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REVIEW

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The development of inovirus-associated vector vaccines using phage-display technologies

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ABSTRACT

Introduction: Inovirus-associated vectors (IAVs) are derived from bacterial filamentous viruses (phages). As vaccine carriers, they have elicited both cellular and humoral responses against a variety of pathogens causing infectious diseases and other non-infectious diseases. By displaying specific antigen epitopes or proteins on their coat proteins, IAVs have merited much study, as their unique abilities are exploited for widespread vaccine development.

Areas covered: The architectural traits of filamentous viruses and their derivatives, IAVs, facilitate the display of specific antigenic peptides which induce antibody production to prevent or curtail infection. Inoviruses provide a foundation for cost-efficient large-scale specific phage display. In this paper, the development of different applications of inovirus-based phage display vaccines across a broad range of pathogens and hosts is reviewed. The references cited in this review were selected from established databases based on the authors' knowledge of the study subject.

Expert commentary: The importance of phage-display technology has been recently highlighted by the Nobel Prize in Chemistry 2018 awarded to George P. Smith and Sir Gregory P. Winter. Furthermore, the symbiotic nature of filamentous viruses infecting intestinal F$^+$ E. coli strains offers an attractive platform for the development of novel vaccines that stimulate mucosal immunity

1. Introduction

Research regarding the potential to use viruses to combat disease has been ongoing for over 100 years [1,2]. While viruses have often been considered pernicious as they co-opt a host for their own survival, often killing the host in the process, new work with viruses that can be exploited as bacteriophages but without the harmful effects has arisen [3]. These viruses, which can be easily manipulated and employed for phage display ability, are being increasingly used for a variety of potent biomedical tools [4]. These filamentous bacterial viruses, which make up the genus *Inovirus* in the family Inoviridae, are thread-like viruses containing single-stranded DNA genomes know as filamentous bacteriophages [5–7]. Over 50 different species of filamentous viruses are known, of which a majority can infect Gram-negative bacteria.

Although inoviruses are now being used for their phage display capabilities, these filamentous viruses have a relationship with the cell that they infect that is more similar to symbiotic non-pathogenic animal viruses than classical phages. Unlike phages, which term comes from the Greek word φάγος for destroyer, inoviruses do not kill their host and only slightly affect cell growth despite yielding titers of up to 10^{13} virions per milliliter of liquid culture. Progeny virions are assembled in the host cell's membrane where single-stranded DNA binding proteins are replaced by major capsid protein subunits before being released into the cell,

resulting in opaque plaques on bacterial lawns [8,9]. Receptor organelles in the bacterial host that are encoded by transmissible plasmids facilitate the interactions between inovirus and cell [5,10]. The functional architecture of inoviruses provides the foundation for their application in vaccine-related projects since inoviruses do not cause harm.

A great number of inovirus species across the world have been isolated and characterized [5]. Despite variation by species, they have the same general physical characteristics. The virions are flexible, thin cylindrical filaments [6,7] under 10 nm in diameter and approximately 1000 nm in length (see Figure 1(a) for details). Most of a single virion is composed of several thousand major capsid or coat protein subunits. These surround a circular single-stranded DNA molecule. At the proximal end of the virion there are a few minor proteins which attach to the cell to initiate infection. At the distal end, there other minor proteins which are used for nucleation and assembly on the host membrane. The structures and life cycles are conserved across different species of inoviruses, resulting in similar functional applications.

Research into inovirus structure and application has been dominated by studies of Ff [11], which infect male (F$^+$) strains of *E. coli*. The most used and best understood of the Ff inoviruses are the closely related fd, f1, and M13 types (for reviews see [9–11]) for which extensive information regarding their life cycle and genetics is known. These nearly identical viruses almost perfectly share all structural motifs, including DNA and protein sequences and gene organization [12–14],

