intestinal epithelium (Rüssmann et al., 1998). Attenuated *Salmonella* species vaccine strains (for example, *S. typhimurium LT2 LB5010*[r-m+]; *S. typhimurium BRD509*[*ΔaroA*, *ΔaroD*] have been used for influenza vaccine development and promoters like *nir15*, *nirB* engineered to express various proteins have been included (Liljebjelke et al., 2010). Salmonella vectors are considered suitable for the delivery of vaccine antigens because they induce T cell immune responses (Atkins et al., 2006) and are able to invade enterocytes, replicate and persist in the M-cells of Peyer's patches, survive in macrophages, allowing antigens to be processed and presented to immune cells (Layton et al., 2009; Jose da Silva et al., 2014). Also they are known to stimulate local immune responses at the site of pathogen entry which can aid in conferring protection (Liljebjelke et al., 2010).

Lactic acid bacteria (*Lactococcus, Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus, Weisella*) have been described as excellent candidates as delivery vectors of therapeutic proteins and mucosal vaccines and could serve as alternative to attenuated live influenza vaccines (Bermudez-Humaran et al., 2011; Pellissery and Nair, 2013). They have been evaluated as mucosal delivery vectors of antigens and cytokines as alternative to oral or intranasal administration of soluble proteins which have been observed to have low immunogenicity (Wells and Mercenier, 2008). *Lactobacillus* spp. possess qualities suitable for use as live vehicles for the delivery of immunogens especially mucosa vaccines. They can transit through the stomach associated with the intestinal

epithelium, have immunomodulatory properties, induce inflammatory responses against infection, increase IgA production, activate monocytic lineages, can regulate the balance of the Th1 and Th2 pathways and have adjuvant-like effects on mucosal and systemic immunity(Wang et al., 2012). The non-pathogenic status of lactic acid bacteria circumvents the need to construct attenuated mutants. Based on these qualities, Wang et al.,(2012) constructed lactobacillus strains vectors(LA4356-pH and DLD17-pH) that expressed HA1 from H5N1 and demonstrated their efficacy in increasing anti-HA IgA in mucosa, anti-HA IgG in serum and increased expression of IL-4. *Lactococcus lactis* vector expressing HA gene (*L. lactis pgsA-HA1 strain*) orally administered with cholera toxin subunit B was also shown to completely protect mice from lethal challenge of H5N1 virus (Lei et al., 2012). Studies to present influenza virus antigens and to improve their immune efficiency in lactic acid bacteria vectors have been explored and mucosal immune route has been regarded as the best to confer protection (Asahi-Ozuki, et al., 2006; Lei et al., 2010). Although the first human trial with a *Lactococcus* strain vector that expresses recombinant interleukin-10 has been completed(Wells and Mercenier, 2008), no lactic acid bacteria based influenza vaccine has yet reached a trial stage that we know. However, studies to present influenza virus antigens and to improve their immune efficiency in lactic acid bacteria vectors are ongoing and there is hope that the advantages in lactic acid bacteria vectors will be harnessed in influenza vaccine development. There are however, safety concerns in the use of lactic acid bacteria vectors and the popular concern like for other organisms is the potential hazard of introducing genetically modified organism to the environment. Pellissery and Nair, (2013) raised the concern of the potential for horizontal transfer of plasmid to other bacteria, thereby posing the potential threat of introducing pathogenic and antibiotic resistance markers to the environment microflora.

