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Adjuvants Used In Animal Vaccines-Their Formulations and Modes of Action: An Overview

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Reviews

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ABSTRACT

Vaccination has proven to be the most effective method for the prevention and control of infectious diseases in animals. The success of vaccination depends on many factors but one of the important factors is the selection of the most suitable and efficient adjuvant. An adjuvant is an ingredient of the vaccine which is used to improve the immune response in the animal's body to vaccines. The inclusion of adjuvant to the vaccine preparation results in the abrupt stimulation of the immune system, production of the stronger immune response, activation of the specific type of immunity and increasing the half-life of vaccine antigen. Although the adjuvants were poorly understood in terms of their structure and mode of action with the enhanced knowledge of the mechanism of action of adjuvants and the immune system, new adjuvants are being formulated that are safe and most effective against existing and emerging animal diseases. In this review, we will discuss the currently available adjuvants for animal vaccines with a focus on their mechanism of action. The attention will be given to commercially important adjuvants, including mineral salts, emulsions, TLR agonists, saponins, liposomes, immunoactive polymers and a combination of adjuvants.

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Introduction

Vaccination has remained as one of the effective and efficient mechanisms for the prevention of infectious diseases. Vaccination has proved to be the single medical intervention that is capable of saving millions of lives. Different types of vaccines have been used in animals and poultry to mitigate the ill effects of various diseases to make their rearing economical. But there are many challenges which are associated with vaccinating the poultry and livestock population, such as animal vaccines should be cost effective, they should be easy to administer, they should have increased stability etc.

Vaccination with only the purified antigens generally results in the stimulation of weak immune response with limited T cell activation, thereby making multiple immunizations necessary to elicit appropriate immune responses. Therefore to improve the efficacy of vaccines certain crucial components are added to them in the form of adjuvants.

They can be defined as the substances which significantly improve vaccine efficacy by (i) Increases the antigen uptake by antigen presenting cells (APCs), (ii) Helps in the activation and resultant maturation of

APCs, (iii) Increases the biological half-life of antigens, (iv) activate inflammasomes, (v) induces the production and proliferation of immunoregulatory cytokines, and (vi) Helps in the dose sparing, thereby reducing the quantity of antigen required to cause the production of target antibody titers.

The name adjuvant is a broader term and has distinct meanings depending on the type of application for which they are being utilized in the vaccination. For instance, adjuvants can be used as specific molecules which directly activate immunostimulatory responses and activate immune receptors. In contrast to this, certain adjuvants may act as delivery systems that do not stimulate the immune system directly but help in the proper presentation of the antigen to the components of the immune system. Some other adjuvant systems, which are the combination of two or more adjuvants may serve both as a delivery system as well as immunostimulatory components. Therefore based on the mechanism of action, adjuvants can be grouped into three broader classes as immunostimulatory molecules, as delivery systems and as combinations of the two or more adjuvants (Table 1). The selection of the Adjuvant for the animal vaccine preparation depends on many important factors, including the type of immune stimulation required, chemical properties of the vaccine antigen, route of animal immunization and above all the species of the target animal involved. Selecting a wrong adjuvant may render a good quality antigen ineffective. Therefore, only the adjuvants with the desired characteristics needed for the effective vaccination of animals need to be selected.

In this review, attention will be given to some commercially important adjuvants that are presently utilized for the production of animal vaccines, including mineral salts, emulsions, TLR agonists, saponins, liposomes, immunoactive polymers and a combination of adjuvants.

Mineral salts

Aluminium containing adjuvants have been used for a pretty long time to enhance the immune response to vaccines. These adjuvants have shown their potential way back in 1926 when they were used in diphtheria toxoid containing vaccines. Since then, aluminium based adjuvants have been added into millions of vaccine doses manufactured. Their level of success is mainly because they have good proven safety profile; low manufacturing cost; compatibility with many known vaccine antigens; less adverse reactions. Previously aluminium adjuvant vaccines were prepared by precipitation of the antigen and alum (Glenny et al., 1926). Precipitation of antigen with aluminium salts has long been replaced by the process of adsorption of antigen to aluminium gels because the process of precipitation does not allow control over the degree of adsorption (Gupta, 1998).