although there are slight variations between the genomes. There are 10 genes and a non-coding intergenic region which are conserved across the Ff species [15–17] as well as DNA replication and transcriptional machinery (for a recent review see [10]). Proteins are encoded by 5 genes in the virion (g3, g6, g7, g8, and g9). Gene 8 proteins (gp8), the major coat protein, make up the vast majority of the virion, occurring in fivefold axial symmetry. Gene proteins 3 and 6 (gp3 and gp6) are located on the proximal end of the virion and are involved in stabilization and infecting the host cell. Gene proteins 7 and 9 (gp7 and gp9) are on the distal end of the virion and perform initiation assembly [18–20]. As shown in the end-to-end model in Figure 1(a), minor coat protein subunits have fivefold axial symmetry. While much research has been done into the structure of the Ff virus over the past 50 years, a conclusive structure has not been determined since the viruses cannot be crystallized. X-ray fiber diffraction studies and physiochemical measurements have revealed the five-star helical symmetry (5-fold rotation axis) of the gp8 subunit and are referred to as Class I [21–23]. However, the structure of the ssDNA and its relationship to the protein sheath is poorly understood, due to the small amount of DNA in individual virions. However, the architecture is sufficiently understood to take advantage of the structures and capabilities of the virus throughout its life cycle.

The life cycle of Ff filamentous viruses begins when an adsorption structure on the proximal end of the virus is adsorbed to the tip of the F^+ specific pilus of *E. coli*. Following binding between the virus and the bacterial cell (for a recent review, see [10]), the major coat proteins of the
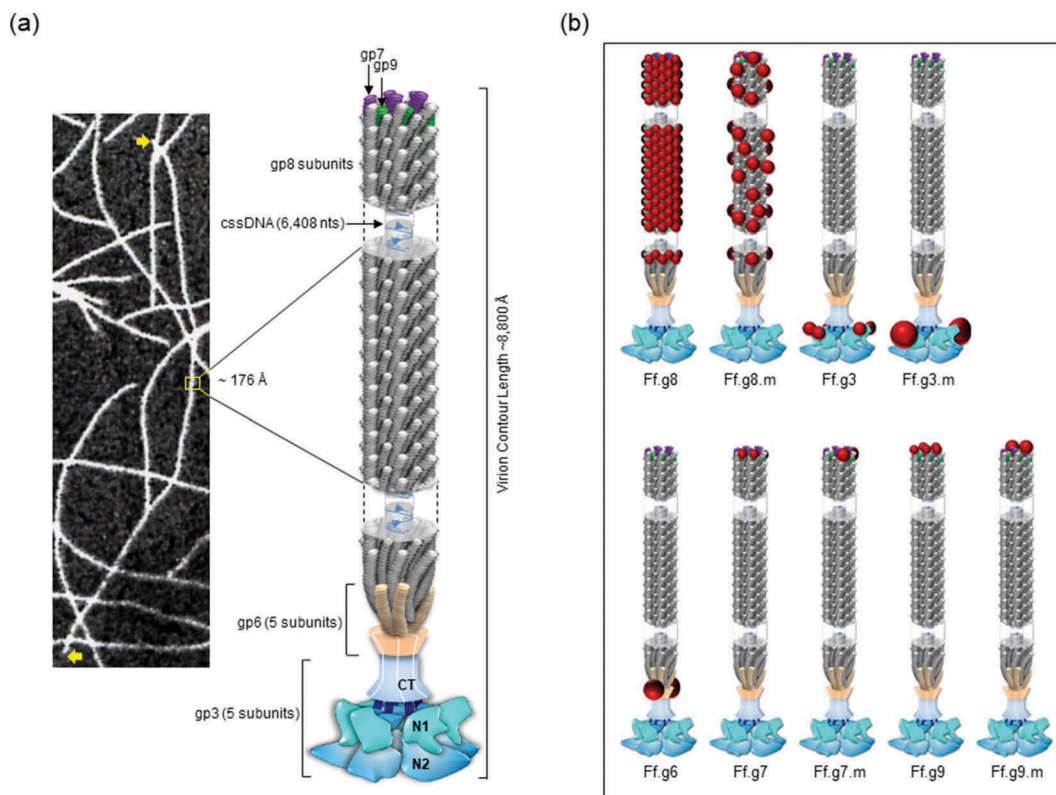
Figure 1. Schematic representation of a filamentous virus and Inovirus-Associated Vectors (IAVs).(a) On the left, a digital scanning transmission electron micrograph (STEM) of unstained filamentous virus (fd). The ends of one complete virion are designated by arrows. On the right, a 3D scale schematic model of an end-to-end virion, based on published physical data. The schematic indicates the circular single-stranded DNA (cssDNA) genome surrounded by the virion capsid (major coat protein pg8) and the four minor coat proteins at the two ends of the virion (gp3 and gp6 at one end and gp7 and gp9 at the other). For recent detailed architectural information, see recent review [31]).(b) Schematic representations of IAVs displaying foreign antigens (red spheres) on their surface. The name designation of each IAV denotes the viral capsid protein by which the antigen is displayed. IAVs denoted by 'm' for 'mosaic' contain both the wild type and antigen display capsid proteins. Adapted with permission from [31].