Several authors have given advantage to *Listeria monocytogenes* in the delivery of heterologous vaccine antigens and DNA vaccines to the host immune systems because of their tropism in a variety of cells and their cytoplasms (Yin et al., 2011; Saxena et al., 2013). They have efficient cytosolic entry of antigen presenting cells, innate immunostimulatory properties, and ability to stimulate vigorous CD4 and CD8 T cell responses. However, there are serious safety concerns associated with recombinant *L. monocytogenes* as vaccine vector due to the high degree of morbidity and mortality that results from naturally acquired infection (Johnson et al., 2011). Strategies to remove these concerns have been developed by deletion of specific virulence genes (Shen et al., 1995; Brockstedt et al., 2004). Some recombinant *L. monocytogenes* vectors developed and evaluated include *L. monocytogenes* ∆*actA/plcB(BMB72)*, *L. monocytogenes* ∆*actA/inlB((BMB54)*, *L. monocytogenes dal dat*, L. monocytogenes DP-L2028(Maciag et al., 2009; Zhao et al., 2005; Ikonomids et al., 1997; Eypper et al., 2013). Ikonomidis et al. (1997) evaluated L. monocytogenes vectors (strain DP-L2840 and DP-L2851) expressing full length NP of H1N1 subtype (A/PR/8/34) and the constructs were able to induce influenza -specific CTL and accelerate viral clearance. Later, Johnson et al. (2011) demonstrated the use of two *L. monocytogenes* vectors (∆*actA/inlB((BMB54*, ∆*actA/plcB(BMB72*) as delivery vectors which were able to secrete heterologous nucleoprotein antigens from influenza and mucosal immune response exhibited. Whereas *L. monocytogenes* vectors are extensively being explored as delivery vectors for many human and animal pathogens, its use for influenza vaccines development is limited. This makes it a fallow ground for influenza vaccines development, considering the advantages accorded it by many authors.

E. coli vectors are extensively utilized in the development of influenza vaccines and several of these vaccines have been shown to be effective in animal models (Song et al., 2008; Wei et al., 2008; Aguilar-Yanez et al., 2010; DuBois et al., 2011). For instance many study groups have produced influenza recombinant HA in *E. coli* which are immunogenic in animals (Treanor et al., 2010; Liu et al., 2009; Taylor et al., 2011). The advantage of *E. coli* as a vaccine vector lies in its speed and capacity as the bacteria can grow to high titre in chemically defined medium and can produce up to 20% of their total protein as recombinant protein (Shaw, 2012). This advantage makes *E. coli* based influenza vaccine attractive in the face of a pandemic. Welsh et al. (2012) demonstrated the suitability of *E. coli* vector for the production of cell-free HA head domain trimer which proved to be effective as an influenza vaccine. *E. coli* vectors are more suitable in the production of subunit proteins such as HA, NA, NP, NS, M in the case of influenza viruses that can elicit specific immune responses (Kemble and Greenberg, 2003). *E. coli* based influenza vaccines though have attractive theoretical advantages, have not progressed rapidly towards licensure due to lingering concerns of potential DNA integration, duration and extent of immunity. Also safety concerns caused by eliciting immune response not induced by natural infection is always entertained.

PLANT BASED INFLUENZA VACCINE TECHNOLOGY

Current biological research works show that plants are being utilized to develop and produce vaccine antigens for influenza (human, birds, mammals), but mainly dwell on subunit-based approach (Chichester et al., 2009). Several authors are of the opinion that plant-based influenza vaccines production platforms offer comparative advantage over other expression systems (Twyman et al., 2005; Yusibov and Rabindran, 2008; Mett et al., 2008; Lopez-Macias et al., 2012; Kalthoff et al., 2010). Major advantages ascribed to this approach include

safety, timely development and production, cost efficiency, target solubility, post- translational modification, scalability and adherence to DIVA principles. Strategies utilized in many studies to produce recombinant proteins in plants include nuclear, chloroplast (transgenic systems), RNA viral vectors-based systems and launch vectors-based approaches (Kumar and Daniell, 2004; Streatfield, 2006; Musiychuk et al., 2007; Marillant et al., 2004). Plant-based approach to influenza vaccine development have been shown to produce native HA antigens with immunogenic characteristics and demonstrated complete protection and cross-protection in animal models (Mett et al., 2008; Shoji et al., 2009).

Kalthoff et al. (2010), showed that Launch Vector strategy using crucifer tobacco mosaic virus vector containing *Arabidopsis thaliana* actin-2 gene promoter could express HA recombinant protein in *Nicotiana benthamiana* and was highly immunogenic and fully protected chickens against lethal challenge infection with H5N1. Kanagarajan et al. (2012) expressed a H7N7 recombinant haemagglutinin (rHA0) in *Nicotiana benthamiana* via a cowpea mosiac virus (CPMV)-based vector (PEAQ-HT). In their report, rHA0 exhibited structural and functional properties of native HA protein and maintained native antigenicity and specificity and thus providing a good antigen to induce immune response in poultry. Also Jul-Larsen et al. (2012) expressed rHA0 in tobacco plants which was shown to be a promising vaccine candidate. Mardanova et al. (2015) reported the production of a chemeric construct of recombinant flagellin of S. typhimurium fused to four tandem copies of M2e peptide(Flg-4M) produced in N. benthamiana that induced high levels of M2e-specific serum antibodies and protected mice against lethal challenge with influenza virus. D'Aoust et al. (2009) in a review summarized that plant-based recombinant HA ectodomain in fusion with carrier proteins or other peptides can be produced by agroinfiltration and can induce haemagglutination inhibition antibody response in model animals.

