PROGRESS OF EDIBLE VACCINE DEVELOPMENT

A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
Masters of Science in Biotechnology

Department of Mathematics and Natural Science
BRAC University

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By

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Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

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Abstract

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Edible vaccines are edible plants genetically engineered to contain a gene that specifies an antigen or a marker sequence sequence of defective gene or protein. The mucous lining comprises one of the first lines of defense during pathogen attack and a vaccine usually triggers the mucosal immune system of the mucous lining. The dissertation will highlight the development framework of the edible vaccine, the timeframe of development along their proposed uses. The encapsulation of the antigen in the plant vehicles' tissues protects the antigen from the acidic nature of the stomach. The immunogen, being in partially intact form, would be able to stimulate an immune response. Edible vaccines could be further used to induce tolerance to allergens. For such cases, the immunogen is in soluble form. Studies have revealed that these vaccines can stimulate the production of secretory IgA along with other antibodies. Currently, six edible vaccines are undergoing clinical trials; however, certain issues need to be solved before their approval. Veterinary edible vaccines have been already approved. Bangladesh has also attempted to design an edible vaccine for pneumonia. It can be inferred that shortly, edible vaccines would be a valuable tool for preventing health conditions.

Keywords: edible vaccines; secretory IgA;antibodies; transformation; GM1-ELISA;

Dedicated to my Parents

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Table of Contents

Declaration		ii
Approval		iii
Abstract		iv
Dedication		v
Acknowledgement		vi
Table of Contents		vii
List of Tables		ix
List of Figures		ix
List of Acronyms		x
Chapter 1	What are Vaccines and vaccination?	1
1.1	Vaccine	1
1.2	Types of vaccine	1
1.3	Vaccination	4
1.4	Immunity and vaccination	5
Chapter 2	A Novel Form of Vaccination	9
2.1	What are edible vaccines?	9
2.2	Why are plants selected for edible vaccines ?	11
2.3	Selecting a candidate for the edible vaccine	15
2.4	Advantages of Fruit-based Edible Vaccines	17
2.5	Can algae be used as Edible Vaccines?	18
Chapter 3	Production Process of Edible Vaccine	19
Chapter 4	The Feasibility of Edible Vaccine- A Case Study	21
4.1	Enterotoxigenic E.coli (ETEC)	21
4.2	Cholera	26
4.3	Rotavirus	32

4.4	Norwalk virus	36
4.5	Human Immunodeficiency Virus (HIV)	38
4.6	Hepatitis B virus (HBV)	42
4.7	Tuberculosis (TB)	47
4.8	Dengue	51
4.9	Rabies	55
Chapter 5	Edible Vaccines for Novel Conditions	59
5.1	Allergens	59
5.2	Cancer	65
5.3	Alzheimer’s Disease	70
Chapter 6	Current Status of Edible Vaccines	74
6.1	Clinical trials	74
6.2	Progress of Edible Vaccine Approval	79
Chapter 7	Biosafety Concerns and Regulatory Affairs	82
Chapter 8	Edible Vaccines and Bangladesh	85
Chapter 9	Edible Vaccines for SARS-COVID2	86
Chapter 10	Discussion and Future Directions	88
References		89

List of Tables

Table 1	Vaccine classification by production process	2
Table 2	List of major advantages and disadvantages of edible vaccines	10
Table 3	Different candidates for edible vaccines	16
Table 4	Progress on the Development of Edible Vaccine	58

List of Figures

Figure 1 :	Timeline for the development of an edible vaccine	57
Figure 2 :	Timeline for the development of edible allergen vaccine	64
Figure 3 :	Timeline for the development of cancer edible vaccine	69
Figure 4 :	Number of published works conducted for each edible vaccine	73
Figure 5:	Summary of the progress of edible vaccine development	81