virus become associated with the inner membrane of the cell [24–26]. The virion's circular single-stranded DNA (cssDNA) is then ejected into the cytoplasm where it is converted to a parental double-stranded replicative form (RF). Using a rolling-circle mechanism, Ff inoviruses replicate their genome. The new virion is assembled through a complex set of interactions that binds the protein subunits to the ssDNA [15–17] and embeds newly synthesized coat proteins into the bacterial membrane [27–29]. ssDNA is passed through the mature coat protein, spanning the bacterial membrane, and additional coat proteins are added on the internal edge of the membrane. Additional proteins are used to package the inovirus and release it into the cell [23,30]. Inovirus assembly on the inner membrane of the bacteria is a harmonized sequential process with both viral-encoded and host proteins playing important roles. For more extensive reviews on inovirus life cycle and replication, see reviews [10,31]. Importantly, this process, which requires both virus and host to complete, does not kill the host, making inovirus replication a sustainable process.

2. Inovirus-associated vectors

Inoviruses are useful for vaccine development because it is possible to insert random oligonucleotides into their genome. This easy genetic manipulation is the basis for inovirus display (phage display) technology [32,33]. Inoviruses that have been genetically modified to display these oligopeptides as fusion proteins on their surface are referred to as inovirus-associated vectors (IAVs). Oligopeptides can be displayed on any capsid protein (gp3, gp6, gp7, gp8, and gp9) following genetic modification. A specific oligonucleotide sequence can be inserted into the viral genome to display the desired oligopeptide as a fusion with capsid proteins gp3, gp7, gp8, or gp9. This results in the oligopeptide's display on every copy of the target capsid protein. However, mosaic inovirus particles can be created where the specific capsid proteins display a mix of wild type and recombinant proteins with the desired oligopeptide [34]. This is done using a phagemid vector which has an extra copy of a capsid protein fused to the specific oligonucleotide. A host exposed to both the phagemid vector and a wild type capsid protein from a deficient helper phage produces mosaic IAVs displaying both wild type and oligopeptide fused capsid proteins. Work using the capsid protein gp6 has resulted only in the production of mosaic IAVs (for reviews see [35–37]) while both mosaic and non-mosaic IAVs have been produced using gp3 and gp8 (for a review see [10]) and gp7 and gp9 (for reviews see [38–40]).

As a result of the different locations, structures, and abundances of the capsid proteins in IAVs, they have differential abilities to display different oligopeptides. Figure 1(b) shows how each of the five capsid proteins of an IAV can display antigens, as have been demonstrated in published literature. The capsid protein that can display the most copies of the desired oligopeptide is gp8 due to the large amount of gp8 in each inovirus. A non-mosaic IAV can display a peptide on each of the approximately 2,700 copies of gp8. However, doing so with large peptides distorts the virus; only peptides of up to 6 amino acids can be displayed in such great quantity without affecting the structure of the inovirus. However, producing a mosaic IAV with oligopeptides on far fewer gp8 units will not cause distortion [33]. While it is theoretically possible to display an entire protein on gp3 [41], presenting on all 5 copies per virion, studies have demonstrated that at most one copy of gp3 will display the desired protein [34].

The ability of IAVs to display different random oligopeptides on their surface has many applications, and is especially useful in for vaccine development. The creation of Random Peptide Libraries (RPL), where random oligopeptides are fused to major capsid proteins (gp3 or gp8) and displayed on individual inovirus clones creating a random variety of IAVs which can be used for vaccine design via epitope mapping using monoclonal or polyclonal antibodies. These random IAVs, whose oligopeptide diversity increases with increased peptide length, can be used to identify the epitopes of specific antibodies through a process called biopanning. This allows for the isolation of IAVs displaying mimotopes which mimic the antibody discontinuous target epitope. By isolating the recombinant inoviruses bearing mimotopes, the DNA and amino acid sequence of the oligopeptides of interest can be determined. Through this breakthrough technology which was the subject matter of the Nobel Prize in Chemistry 2018 (see 'Expert Commentary' below), inovriuses displaying oligopeptides mimicking antigens (or specific epitopes of an antigen) can be used to vaccinate hosts thus inducing the desired antibody production. Vaccines of this sort have been developed in a variety of organisms against many different diseases (See tables 1 and 2 in [31] for a list of inovirus-based vaccines), as the introduction of the inoviruses can induce the production of specific antibodies. While there have already been various successes, this method can potentially open the door to the development of many novel vaccines and vaccine methodologies.