However, they observed that in all the plant-based research works reviewed, high dosage and multiple injections were required to induce immune response which is a drawback to this approach. It is important however to analyze that compared to the safety concerns associated with the direct use of influenza virus in vaccine development, high dosage administration is a lesser evil as rHA0 antigens can easily be mass produced in plants to meet demand. Purification of the recombinant HA antigen and its administration together with other recombinant influenza virus derived antigens such as NA, NP might be a solution to the high dosage drawback.

Another attractive approach developed by some authors is the plant-based transient influenza virus-like particle formulation capable of producing VLPs with unprecedented speed. It has been discovered that rHA0 easily accumulates in the producing tissue as VLPs and this led D'Aoust et al., (2008) and D'Aoust et al. (2009) to study extensively the assembly of rHA0 derived from different subtypes of influenza viruses into VLPs upon expression in *N. benthamiana.* They showed that these were true VLPs with lipid bilayer envelope supporting the presentation of HA trimer. In animal studies using mice, these authors showed that the VLPs protected mice against heterologous lethal challenge with A/Turkey/582/06 (H5N1).

It is now evident that plants are ideal hosts for the production of influenza VLPs from the sole expression of HA because sialylated substrates for the attachment of HA to the cell surface are absent (Saint-Jore-Dupas et al., 2007; Seveno et al., 2004). The plant-based strategy for influenza vaccine production in all the studies proved the advantage of speed, productivity and safety which are ideal for the developing countries or regions like Africa especially in the face of a pandemic. The plant-based transient influenza VLPs as we observed is near ideal for African situation as safety concerns, high cost of development and production and speed are all removed. Preclinical and human clinical trials of a number of plant-based influenza vaccine candidates especially the HA VLP vaccine candidates have been going on for some years. For instances, the preclinical and clinical trials of the H5 and H7 HA VLP plant-based vaccine candidates produced by Medicago Inc., North Carolina USA was reported since 2012 (Rybicki, 2014).

GENOMICS-BASED INFLUENZA VACCINE TECHNOLOGY

Genomics-based technology utilizes reverse genetics and sometimes coupled with in vitro mutagenesis to generate recombinant vaccines and gene delivery vaccine vectors. At a glance, the technology seems not suitable for the developing countries. However, the generation of gene delivery vectors particularly viral vectors is quite a useful technology as these vectors can be utilized for delivery or expression of influenza genes known to give immunogenic protection. The technology is used in the development of candidate vaccine viruses especially from highly pathogenic avian influenza viruses such as H5N1 and H7N7 in poultry (Dong et al., 2009; Wood and Robertson, 2007). Viral vectors can be constructed from single-stranded, negative-sense RNA viruses that can express foreign epitopes with the potential to combat influenza infections in both humans and animals (Jones et al., 2005; Schwartz et al., 2007). Peste des petits ruminants virus(PPRV), rinderpest virus(RPV) and Newcastle disease virus (NDV) recombinant vectors have been used to construct viral vectored influenza vaccines which have been tested to be extraordinarily good inducers of humoral and cellular

immune responses (Mebatsion et al., 2002; Ge et al., 2007). These authors observed that experimental animals were completely protected from homologous virus challenge with no virus shading and no signs of disease.