The commonly used aluminium adjuvants used in vaccines are aluminium phosphate adjuvant and aluminium hydroxide adjuvant (Dandashli et al., 2002; Yau et al., 2006). Although the use of aluminium adjuvants in vaccines has been traditionally done for quite a long time now, the exact mechanism of action through which they activate the host immune system is not fully understood. Aluminium is known to bind antigens with high affinity and performs its function by the genesis of depot formation at the site of vaccine application. Depot formation allows maintenance of high antigen concentrations at the injection site, thus allowing continuous dispersion of antigen from the aluminium particles (Kool et al., 2012).

Table 1. Classification of Adjuvants

Type	Adjuvant
Immune Stimulators	TLR3 Agonists: Polyinosinic and polycytidylic acid
	TLR4 Agonist: Monophosphoryl lipid A (MPL-A)
	TLR5 Agonist: Flagellin
	TLR 7/8 Agonists: Imiquimod and Resiquimod
	TLR 9 Agonist: CpG oligodeoxynucleotides (ODN)
	NOD Agonist: Muramyl dipeptide (MDP)
	Saponins (QS-21)
Delivery Systems	Aluminium Salts (Alum)
	Calcium phosphate
	MF59
	AS03, AS04
	Incomplete Freund's Adjuvant
	Polylactic Acid (PLA)
	Virus like particles
Combination of Adjuvants	Cpg ODN and Emulsions
	MF59 AND AS03
	Polyphosphazenes and Polyinosinic acid
	Saponins and Emusions
	MF59 and Carbopol 971P
	TLR4 and TLR 7/8 Agonists

Aluminium adjuvant has been seen to show its by inducing the secretion of interleukin (IL-4) cytokine, which in turn stimulates T helper 2 (Th 2) type immune response. This process promotes the formation of IgG1 and IgE immunoglobins and eosinophills which makes these adjuvants good for antibacterial vaccines (HogenEsch, 2002; Ulanova et al., 2021).

The amount of aluminium adjuvant in vaccines is important from the perspective of safety and necessity to induce the desired effect. The quantity of aluminium adjuvant present in vaccine formulation is usually expressed as the concentrations of elemental aluminium per dose. One milligram of Al3+ is equivalent to 2,2 mg of aluminium hydroxide adjuvant and 4,5 mg of aluminium adjuvant (Vecchi et al., 2012).

Aluminium adjuvants have a large adsorptive capacity, thus only a small amount of aluminium adjuvant is required for the absorption of vaccine antigens. Examples in animals where aluminium adjuvants in vaccines have been used include some commercially important diseases like Newcastle disease and foot and mouth disease (Pini et al., 1965; Sellers and Herniman, 1974).

Another important mineral-based adjuvant is calcium phosphate (CaP), which was initially used as an adjuvant in vaccines for some potential human diseases like poliomyelitis, pertusis, diphtheria and tetanus but its use as an adjuvant was completely replaced by the aluminum-based adjuvants in the late 80's. Now, it is again realized that it has the potential to become a good substitute for the aluminium adjuvants (Sesardic et al., 2007).

One of the advantages of the CaP is that it is naturally present as a constituent in tissues of the organism, therefore it is safe and biocompatible. The absorptive capacity of CaP is equal or higher than the aluminium hydroxide, because of the zeta potential which is a difference in electrokinetic potential (Olmedo et al., 2014). Also, the administration of CaP as an adjuvant causes fewer local reactions which are mainly due to reduced IgE immunoglobulin production (He et al., 2000). The use of calcium phosphate as a nanoparticle adjuvant has elicited sufficient immune response to protect laboratory animals against laboratory-induced FMD disease (Joyappa et al., 2009). Although the mineral salts as adjuvants are relatively safer and also show better adjuvanticity in vaccines against extracellular pathogens, their utilization in vaccines against intracellular pathogens is limited.

Emulsions

The emulsions have long been used as vaccine delivery agents mainly because of their ability to induce depot formation at the injection site and thus facilitating the slow release of antigen over a while. The emulsion provides an aqueous phase which traps the antigen and with the progressive emulsion degradation, there occurs slow and spontaneous release of the antigen.

Depotformation protects the small protein antigens from degradation and also helps in the uptake of antigen molecules by antigen presenting cell (Khong and Overwijk, 2016). Traditional oil-based emulsion adjuvants like Freund's complete or incomplete adjuvant had been shown to have limited capability in inducing T cell-based immune responses and also have adverse post immunization reactions (Suzuki and Hazama, 2017).