3. Inovirus display technology in vaccine design

IAVs have been successfully used as vaccine carriers for a variety of species and diseases across a range of vaccine studies (as shown in [31]). These vaccines have been effective against infections agents such as viral, protozoan, and worm parasites as well as non-infectious diseases including Alzheimer's and a variety of cancers. Inovirus display technology has been used in two different ways to develop vaccines. The first method employs inovirus display technology to screen RPLs with monoclonal antibodies to determine which peptides can be used. These immunogenic peptides are used as vaccines either with carrier proteins or in their soluble forms to elicit an immune response [42–55]. The second method not only employs inoviruses for epitope mapping, but also utilizes inoviruses to serve as the carrier for the isolated immunogenic peptide [56–83]. Directly using IAVs to present the peptide has been more consistently successful in producing the production of the proper antibody than the introduction of soluble peptides. Inovirus-bound peptides (on IAVs) consistently retain their 3D structure and are more stable than soluble peptides, which do not always reflect the desired antigen epitope [84]. IAVs are structurally simple, which allows the immune system to respond to the fused peptides rather than the viral coat.

Combined with ability to display many copies of the desired peptide, IAVs are highly immunogenic, creating effective vaccines [85]. Because the inoviruses replicate in *E. coli* cultures, the production of large numbers of vaccines is cost-efficient. IAV's structural simplicity, high immunogenicity, and economical production make them an efficient and attainable system for creating a variety of effective vaccines.

Much of the research involving inovirus-based vaccines has been to target infectious diseases in animals. Studies have shown that IAVs have been constructed to protect vaccinated animals against a variety of infectious diseases by eliciting both humoral [82] and cellular [86] immune responses. To test the efficacy of IAV vaccines against target pathogens, studies were conducted in which animals were challenged with a specific pathogen following IAV vaccination. In these studies, IAVs were shown to mitigate or prevent infection from viruses and parasites. In one study, mice were completely vaccinated against Human Respiratory Syncytial Virus (RSV) by binding a 15-mer linear epitope to gp3, which induced a humoral response [56]. Monoclonal antibodies against various viral diseases have also been successfully screened against RPLs to produce recombinant inovirus vaccines against Herpes Simplex Virus type 2 (HSV-2) using a 15-mer peptide [57] and against Neurotropic Murine Coronavirus sing a 13-mer peptide [58]. These inovirus vaccines induced humoral responses in mice, greatly reducing mortality and providing protection relative to the dose of the vaccine [57].

IAV vaccines have also been developed against fungal parasites in animals, providing both individual protection and large scale vaccination success. In 2005, Yang et al. and, in 2006, Wang et al. used inoviruses to vaccinate against systemic candidiasis, a fungal infection caused by *Candida albicans*. They used the inoviral coat protein gp8 to display a 6 amino acid peptide epitope of the fungal heat shock protein 90 to vaccinate against the infection [59,60]. Not only did vaccination lower the burden of infection, but it also increased the lifespan in the infected mice [59]. In 2004, Manoutsarian et al. used IAVs to vaccinate pigs against *Taenia solium*, a parasitic worm which causes neurocysticercosis in humans but uses pigs as intermediate hosts. Unlike previous studies, which used a single specific peptide fused to a inovirus, four different antigenic peptides were displayed by inoviruses in a cocktail of recombinant IAVs. The induction of a cellular response completely vaccinated 1/3 of the pigs in the study and reduced the number of cysticerci in all other pigs [61]. Following the success of this study, a large-scale vaccination of 1047 pigs in Mexico was undertaken in 2008. The pigs in a natural environment were successfully immunized, reducing parasite presence in the vaccinated pigs, and providing a more cost-efficient alternative to vaccination with synthetic peptides [87]. Many additional vaccines have been developed that induced both humoral and cellular responses against parasites in animals providing partial protection against the infection and reducing infection burden. Many of these studies were done by screening monoclonal and polyclonal antibodies against large RPL (for a review of more inovirus vaccine studies see [31]).