Reverse genetics is a technology used in the creation of a virus from a full-length cDNA copy of the viral genome, referred to as an infectious clone (Lee and Suarez, 2008). It can also be used to engineer deliberate genetic change into a viral genome. This technology is important to the development of avian influenza vaccines in developing countries as recombinant viral vectors other than influenza viruses can be utilized in the design of new vaccines. Viruses known not to infect poultry can be used to design viral vectors to express avian influenza epitopes which confer protection. A good example is the avirulent poultry virus LASOTA already used as vaccine against NDV. Nayak et al. (2009) using RG constructed recombinant NDV(rNDV) expressing H5N1 HA which was demonstrated to serve as a bivalent vaccine for NDV and HPAIV in experimental infections. This technology is considered potentially safe, effective, convenient and affordable. Such vaccines will permit serological differentiation of vaccinated and avian influenza field virus infected animals. The use of avirulent viruses for recombinant vectors construction makes the technology safe for development in African undeveloped environment. Cost effectiveness is also an advantage when the vector used is a vaccine virus for another poultry disease, thus a bivalent vaccine is the target.

VIRUS-LIKE PARTICLES AND NANOPARTICLES VACCINE TECHNOLOGY

In the simplest language, virus-like particles (VLPs) are structural influenza viral proteins which can self-assemble and mimic the morphology of the natural pathogen, but is non-infectious and non-replicating. Many properties brought VLPs to the lime light in vaccinology which include the following: capacity to enter target cells, easy release from infected cells to entice host immune response, structural stability, tolerance towards manipulation to carry and display heterologous molecules, the potential to serve as building blocks for novel nanomaterials, adjuvant properties that can induce innate and humoral immune responses (Ludwig and Wagner, 2007; Kushnir et al., 2012). VLPs are also known to display antigenic epitopes in the correct conformation and in a highly repetitive manner (D'Aoust et al., 2008).

VLP is a major advancement in the development of subunit vaccines and now has diverse applications including vaccines development, targeted drug delivery, gene therapy and immune therapy. It is a better alternative to isolated (soluble) antigens in stimulating strong immune response and its protective advantage is now seen in VLP-based vaccines against hepatitis B virus (HBV) and the human papiloma virus (D'Aoust et al., 2010).

Initially, VLPs formation involved curing viruses of their genomic DNA or RNA and purifying the protein coat which can self-assemble. Today VLPs are produced by recombinant protein expression in vitro using different expression systems. In influenza VLPs vaccines technology, a set of structural protein genes (for example, HA, NA M) are isolated and amplified using techniques like cDNA cloning, RT-PCR and PCR. Influenza virus genes can also be obtained from the Influenza Sequence Database and synthesized e.g. using GeneArt (Germany), the product cloned and subsequently expressed for VLPs (Mahmood et al., 2008). The proteins are subsequently expressed using suitable expression systems, cultured in cells e.g. yeast and can be purified for VLP formation by self-assembly (Plummer and Manchester, 2011). The production of influenza VLPs requires co-expression of several structural proteins, their assembly into particles, incorporation into host membranes and the release of particles from cell membrane. A typical example is the co-expression of HA, NA, M1 and M2 of H3N2 and the VLPs produced in insect cells (Latham and Galarza, 2001). Pushko et al. (2005) and Galarza et al. (2005) also produced VLPs containing HA, NA and M in baculovirus expression system. There are VLPs-based influenza virus vaccines that have undergone clinical evaluation (Landry et al., 2010; Song et al., 2011; Lopez-Macias et al., 2011). Influenza VLPs have been shown to elicit HA-specific serum antibodies and completely protected experimental animals against lethal challenges with influenza viruses (Pushko et al., 2005; Pushko et al., 2007; Khurana et al., 2011).

VLPs-based influenza vaccines technology takes care of a delicate balance of protection and safety as influenza viruses propagation is not involved in the processes and VLPs are not known to be infectious or replicating. The VLPs display antigenic epitopes in the correct conformation and induce strong humoral and cellular immune responses capable of giving complete protection. It is a technology that is suitable for the African situation as it can easily be developed or adopted, production is easily scalable and the current epidemic virus genes can be utilized to give the best protection. VLPs can serve as platforms for attachment or display of foreign epitopes and hence the vaccine can be produced to observe the principle of DIVA in poultry vaccines. Epitopes can be attached through fusion or genetic insertion into capsid for the purpose of mounting immune response to the protein or peptide attached. However, the technology has a drawback as it is difficult to add large epitopes and such proteins when added might hinder VLP assembly.