List of Acronyms

AIDS- Acquired ImmunoDeficiency Syndrome

ASC – antibody secreting cells

BCG - Bacillus Calmette–Guérin

Cry j - *Cryptomeria japonica* allergen

DNA – deoxyribonucleic acid

DPT - diphtheria, pertussis (whooping cough), and tetanus

E.coli - Escherichia coli

ED- envelope protein domain of dengue virus

ELISA- enzyme-linked immuno-absorbent assay

ELISPOT - enzyme-linked immuno-sorbent spot

ETEC - Enterotoxigenic *Escherichia coli*

GE – genetically- engineered

HBV – hepatitis B virus

HCV - hepatitis C virus

HIV- human immunodeficiency virus

HPV - Human Papillomavirus

Hib - *Haemophilus influenzae* type b

IFN – interferon

IgG – immunoglobulin G

IgA – immunoglobulin A

sIgA – secretory IgA

IL- interleukin

LTB - Heat-labile enterotoxin B subunit

MIS – mucosal immune system

MMR - measles, mumps, and rubella

MVH - measles virus hemagglutinin

NVCP – Norwalk Virus capsid protein

SARS- Severe acute respiratory syndrome

SIV – Simian Immunodeficiency Virus

Ti plasmid – tumor-inducing plasmid

T-DNA - Transfer DNA

TB - tuberculosis

TBV – transmission blocking vaccine

VLP- virus-like particles

Chapter 1

What are vaccines and vaccination?

1.1 Vaccine

A vaccine is any biological preparation that serves as a means to impart immunity. The vaccine usually consists of a modified form of the natural pathogen's antigenic determinant or epitopes. A vaccine does not cause the disease itself; it introduces a minimum level of the pathogen so that the body can identify and produce an immune response against the pathogen. It is a form of active immunization.

1.2 Types of vaccine

Vaccines can be subdivided into two categories depending on their use. (World Health Organization, 2012). The first category is the prophylactic vaccine, which is administered to prevent future infection. Prophylactic vaccines are usually a part of national immunization programs that protect children against enteric pathogens. However, some prophylactic vaccines are also used during epidemics. Most prophylactic vaccines are administered during infancy, the logic behind this being that it will be early enough to establish the onset of immunity but it is not late enough to elicit tolerance to the vaccine. Booster doses of the vaccines are also scheduled to be given at predetermined time points later in childhood. In order to minimize the number of shots that are needed to be taken, combination vaccines are also designed; a combination vaccine contains many different antigens. The number of antigens is determined by their valency.(Simpson, 2014) The second category is the therapeutic vaccine which is used for treating a disease. The therapeutic vaccine is given after the onset of an infection and in order to counteract the pathogen or anomaly directly. Therapeutic vaccines are most commonly used for viral infections, autoimmune disorders, allergies, and malignant disorders of the body. There is much interest in the development of therapeutic vaccines nowadays (Shimasaki, 2014).

Based on the production procedures, vaccines can be classified into various forms:

Table 1 - Vaccine classification by production process(Simpson, 2014)

Vaccine types	Production process	Comments
Live attenuated vaccine Example :Sabin oral polio vaccine, BCG vaccine, MMR vaccine	The microbe is usually cultured in suboptimal conditions- in extreme temperatures or pH or by growing in a different host. The cells are weakened by passaging it through hosts other than the native one. Sabin vaccine- monkey kidney cells, BCG vaccine- bile salt	The change in optimal conditions usually introduces a selection pressure in the microbes; some of the microbes undergo mutations that allow them to survive and grow well in new conditions; As a result, the cell lines become less adapted to grow well in their native host
Inactivated vaccine Example : Salk vaccine, Cholera vaccine	Microorganisms are killed or inactivated by heat, radiation, or chemicals like formalin. Salk vaccine-formalin, cholera vaccine- both heat and formalin	The technique is used in such a way that the epitope is usually preserved but the cell is effectively nonviable.
Toxoid vaccine Example- diphtheria vaccine and the tetanus vaccine	Toxoid vaccines consist of inactivated toxins or toxoids that are administered to counteract the effects of the endotoxins.	Both of the toxoids are used to form conjugate and then used as vaccines. (Hermanson, 2013)
Subunit vaccine	They are usually formed from the purified peptide or polysaccharide epitope of the pathogen. They are synthesized using recombinant DNA technology.	