In addition to their success against infectious diseases, inovirus display technology has been successfully used to design vaccines which prevent or mitigate the progression of non-infectious diseases. IAVs have been used to display antigen epitopes which elicit immune responses against tumors and other non-infectious diseases. An epitope of the Melanoma Antigen A1 (MAGE A1) displayed on gp8 induced a cellular immune response against the melanoma tumor in vaccinated mice. Not only did vaccination inhibit tumor growth, but it reduced mortality in the targeted mice [88]. Induction of a cellular response, decreased tumor growth, and higher survival rate was observed in mice vaccinated against murine mastocytoma P815 in a later study [89]. The highly immunogenic characteristics of inovirus display technology has also been used to activate immune responses against colorectal cancer tumors, which often evade antitumor immune responses, and to reduce tumor growth [90]. Inovirus-based vaccines have also been used against diseases such as Alzheimer's. The same methods have been used to induct antibodies against β-amyloid plaques by displaying a specific 4 amino acid antigenic epitope on the surface of the inovirus. Multiple studies in mice using recombinant inoviruses have induced a humoral response and reduced β-amyloid plaque burden [91–94]. While not used preventatively as has been done with infectious diseases, IAVs can vaccinate animals against further disease progression in non-infectious diseases. These findings suggest that there will be future development of inovirus-based vaccines against non-infectious diseases that mitigate the damage done to both animals and humans.

Despite the advances in the use of IAVs to combat disease, the consistent development of inovirus-based vaccines still faces challenges. Even when inoviruses have been screened with specific antibodies to bear the desired peptide mimotopes, immunization using IAVs does not always produce the expected immune response. Keller et al. in 1993 were unable to induce the production of broadly neutralizing antibodies against Human Immunodeficiency Virus Type-1 (HIV-1) in rabbits despite having screened for the proper mimotopes [66]. Dorgham et al. in 2005 screened a 15-mer RPL using a broadly neutralizing antibody and, using the selected inoviruses, were able to induce antibodies in vaccinated mice. However, the induced antibodies did not exhibit neutralizing activity against HIV-1 [68]. Other RPLs screened against monoclonal and broadly neutralizing antibodies have produced antibodies that do not have neutralizing capabilities [55,70]. In one such case, crystallization of the specific mimotope revealed that the oligopeptide borne by the inovirus was structurally different that the natural antibody epitope [69]. This structural issue is likely at the foundation of many of the issues involving ineffectual inovirus-based vaccines. When IAVs fail, it is probable that the chosen mimotopes poorly resemble the original antibody epitopes and cannot function in the desired fashion, either failing to induce antibodies or inducing of antibodies without neutralizing capabilities.

Despite the efficacy of these inovirus-based vaccines against a variety of infectious and non-infectious diseases, there are still many diseases for which effective vaccines have not been developed using phage display or other methods. For example as discussed above, despite extensive work with inoviruses beginning in 1993 by Keller et al. [66], there has been very little success developing a (HIV-1) vaccine [95]. While four major epitopes on

the HIV-1 envelope gp41 and gp120 glycoproteins have been identified [96–98], inducing the production of effective antibodies has not been successful [70]. While current techniques have not succeeded, both humoral and cellular responses to HIV-1 through inovirus-based vaccines are being researched [99,100]. However, the groundwork for future work involving HIV-1 and other diseases has been laid out, paving the way for a multitude of various new inovirus-based vaccines.

While there have been many vaccine studies targeting HIV-1 using IAVs, none have been completely successful. Studies involving inovirus based-vaccines targeting HIV-1 initially only used the broadly neutralizing monoclonal antibodies 2F5, 2G12 and b12 [55,66–70]. However, as discussed above, these studies have either failed to induce antibodies or induced non-neutralizing antibodies, despite often inducing a humoral response. In other studies, polyclonal sera from HIV-1-infected individuals have been used to screen RPLs to search for new broadly neutralizing monoclonal anti-HIV-1 antibodies [71–75,101]. These studies have used IAVs to induce the protection of neutralizing antibodies in mice [73] and macaques (against Simian-Human Immunodeficiency Virus) [75]. Inovirus-based vaccines targeting HIV-1 have been able to control viral load in a variety of non-human mammals after a challenge by HIV-1 or induce neutralizing antibodies, but not inhibit novel infection or induce broadly neutralizing antibodies. Additionally, inovirus-based HIV-1 vaccines have often failed due to the autoreactivity of the monoclonal antibodies that can hopefully be avoided with the new 'next generation' broadly neutralizing antibodies [102–106].