An off-shot of VLPs-based technology is the nanoparticles platform and there are many tools available for the generation of nanoparticles which include: chemical conjugation, liposomes, emulsion systems, virus-

nanoparticles (VNPs), dendrites and micelles formation (Cui and Mumper, 2002; Petros and DeSimone, 2010; Saroja et al., 2011). Presentation of vaccine antigens in particulate form has advantages over soluble antigens alone as seen in vaccines such as the hepatitis B and the human papilloma virus vaccines. Knowledge of protein structure is now used to design self-assembling nanoparticles that elicit broader and more potent immunity than the traditional influenza vaccines. This approach efficiently formulates existing purified antigen(s), proteins or other biological molecules, into nanoparticles for immunogenicity trials (Gregory et al., 2013). Vaccine development has progressed toward creating highly defined recombinant proteins and synthetic molecules as vaccine antigens (Toussi and Massari (2014). Kanekiyo et al. (2013) genetically fused HA to ferritin to form a self-assembling nanoparticle vaccine that protected ferrets against 2007 H1N1 virus challenge. These authors used a new innovation called PRINT® Technology which can generate particles of broad range of target sizes, shapes and composition. Szurgot et al. (2013) constructed an adenovirus based protein polyvalent and biodegradable nanoparticles with Influenza M1 dominant epitopes which induced cellular immunity in vivo in chickens. Although for humans, Galloway et al. (2013) used the PRINT® technology to formulate nanoparticles containing HA that was effective in murine models. The drawback of the earlier tools is lack of flexibility to control particle size, shape and composition. These show that VLPs and nanoparticles platforms are quite ideal for the development of influenza vaccines in Africa as the equipment and processes involved have low cost.

UNIVERSAL INFLUENZA VACCINE TECHNOLOGY

A new approach to influenza vaccine development is a design based on conserved viral epitopes(matrix 2 ectodomain - M2e, nucleoprotein -NP, matrix1 -M1, C-terminal segment of HA0 -HA2) discovered to exhibit protective immune response(Ekiert et al., 2009; Epstein et al., 2005; Chen et al., 2009; Thomas et al., 2006). The approach is hinged on the fact that these epitopes are conserved across most subtypes of influenza viruses as opposed to the variant HA and NA (Fouchier et al., 2005; Laver et al., 1984). HA2 has been shown to be a potential inducer of protective heterosubtypic immunity which is related to its broad cross-reactivity (Gerhard et al., 2006; Gocník et al., 2007; Varecková et al., 2008). Quite a number of authors(Imai et al., 1998; Okuno et al., 1993; Vareckova et al., 2003; Edwards and Dimmock, 2001) demonstrated that HA2 induced antibodies inhibit the fusion of viral and endosomal membranes, prevent conformational change of HA due to low pH and block the insertion of fusion peptide into the endosomal membrane. Monoclonal antibodies against HA2 subunit and its fusion peptides have also been shown to have broad spectrum protection as a universal passive immunotherapeutic agent against seasonal and pandemic influenza viruses(Throsby et al., 2008; Wang et al., 2010). A combination of the matrix protein 2 and the stalk domain of the hemagglutinin has also drawn general interest for improved antigen design (Zhang et al., 2014). A universal vaccine approach made up of chemeric hemagglutinin construct of unique head and stalk combination was also shown to protect against challenge with H5 subtypes virus and hence a potential of broad protection against a variety of influenza virus subtypes (Krammer et al., 2013).

Extracellular N-terminal domain of M2 protein(matrix 2 ectodomain- M2e) is a 23 amino acid peptide which is highly conserved in influenza A subtypes(Cheung and Poon, 2007), making it an attractive target for preparation of a universal influenza A vaccine. An earlier investigated approach is the fusion of multiple copies of M2e to a carrier protein (for example, hepatitis B virus core protein) which is able to aggregate into VLPs and typical formulations were shown to have long lasting protection against influenza virus experimental infections (Neirynck et al., 1999; Fiers et al., 2004). De Filette et al. (2006) improved the efficacy of M2e-HBc construct by including an adjuvant known as CTA1-DD. Many constructs of M2e engineered by conjugation to other carrier proteins were evaluated by some authors and were found to protect experimental animals against lethal challenges of homologous and heterologous HA subtypes (Ernst et al., 2006; Fan et al., 2004; Hueatt et al., 2008; Liu et al., 2004). These approaches and many more show that M2e is a potential vaccine candidate to induce protective immunity against any strain of influenza A virus and gives a promise for inventing universal vaccine against human and animal influenza. Nucleoprotein (NP) and matrix protein (M1) are also seen as potential contributors to a successful development of a universal influenza vaccine. Although these internal influenza antigens produce antibody responses that do not confer protection, they play important role in cellular immune response. NP and M1-specific Th cells could augment protective antibody response aiding the B cells to produce antibodies specific HA (Lamb et al., 1982).