Two broader types of emulsion adjuvants which are used in vaccine formulations are water-in-oil (w/o) emulsion and oil-in-water (o/w) emulsion. In a water-in-oil emulsion, the antigen is entrapped in an aqueous phase which is surrounded by an uninterrupted oil phase. The classical example of water in oil emulsion adjuvant is Freund's complete and Freund's incomplete adjuvant (without mycobacteria) (Freund et al., 1937). However in the case of water in oil emulsions, several adverse reactions had been seen like local inflammation, the formation of ulcers and granulomas at the injection site (Petrovsky, 2015). Over the years few successful water-in-oil emulsions have been developed for veterinary vaccines which have fewer adverse effects. One such example is Incomplete SEPPIC adjuvant (ISA). This adjuvant has been used in vaccines against porcine enzootic pneumonia (Jorge et al., 2014) and FMD in cattle (Khorasani et al., 2016).

Most of the successful out of the two broader classes of emulsion adjuvants is oil-in-water (o/w) emulsion, which is formed by dispersing oil droplets in the aqueous phase. Oil-in-water emulsion does not form depot at the injection site and stimulate the immune system by activating the differentiation of antigen presenting cells (APC) like macrophages and dendritic cells. Oil-in-water based emulsion such as MF59 which is a squalene based emulsion have good performance by enhancing antigen immunogenicity and causing less

adverse reactions (Dell'Era et al., 2012). Several oil-in-water based adjuvants are commercially available for use in animal vaccines including Emulsigen-D, Montanide adjuvant and Metastim.

Emulsion-based adjuvants are a good selection for animal vaccine preparations because they can be prepared from economical and easily available materials. The exact mechanism of how emulsion-based adjuvants efficiently stimulate the host immune system is not fully recognized and needs more elaborative research.

Liposomes

Liposomes are spherical structures consisting of vesicles which are composed of several concentric lipid bilayers alternating with aqueous phases (Gregoriadis, 2008).

Based on the structure of lipid bilayer, liposomes can be distinguished as unilamellar (ULV) or multilamellar (MLV) vesicles (Bozzuto, 2015). The single lipid bilayer present in unilamellar vesicles is a perfect structure for the encapsulation of hydrophilic molecules while as the concentric lipid bilayers present in multilamellar vesicles are ideal for the encapsulation of lipid soluble antigens.

Liposomes have proved to be efficient vaccine carriers acting as vaccine adjuvant-delivery system (VADS), because of their diverse nature, unique surface bearing structures and good bio-compatibility (Weissig, 2017).Liposomes entrap the antigen thus protecting them from the environmental hazards, delivering antigen to the specific lymphocyte locations and also potentiate the initiation of antigen specific immune stimulation (Moyer et al., 2016).

Use of liposomes have proven to be an efficient alternative as a delivery system for a number of therapeutic agents like antibacterial, antiviral, vaccines, anti-carcinogenic therapies and hormones. Liposomes can be administrated through various routes like intramuscular, subcutaneous, intravenous, naso-oral and as a topical application (Allen and Cullis, 2013).

Application of liposome as a vaccine adjuvant have shown many promising features including no granuloma formation, no hypersensitivity reation at the site of injection, development of strong humoral immune response and wide range of adjuventicity effects (Perrie et al., 2016).

Liposomes have been seen to be equipped with pathogen associated molecular patterns (PAMPs), thus helps in attracting antigen presenting cells (APCs), which in turn interacts with them through pattern recognition receptors (PRRs) resulting in the subsequent triggering of adaptive immune system (Geijtenbeek and Gringhuis, 2016).

The potential characteristics of liposome based vaccinations against wide range of animal pathogens has made liposomes as one of the prospective adjuvant for animal vaccines. Liposomes have been used with a variety of antigens in the different animal species including both the clinical vaccines and experimental vaccine candidates (Korsholm et al., 2012).

Liposome coated nano-particles had been used in vaccines against Newcastle disease virus (NDV) (30). It was seen that the birds immunized with the liposomal Newcastle disease vaccine have higher number of cell counts and also antibody production. Similarly, liposome based vaccine in the form of encapsulated avian pathogenic E.coli (APEC) had been used for control of Colibacillosis in poultry birds (Yaguchi et al., 2009).The immunized birds were found to have lower concentration of APEC based bacteria in their blood.