Vaccine types	Production process	Comments
Protein subunit vaccine Example – HPV vaccine	The gene encoding the epitope is introduced into the genome of a harmless host-microbe, then cultured in large quantities and harvested. The gene can be manipulated to enhance immunogenic properties as well as for easier application into the host	
Polysaccharide subunit vaccine Example – diphtheria vaccine, tetanus vaccine, Hib vaccine, influenza vaccine	Polysaccharides from potent pathogens can be purified and used as a vaccine	The efficiency of the vaccine in its pure form is increased by bonding it to carrier proteins, thus initiating the production of conjugate vaccines
Peptide vaccines Example - the tumor vaccine containing the tumor-associated antigen, allergy vaccines, and Alzheimer's vaccine(H. Yang & Kim, 2015)	The peptide sequences of some antigens can act as vaccines. They usually consist of 20-30 amino acids. They can be both native and synthetic. For native peptides, the peptide is extracted and purified. For synthetic ones, the sequence of the peptide is used and the peptide is arranged in a synthesis reaction.	
DNA vaccine – recombinant DNA and naked DNA Examples – vaccine for *P. falciparum*(Smooker et al., 2004).	Recombinant DNA vaccine: The DNA is usually loaded onto an expression vector, which is an unrelated microbe Naked DNA vaccines: The raw DNA is encoded in a vector and will be flanked by sequences that will facilitate the transport, uptake, integration, and expression of the epitope in the host cell	DNA vaccines are mostly used against intracellular parasites like – *Leishmania, Toxoplasma, Entamoeba,*and*Tryponasoma*.

1.3 Vaccination

Vaccination is the practice of delivering antigens to the body in order to induce an immune response. To begin with, during an infection there is a particular time lapse during the onset of an infection and the production of antibodies. The body's immune cells take some time to identify the epitope of the pathogen and produce specific antibodies. This time lapse can be life-threatening for patients suffering from infections of highly-potent pathogens. However, the antibody titers are usually very low. This is known as the primary response. A vaccine usually confers antibody production at a faster rate and at higher levels. This is called the secondary response. So the infection is rapidly subsided. Vaccines also produce immunogenic memory- memory cells record the configuration of the antigen and remember them for future infections.

Vaccination is very important for the individual as well as the country. By getting vaccinated, the individual is being protected from serious and life-threatening diseases that could occur in the future. However, it could be argued that the chance of contracting such diseases is low; vaccination helps to lower the risk of contracting such diseases even more. Many serious diseases like polio have been eradicated due to successful vaccination schemes. In addition to this, vaccination provides herd immunity. It could be possible that a few individuals in the community would not get vaccinated due to being too young, medical conditions or fear of side effects. If most of the individuals are vaccinated, then the infection could be restricted from spreading in the community. (World Health Organization, 2012)

1.4 Immunity and Vaccination

Immunity is the body's ability to ward off and eliminate non-self invasion. The body's immune system is composed of two parts- innate immune system and acquired immune system.

Innate immune system is present in our body from the time of birth. The innate immune system consist of physical barriers- skin, gastro-intestinal tract, respiratory tract, cilia and hair structures of the body, chemical barriers like various secretions and cytokines and phagocytic cells- macrophages, mast cells, neutrophils, eosinophils and basophils; natural killer cells and dendritic cells. The cells which contain toxic granules in cytoplasm are called granulocytes- neutrophils for unicellular pathogens, eosinophils for multicellular parasites and histamine-secreting basophils. The macrophages engulf pathogens and cell debris; they are crucial for antigen-presentation. In a similar manner, dendritic cells are also involved in antigen presentation. Natural killer cells aid in the immunity by destroying infected cells to reduce their transmission. Mast cells produce chemokines like histamine and cytokines to mount an inflammatory response. The complement system present in the blood also helps in the fight against invading pathogens. The innate immunity is non-specific – it targets any cell that is recognized by the immune system as foreign particle. The innate immunity is also short termed and does not initiate the development of a memory response. However, the innate immune system is very effective in eliminating any foreign body rapidly in its first encounter.

The adaptive immune system is very specific and has a developed immunogenic memory. Even though at the earlier stages during combating an immunogen, the response is slower because the immunogen needs to be sampled and matched to the correct receptors of the immune cell. After that, an antigen specific response is generated rapidly which is known as the primary response. If the same immunogen is encountered again in the future, the recognizing ability of the receptor, termed as immunogenic memory, will lead to a specific, rapid response.