While these conserved viral epitopes individually have shown their potentials as universal influenza candidates, a new innovation is the combination of these epitopes as a vaccine. A combination of these epitopes is seen to have the potential to give a universal influenza vaccine that can give cross-protection against subtypes of influenza viruses. Pushko et al. (2005), Mahmood et al. (2008) and Tao et al.,(2009) all demonstrated protective immune responses by VLPs made of HA1, NA and M1 at different times and locations. Recently Price et al. (2014) showed that a candidate "universal" vaccine based on NP and M2 formulation protected animals against lethal infection and reduced transmission of influenza virus to

co-housed unimmunized mice.

PERSPECTIVE AND CONCLUSION

Obviously, the history and epidemiology of avian influenza outbreaks in Africa as reported by many authors calls for a comprehensive control programme. As earlier stated, the complexity of AI epidemiology in Africa was linked to informal and formal movement of poultry commodities across boundaries with little or no adherence to standards of international trade. This is a risk factor that exposes Africa to reintroduction of HPAI as is being experienced this year, 2015. The introduction of multiple clades during the last outbreaks gave rise to reassortment activities that produced new sublineages, an indication of the possibility of emergence of more virulent or pandemic subtypes in Africa. The increased evolution of HPAI in birds and exceptional virulence in humans singles out HPAI H5N1 as a pandemic threat (Maines et al., 2008; Fusaro et al., 2009) and it is imperative that a comprehensive control programme should be in place at all times. Birds to human infection was minimal except for Egypt which had up to fifty-four confirmed human cases, as the other two African countries (Nigeria and Djibouti) had only one confirmed human case each. The detection of H5N2 virus in apparently healthy white-faced whistling duck (*Dendrocygna viduata*) in Nigeria suggests the possibility of introduction through migratory wild birds (Gaidet et al., 2008).

To avoid huge economic loss and human fatality due to outbreaks of HPAI H5N1 in Africa, we are of the view that comprehensive control programmes should be established by countries or regions to prevent reintroduction. WHO, FAO and OIE agreed comprehensive control strategies for AI include: education, biosecurity, diagnosis and surveillance, elimination of infected poultry and decrease susceptibility including vaccination of poultry flocks (OIE, 2009; WHO, 2005). Despite the huge economic loss, authors still advocate culling as the time-honored method to control or eradicate AI outbreak and the best way to prevent transmission to humans. However, when the viruses are spread over a wide area and have infected multiple avian species, culling and physical containment alone are not likely to be successful (Marangon et al., 2008; Rao et al., 2009). International organizations such as the World Organization of Animal Health (OIE) and Food and Agricultural Organization (FAO) now recommend vaccination of poultry for the control of AIV and countries like China and Italy have accepted vaccination as a component of AIV control programme.

Inclusion of decrease susceptibility which includes vaccination of poultry flocks makes a control strategy comprehensive in wide spread infections involving multiple avian species. Handy new avian influenza vaccine technology that gives vaccines of high potency, efficacy and complete protection is needed.