The liposome associated fimbriae antigens of Salmonella enterica, a common pathogen associated with the poultry and poultry products had been utilized as a vector for the preparation of subunit vaccines against salmonellosis in poultry (Li et al., 2004). Immunization of chicken with these fimbrial antigens helped in the development of notable increase in the immunoglobulin concentrations of IgG and IgA with the resultant decrease in the excretion of bacteria in the faeces of the immunized birds. Liposome based vaccines against parasitic pathogens in livestock animals had been demonstrated to elicit strong immune response against the invading parasites. One such example is the use of liposome-DNA based complexes having plasmid coding for microneme protein, thus providing the effective immune response against Toxoplasma gondii parasite (Hiszczynska-Sawicka et al., 2012).

Saponins

Saponins are naturally occurring substances widely distributed in higher plants having many pharmaceutical applications. Chemically saponins are composed of one or more hydrophillic moieties which are attached to the lipophillic triterpene derivative. Due to their anti-microbial, anti-fungal activities saponins are believed to be involved in plant defence mechanisms (Sparg et al., 2004). The ability of various plant derived saponins to stimulate the mammalian immune system has promoted their application as an adjuvant in number of animal vaccines. (Kensil, 1996).

The saponins currently used in animal vaccines are all extracted from the bark of the South American tree Quillaia saponaria. The most prominent saponin-based adjuvant for animal vaccines is Quil A and its purified faction QS-2 (Santos et al., 2007; Cunha et al., 2012).

Administration of quil A as an adjuvant stimulates both cellular and humoral responses, with the subsequent generation of cytotoxic T lymphocytes responses (Brunner et al., 2010). This type of immune response activated by the Quil A has made it to be ideal adjuvant for the vaccines against coccidial parasites and also against toxoplasmosis (Sun et al., 2009; Zulpo et al., 2012). The other important saponin is QS-2, is the of promising adjuvant which is currently in clinical trails for production of vaccines for many infectious diseases (Zhu and Tuo, 2015).

Adjuvants are substances used to improve the immune response to vaccines; Earlier immunization leads to the development of a more robust immune response (Gerdts, 2015). Saponin complexes as ISCOMs are able to effectively stimulate both CD8+ and CD4+ T cell responses in mammals (Coffman et al., 2010).

The immunological functions of saponins are mainly because of certain structural components present on their surface. It has been seen that sugar moieties present on saponin surfaces attach to the antigen presenting cells (APCs). This attachment stimulates APCs to secrete cytokines resulting in the activation of both cell-mediated and humoral immune responses (Marciani, 2003) Since saponins have less sustainable immunity and are also toxic in their natural form, there is an effort for the production of synthetic analogs of naturally occurring saponin. One such example is the extract from Q. brasiliensis leaves which show sustained immune response and are also less toxic when compared with naturally occurring saponin extracted from Quillaia saponaria (Silveira et al., 2011).

TLRs Receptors

The TLR receptors form an important group of pattern recognition receptors (PRRs), which form a connecting link between adaptive and innate immune responses of mammalian species (Iwasaki and Medzhitov, 2004). PRR is a pattern recognition strategy of host mammalian species to identify the characteristic molecular patterns of the invading micro-organisms. TLRs functions as receptors which recognise conserved molecular patterns of invading microbes and result in eliciting the first level of defence, the innate host defence mechanism. TLRs are expressed by a variety of cell types (Dowling and Dellacasagrande, 2016). The expression of TLRs on macrophages elicit only innate immune response, while the expression of TLRs on dendritic cells result in the activation of both innate and adaptive immune mechanisms. The TLRs can be present either on the cell surface or can be present inside the cell. TLRs present on the cell surface recognise the bacterial products present in the extracellular space while the intracellular TLRs recognise the genetic particles of both the bacterial and viral pathogens (Table 2).

TLRs have emerged under great selection pressure, and have been being preserved in all the vertebrate species (Brownlie and Allan, 2011). Sequence information of many TLRs is available for a number of domestic animals, including sheep, cattle, pig, horse, dog and chicken (Jungi et al., 2011; Uenishi et al., 2009). Many studies have been made to analyze the expression patterns of different TLRs by using the species specific anti-TLR antibodies especially in bovines and ovines (Kwong et al., 2011).