The adaptive immune system can be of two types- active and passive. Active immunity is when the immune cells of the body produce antibodies during an infection or after getting a vaccination. Passive immunity is when preformed antibodies are introduced into the body by means of blood or sera, or when the antibodies cross the placenta or are passed into a neonate in the colostrums.

The adaptive immune system is also known as the acquired immunity. Two components form the most crucial components- the T-cells and the B-cells.

The T-cells possess special receptors – T cell receptors or TCR on their membranes. The T-cells will either have CD4 receptors or CD8 receptors. Some specialized T cells will also have CD25 receptors. The T –cells can bind to an antigen when it is presented by antigen presenting cell and combined to its Major Histocompatibility Complex- MHC. The T cells with CD4 receptors are called T helper cells. The T cells with CD4 and another receptor CD25 are T regulatory cells. The T cell with CD8 receptors are T killer cells or cytotoxic T cells.

After their maturation in the thymus, the T cells that are released are known as naive T cells. They need an antigen- presenting cell for activation. Activation of T killer cells occurs when they bind to MHC -I molecule of the antigen-presenting cells like dendritic cells and forms a stable complex. The killer T cells also need assistance from helper T cells that produce signaling molecules. Naive T killer cells are in inactive state and they can rapidly activate and proliferate by antigen presentation into effecter cells when they encounter an antigen homologous to its receptor. These cells undergo clonal expansion and differentiation to form short-lived cells. The cytotoxic or killer T cells then combat any intracellular pathogen or tumor. The killer T cells release cytotoxic granules on contact with infected cells and cause their lysis. Some of the T killer cells remain in the circulation after the condition is cleared with specific antigen-binding ability and reverting to cytotoxic nature after re-encounter with the immunogen. They are called memory T cells and they convert into effector T cell with vast clonal expansion ability. Some mature T killer cells recalculate the body in search of their specific antigens.

Similarly, the naive T helper cells are activated by forming a stable complex with MHC–II molecule and undergoing clonal expansion into helper T cells, effector cells and memory cells. Then they are differentiated into Th1 or Th2 cells. The Th1 cells differentiate by the presence of the cytokines IFN-Ɣ and IL-12. The memory Th1 cells are involved in the faster response with re-encountering immunogen. The effector Th1 cells are able to stimulate the development of killer T cells. The Th1 cells are responsible for cellular immune response. The Th2 cells are differentiated by additional activation by co-stimulatory CD20 molecules and the cytokines IL-2 and IL-4. The Th2 cells secrete a wide variety of cytokines as well as stimulate production of antibodies.

The ratios of CD4 and CD8 T cells play a crucial role in the balance of these two T cells titers in the body. However another category of T cell known as regulatory T cells. They are responsible for maintaining the ratios of the other two T cell levels, preventing unsolicited attacks to the body's cells and suppression of immune response once the condition has subsided. They are differentiated by MHC-II molecules along with a wide variety of transcription factors. They can release inhibitory cytokines.

The bone marrow derived B cells produce highly specific antibodies in adaptive immune system. Their activation and proliferation usually depends on many factors like cytokines and activation and silencing of B cell-specific genes. B cell can be activated either by the help of T cells or without it. For activation by T cells, they have to bind to antigens on pathogens or in body fluids and then they interact with CD40 receptors of T cell. For activation without T cells, signaling molecules usually bind to membrane antibody like B cell receptor or BCR and toll-like receptors. The two main types of B cells are plasma cells and memory cells. Plasma cells are usually activated by T helper cells, undergoing somatic mutations and affinity maturation, which enables them to specific antibodies and undergo isotype class switching. Memory cells are antigen-specific plasma cells that are long-lived and on re-encounter with the same antigen, they are able to produce immediate and rapid immune response with highly-specific antibodies in very high levels.