Risk of reversion to virulent subtypes, escape into the environment and exposure of laboratory personnel have been the greatest concerns of many countries in introducing vaccination of poultry flocks as part of control strategies (Plummer and Manchester, 2011). The new and emerging influenza vaccine technologies have drastically reduced these concerns because the direct use of influenza viruses in the development and production systems are removed. For instance VLPs-based and universal vaccines technologies are very much promising because influenza viruses propagation is not involved in the processes and VLPs are not known to be infectious or replicating(Ludwig and Wagner, 2007; Kushnir et al., 2012). Plant-based and recombinant NDV (rNDV) technologies are also suitable for the developing countries with enormous advantages such as safety, timely development and production, scalability, and adherence to DIVA principle (Subbarao and Matsuoka, 2013). Recombinant NDV vector based influenza vaccines have been used experimentally as bivalent NDV-AI vaccines and could be administered through aerosol sprays, drinking water or eye drops (Rao et al., 2009). Hence safety issues cannot be a major hindrance to the development and production of influenza vaccine in a developing country or region using these technologies. It has been established that influenza vaccine developed based on the current circulating subtype in an area, country or region gives the best protection because of the phenomenon of antigenic variation or drift. Hence the best influenza vaccine for an area, region or country is the one developed using the current subtype in the location. We are of the opinion that the vaccine developed within a country or region will be more cost effective and potent as routine surveillance can be undertaken to obtain the current circulating subtype which serves as a base for development. It is easier to carry out yearly update of vaccine production as required by international regulatory agencies when it is produced in the country or region of administration.

Another demerit of conventional influenza vaccines is that they have been found not to give absolute immunity in the field and thus vaccination may not be optimal (Marangon et al., 2008; Swayne, 2009). "No Vaccination" proponents do argue that since vaccination can only reduce virus shading and no single vaccine is protective against all subtypes, it is better to exclude vaccination of poultry flocks from the control strategy. It is important to know that a comprehensive control programme for AI is designed to achieve three broad goals: prevention, management and eradication. As intervention strategy, vaccination and use of antiviral drugs could prevent or lessen the severity of the disease. It is now well established that emergency vaccination must be carried out in the face of an outbreak in a well-defined compartment. Also preventative vaccination is considered

when the risk of introduction persists, while routine vaccination be the tool of last option in disease endemic areas (Swayne, 2009). It is quite essential that vaccination is part of a comprehensive control programme. Vaccination of poultry flocks with conventional vaccines has been known to protect against clinical signs and death, decrease virus shading, prevent major outbreaks and provide at least 20 weeks protection after a single dose (Swayne, 2006; van der Goot et al., 2005; Tian et al., 2005; Capua et al., 2004). The new influenza vaccine technologies seem promising to overcome the two major drawbacks of conventional influenza vaccines (lack of complete prevention of virus shading and conferring long lasting protection). VLP vaccines are now known to have broadened immune response and have demonstrated greater immunogenicity when compared with recombinant HA antigen and inactivated influenza virus (Wang et al., 2010). Kushnir et al. (2012) demonstrated that inclusion of M2 in the construction of VLPs showed a broader and improved protection against multiple subtypes of influenza virus. There is a renewed interest in the development of universal vaccines especially for influenza A viruses as a result of the discovery of heterosubtypic protection by conserved epitopes: M2e, HA stalk region, M1 (Corti and Lanzavecchia, 2013; Zhang et al., 2014). These epitopes have been presented in a variety of platforms such as soluble proteins with adjuvants, subunit or domain epitopes fused to carrier proteins, VLPs and nanoparticles and exhibiting cross-protective antibodies (Subbarao and Matsuoka, 2013). There are indications that universal influenza vaccines have the potentials to give long lasting protection.

Developing countries especially in Africa are bedeviled by numerous emerging infectious disease outbreaks and epidemics of both humans and animals. It is glaring that Africa needs to pursue the development and production of vaccines and therapeutics which in our opinion it needs more than any other part of the world. We subscribe to the fact that new influenza vaccine technologies will give vaccines of better efficacy, potency, safety and lower cost of production and administration. As a result of the high cost of vaccine development and production, such a venture can be established as a regional or continental venture under the umbrella of ECOWAS or AU for a start. Better understanding of potential economic value of the new influenza vaccine technologies can help guide investment and development policies of governments, investors, scientists and other decision makers. This is the main thrust of this paper, that at the end those who move the wheels will see the need to embrace not just the new and emerging influenza vaccine technologies but the big value of vaccine development and production to control emerging infectious diseases that thrive so well in Africa. In the quest to become developed countries some days, it is important to know that self-reliance in meeting the health needs of the people is one of the prerequisites of assuming the status of a developed country.

Conflict of interests

The authors did not declare any conflict of interest.

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