The identification of the characteristic recognition patterns of the invading micro-organism by the specific TLRs promotes the activation of various signalling pathways resulting in the increased production of messenger molecules like chemokines, cytokines and many other stimulatory molecules. Various components of pathogens have been utilized as TLR agonists which cause expression of cytokines involved in T cell differentiation. Examples of TLR agonists include bacterial lipoprotein recognised as a TLR2 agonist, bacterial lipopolysaccharides (LPS), component of Gram-negative bacteria surface membrane, is recognised as a TLR4 agonist, bacterial flagellin is recognised as a TLR5 agonist. Similarly cytosine phosphate guanine (CpG motifs) of bacterial DNA are recognised as a TLR9 agonist (Takeda et al., 2006).

Polymers

Over the recent years, research has been expanding tremendously to formulate and design novel entities to act as an efficient adjuvant and bio-polymers have proved to be the promising candidate for increasing the antigen potential against several diseases. Several naturally occurring polymers (Table 3). Likewise, numerous synthetic polymers have been investigated for vaccine development (Sahdev et al., 2014).

Polymeric biomaterials as an adjuvant enhance immune responses in several ways. They prevent the degradation of antigen by encapsulating it, thereby increasing the stability of the antigen (Leleux and Roy, 2013). There is a sustained release of vaccine from a polymeric matrix (Jaganathan et al., 2004). Multiple antigens can be inserted together with the polymeric adjuvant which are effectively processed by the antigen presenting cells (APCs). Flexibility in the surface structure of polymeric biomolecules helps in easy activation of pathogenic pattern recognition receptors (PPRs) or endosomal TLRs resulting in efficient modulation of immune responses (Bento et al., 2015).

There are many examples of immunoactive polymeric biomaterials which have used as animal vaccine adjuvants. The natural polymer chitosan is employed not only for drug delivery, but has also been utilized in antibacterial applications (Muzzarelli, 2010). Because of the bioadhesive nature and good biocompatibility of chitosans, they have potential for mucosal vaccine delivery systems (Cordeiro et al., 2015). This mucoadhesive property causes the sustained release of antigens in the mucosal associated lymphoid tissues (MALTs) (Xia et al., 2015).

The simultaneous administration of attenuated Newcastle disease viral antigen with chitosan molecules have been found to enchance the antigen specific cell mediated immune response in chickens (Rauw et al., 2010). Similarly, cattle which were immunized with chitosan based FMD vaccine were protected from the FMDV (Pan et al., 2014). Many synthetic polymers have also been tested as animal vaccine adjuvants. Polyacrylic acid polymers such as Carbopol are the most important and widely used as synthetic polymer adjuvant. Carbopol has been employed as adjuvant in vector based inactivated vaccines against the influenza virus for horses (Paillot, 2014). The use of Carbopol in swine vaccines has been shown to effectively promote T cell proliferation and interferon gammaproduction in swines which have been vaccinated with the modified live PRRS virus vaccine (Mair et al., 2015). Another important synthetic polymer based adjuvant is polyphosphazene which have shown promising results in the laboratory model animals. It was reported that polyphosphazene adjuvanted Actinobacillus pleuropneumoniae vaccine in pigs can promote IFN-γ production with simultaneous increase in IgG antibody (Dar et al., 2012).

When compared with other adjuvants, polymers are better in terms of safety parameters. Because of their ability to stimulate the host cell-mediated immunity, they are especially effective against some of the important animal viral pathogens.

Combination of Adjuvants

To have all the properties of a perfect adjuvant in a single molecule is quite impossible. Therefore, current adjuvant formulations often consist of two or more adjuvant molecules, that when combined together act synergistically by stimulating a variety of cells and immune mechanisms (Lee and Nguyen, 2015).

In view of the number of animal diseases emerging and re-emerging and to target varied species of animals and birds, preparation of combination adjuvants is of utmost importance to address all requirements of a sucessful animal vaccination programmes. Different combinations of adjuvants comprising of TLR ligands, emulsions, liposomes, saponins and synthetic polymers have been utilized for the formulation of combination adjuvants.