The killer T cells usually recognize molecules present on all nucleated cells, which is the MHC I pattern. The helper T cells usually recognize molecules that are mainly found in non-nucleated cells like some immune cells, which facilitates in their interaction with the other immune cells, the MHC II pattern. It can be said that MHC I usually displays antigens derived infected cells and the MHC II displays antigens that are phagocytosed. (Murphy & Weaver, 2016)

Helper T cell assistance is required for the formation of memory T cells, by aiding the CD40 signal to recognize and bind to the effecter killer T cells. (Punt, 2013)

T helper cell mediated Th2 vaccination response begins as soon as the dendritic cells take up antigenic epitopes. For non-self epitopes that have been consumed by phagocytes, usually MHC II complex is suitable. The dendritic cells migrate to lymph nodes of t regions to match the antigen to the T cells, and the MHC II usually forms a complex with the helper T cell. They also secrete a lot of co-stimulatory signals. Only naive helper T cells are able to bind to the dendritic cells and get activated. The activated helper T cells send stimulatory signals like CD40L, which activates the B cells.(Siegrist, 2018) The B cells will then migrate to the helper T cells and recognize a helper T cell that has the same antigen specificity as the B cell.(Punt, 2013) Vaccine –induced regulatory T cells subdue T cell response in a variety of ways- they can drain the lymphatic fluid to prevent cellular migration and antigen presentation, block the priming of naive cells or destroy antigen-presenting cells. The regulatory T cells are stimulated after the imminent threat has been subsided to prevent T cells from attacking body cells and eliciting inflammatory response. The proportions of memory T cells formation depends on the levels of activated effecter T cells and the continuing presence of antigens. The memory T cells either stay in the lymph nodes or migrate to peripheral sites. (Siegrist, 2018)

Chapter 2

A Novel Form of Vaccination

The concept of edible vaccine had been present in the scientific community for the past few decades. It had currently been considered as a subject of research by demonstrating its versatile usage against a wide plethora of health hazards. Advancements in plant biotechnology have made it possible to introduce pathogenic antigens in plant vectors that would enable it to manufacture the antigens in plant parts. Additionally, plant-based edible vaccination imparts a crucial advantage, unlike other vaccines. The vaccine could be consumed as food. In places of the world where there are not enough facilities to provide overall vaccination coverage, edible vaccination could act as a promising candidate. The edible vaccine is recently considered the most promising type. This is when the plant part generating the vaccine molecule becomes the vehicle of transmission. Mostly edible plants are used for the preparation of the vaccine

2.1 What are edible vaccines?

Edible vaccines are immugen molecules synthesizing edible plants. The vaccine-producing gene is inserted into the host genome using modern biotechnology tools. These vaccines can be directly consumed. The edible vaccine also has an important role by forming the first-line defense against pathogens attacking the mucosa. The mucosal immune system (MIS) forms an important route for defense for gaining immunity through vaccination. The mucosal immunity is stimulated by the recognition of antigen by a group of specialized cells called the M-cells. These cells are usually present in the mucosal linings of the intestines. The M-cells pass on the antigen to the antigen-presenting cells that are adjacent underneath it for processing. (Abeysundara et al., 2017) The physical form of the immunogen is vital for the type of response the edible vaccine will develop. If the vaccine molecule is intact or partially intact, then an immune response would be developed specific to the vaccine. If the vaccine molecule is digested into soluvle particles by the body, then oral tolerance will be developed.(Tordesillas & Berin, 2018) So choosing an approprite palnt or palnt part is absolutely necessary to achieve the targeted effects of the edibe vaccine.

Table 2– List of major advantages and disadvantages of edible vaccines (Abeysundara et al., 2017)

Advantages	Disadvantages
1. Efficient mode of action- do not require adjuvant for stimulating an immune response	1. Tolerance can be developed to vaccine molecules
2. Provides mucosal immunity	2. The dose of the vaccine will vary depending on plant factors
3. Cost- effective – no need to store in cold chain storage	3. Dosage of vaccine need to be determined
4. Can be stored for future use as seeds- contains less moisture ,easily dried	4. Some plants cannot be eaten raw- cooking may degrade immunogen
5. Economical - can be easily grown as normal plants	5. Vaccine may get infected by plant pathogens
6. Widely accepted	
7. No need for sterilization- less specialized personnel needed	
8. Second –generation vaccines can be produced by integrating more antigens	
9. Growth rate can be increased by breeding with suitable species	

The above table summarizes the main pros and cons of edible vaccines. It can be deduced from the table that the benefits of edible vaccines greatly outweigh the shortcomings.