4. Conclusion

Inoviral vectors have been used extensively in the development of vaccines against a variety of infectious and non-infectious diseases over the past two decades. As shown above, the IAVs have successfully induced a humoral or cellular response, or both. In the studies, the vaccines could provide partial or complete protection against pathogens causing infectious disease or merely lower the burden of infection. Against non-infectious diseases, inovirus-based vaccines have been shown to be effective at mitigating disease development by initiating productive immune responses. There have already been many applications of vaccines against infectious and non-infectious diseases using inoviral vectors and IAVs have distinct characteristics that make them more applicable for the development of new vaccines than other viral vectors.

5. Expert opinion

Inoviruses and by extent IAVs are highly immunogenic and do not require adjuvants, facilitating the ease and simplicity of usage as vaccines. They can display multiple (from few to thousands) copies of a peptide on their surface while still maintaining the desired structure and conformation as well as infectivity with their bacterial hosts. IAVs, by displaying only particular peptides, allow the immune system to interact with a specific epitope rather than a larger, more complex protein, yielding a more targeted response. They are capable of stimulating humoral and cellular immune responses and do not

pose known health risks to animals or humans. IAVs can be administered safely in high doses through multiple routes, enhancing their usage in a variety of contexts [107]. With their cost-efficient production, structural simplicity, and record of success in a variety of contexts, IAVs have the potential to be exploited for many new vaccines in humans and animals against a variety of diseases.

Advances in inovirus research and phage display technology are making the development of new vaccines for a variety of diseases feasible in the near future. Currently, there are many diseases without vaccines or for which treatment is highly invasive. Further development involving IAVs will be likely to introduce more, better, and increasingly cost-effective vaccines. As the structure and tools for genetic manipulation of inoviruses are being better developed, IAVs will likely begin to be more extensively introduced into animal and human use. The current research is only revealing the tip of the iceberg of the extensive ways in which inovirus-based vaccines will be applied in future use. Accordingly, there are aspects of the genetic engineering of phage-display that need to be further expanded. Specifically, further studies should be conducted to evaluate the maximum antigenic load capacity of the gp8 coat protein without jeopardizing the architectural integrity and infectivity of the IAV (Ff.g8, see Figure 1). Furthermore, additional research about the potential of using the other capsid proteins (gp3, gp6, gp7, and gp9) or combinations of any of the capsid proteins for antigen display is needed. Additionally, future studies could elucidate the nuances of the uses of individual capsid proteins for specialized applications such as antigen display, penetration, targeting, etc. An additional aspect of IAVs that needs to be further explored is how antigenic display on the surface of IAVs induces immunogenicity. This predictive work needs to be undertaken by experimental and computational (in silico) research.

Further research will involve the development of new vaccines and improving the efficacy of current vaccines. Additional undertakings should be done into identifying the structure of the viruses, since this will better be able to inform how the phage-display technology will best be applied as more complex work is being done using monoclonal and polyclonal antibodies to create more nuanced vaccines. The future has a dual focus: the development of vaccines for infectious diseases and the induction of immune responses in non-infectious diseases to limit the harm that they do (or eliminate them).

The underlying principle of future IAV-based vaccine development could be based on the unique natural symbiosis, which is known to exist between humans, non-pathogenic bacteria such as E. coli, and inoviruses. It is well established that humans or other warm-blooded animals acquire E.coli, including F+ strains, during their first few days in life or even before birth and that they are never thereafter without it. F-specific inoviruses (Ff inoviruses) cannot infect humans, but they propagate at high viral loads in specific strains of E. coli including non-pathogenic strains which are symbiotic to humans. Future IAV-based vaccines within the next five to ten years could take advantage of the triple symbiotic nature between humans, enteric F + E. coli strains and F+-specific

inoviruses to target the induction of mucosal immunity. This approach will be particularly effective against sexually transmitted pathogens such as HIV-1.

Much future study lies the further development of IAVs and how they can induce immune responses on a broad scale. Research regarding the development and advantages of phage display technology was recently recognized by the Nobel Committee. George P. Smith and Gregory P. Winter were awarded the Nobel Prize in Chemistry 2018 for the 'elegant method known as phage display, where a bacteriophage – a virus that infects bacteria – can be used to evolve new proteins … [This] revolution … is bringing and will bring the greatest benefit to humankind' [108]. The development of more and better inovirus-based vaccines will continue to advance preventative treatment against diseases which have not been able to be combatted.

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Declaration of interest

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