Table 2. Use of TLRs agonists in animal vaccines

TLR Agonist	Mechanism of Action	Disease Target
Escherichia *coli* heat-labile Enterotoxin	Induction of specific cellular immunity by up-regulating the expression of IL-4, IL-8 and IL-1β,	Swine Influenza A
Bacterial protein Flagellin	Production of higher levels of anti-FMDV neutralizing antibodies	Foot-and-Mouth Disease Virus
Combination of Flagellin and heat-labile Bacterial Enterotoxin	Promotes the production of neutralizing antibodies specific for the virus and causes the enchanced production of CD4+ and CD8+ T	Rabies Virus
Membrane Lipoprotein	Promotes T cell and humoral responses through TLR2 mediated activation and maturation of APCs.	Classical Swine Fever
CpG Oligodeoxynucleotides (ODNs)	Interact with TLR9 and stimulate Th1 response through the induction of IL-12 and IFN-γ.	Avian Influenza Virus
Combination of CpG Motifs	They act by causing the immunosynergistic effect resulting in the production of B cells and secretion of Type I/II-IFN cytokines.	Chicken Anemia Virus

Table 3. Natural and Synthetic Polymers used as adjuvants and their mechanism of action

Source	Polymer	Mechanism of action
Natrural Polymers	Chitosan	Acts through the activation of macrophages with subsequent production of cytokines,resulting in increased synthesis of antibodies.
	Mannan	Causes increased phagocytosis of the invading antigen through the activation of complement pathways.
	Lentinan	Causes the stimulation of macrophages with the resultant respiratory burst and increased interleukin-6 production.
	Dextran	Acts through the activation of both inflammatory cutokines and toll like receptor pathways.
	Inulin	Activation of the complement cascades
Synthetic Polymers	Polyphosphazenes	Increased secretion of proinflammatory cytokines and chemokines at the injection site
	Polyanhydrides	Acts by causing the increased expression of surface markers CD86, CD40 and MHC class II on antigen presenting cells. Also induces specific proliferation of CD4þ and CD8þ T-cells.
	Polycaprolactones	Activation of the Th1-type of immune responses. Also causes increased release of IFN-g and IL-2 cytokines.
	Non-ionic block polymers	Causes macrophage activation and high level expression of MHC class II molecules.
	Polyelectrolytes	They act by increasing the uptake and presentation of antigens.They also enchance the expression cell surface receptors and release of pro-inflammatory cytokines.

A novel adjuvant combination comprising of three components, namely polyphosphazenes, host cell peptides, and a TLR ligand, either CpG or ODN, when co-formulated with vaccine antigens resulted in the effective and quick immune response (Gracia et al., 2011). This combination provided immunity against varied infections including respiratory syncytial virus, swine influenza and Bordetella pertussis (Garg et al.,

2015; Garlapati et al., 2012). Another adjuvant combination consisting of the of CpG oligodeoxynucleotide motifs and different emulsions such as montanide ISA 206 as adjuvant system have successfully resulted in the augmentation of immune response against FMD (Ren et al., 2011).

This adjuvant combination induces the production of neutralizing antibodies in higher amounts than the adjuvants used separately. The combination of emulsion and saponin as adjuvant was also seen to improve the effectiveness of vaccines against foot and mouth disease. Ingredient of this adjuvant system, Saponin QS-21 was found to stimulate the production of higher titers of IgG1, IgG2a, IgG2b, and IgG3 antibodies against FMD (Cokcaliskan et al., 2016).

Conclusion

Adjuvants form an important part of vaccine formulations and have been used to improve the immunogenicity of animal vaccines for a long period now. With the improvement in the knowledge of the immune system and its interactions, our understanding regarding the mechanism of action of both natural and synthetic adjuvants has greatly improved. Selection of proper adjuvant for animal vaccines depends on many factors like induction of rapid and sustainable protective immunity, safety, effectiveness, meeting food safety standards and above all should be economical. The use of desirable adjuvant for the animal vaccine has an important impact on human health as it can reduce the irrational use of antibiotics in animal health practices.

Recent advances in adjuvant research has shifted from traditional empirical formulations to more rational and targeted formulations allowing for the development of effective animal vaccine formulations. Molecular designing is the way forward to design the vaccine adjuvants that can act individually or synergistically with other adjuvants to stimulate the immune system in a very specific and calculated manner. Furthermore, the production of combination adjuvants for multiple antigens should be the thrust vaccine research area, thereby making vaccination strategy in animal health management an economical affair.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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