Moreover, transgenic crops can also decrease the processing costs of edible vaccines. The transportation and storage costs would decrease as well. The development process of such vaccines use standard modern biotechnology tools. These vaccines would be ideal for use in less-developed countries. However, the short shelf life of such vaccines may pose a problem. (Athulya & Vethamoni, 2018)

(Streatfield et al., 2001) states that edible vaccines are relatively safer than conventional vaccines. The vaccine consists of a relatively small portion of antigen, so it does not pose risk of infection or toxicity. Moreover, human or animal pathogens cannot infect plant cells, so it eliminates the risk of contamination. When expressed proteins are naturally packed with plant tissues, the stability of the proteins is enhanced. Furthermore, the cultivation process of transgenic vaccine plants is the same as normal plants, so it is cost-effective and does not rely on sophisticated technology.

Based on (Shah et al., 2011) , edible vaccines do not require adjuvant for effective delivery because the plant tissue acts as the carrier vehicle. These vaccines are also stable at room temperatures. Since edible vaccines are exposed to the mucosal membranes, they stimulate mucosal immunity in addition to systemic immunity. These vaccines could be mass cultivated to cater for nationwide immunization campaigns. However, concerns arise because of the different glycosylation patterns of plants and animals, which could affect vaccine efficiency.

2.2 Why are Plants selected for Edible Vaccines?

The main criteria for the use of plants as edible vaccines are that most of them can be eaten raw. In the case of edible vaccines where the tissue can be used as a mode of administration, vaccine molecules can be pressed be expressed in fresh plant tissues like foliage, leaf fronds of aquatic plants, and seedlings. They can also be expressed in dry tissues like cereals and seeds. Expression in dehydrated tissues has the advantage of being well preserved and effectively stored in the tissues, while fresh tissues require processing.(Joshi et al., 2008) When producing vaccines from edible plants, certain factors need to be considered as the choice of the plant, type of vaccine that would be produced, the storage conditions of the vaccine, and the plant part that would be targeted. Banana, potato, tomato, rice, wheat, maize, and barley are considered ideal options because can be easily stored and transported and are consumed by the mass population of all ages.(T. G. Kim & Yang, 2010)

The edible vaccine which is most practical is when the plant parts become the vehicle containing the vaccine. The prime candidates for such vaccines are vegetables, fruits, food crops, seeds, and legumes. They are the best options because fruits and vegetables can be consumed raw or after being semi-processed. Therefore, the disintegration of the vaccine molecule will be at a minimum(Jelaska et al., 2014).

(Han et al., 2006) and the team has proposed a variety of reasons for use of plants to produce edible vaccines that will function as a vehicle as well. For the use of vegetables, it has been

stated that vegetables taste good and they are free from harmful additives. Vegetables are naturally loaded with nutrition and fibers that would help to boost up the body's immunity. They are best when eaten fresh. Of all the edible vegetables, researchers are especially focused on potatoes, tomatoes, and carrots.

Potato is considered the ideal model for an edible vaccine that will also act as a vehicle. Potatoes are being used for many of the edible vaccines that have been successfully administered during clinical trials. In addition to this, enteric pathogens like ETEC LT-B and Norwalk virus capsid proteins are successfully expressed in potato tubers. Trials showed that a low dose of vaccine in raw potato tubers was able to produce remarkable amounts of both systemic and mucosal antibodies. The HBV vaccine is additionally being developed in potatoes. (Jelaska et al., 2014) Apart from this, vaccines for enteric pathogens like cholera, *E.coli,* and rotavirus are also being developed in potatoes. The human papillomavirus surface antigens are also expressed in potato tubers. Despite this, potato is not as immunogenic as other edible vaccines (Rybicki, 2010). However, it has been found that antigenicity is sufficiently preserved in cooked potatoes. When edible vaccine potatoes were boiled, the process only accounted for 50% loss of the vaccine. Therefore, it is essential to determine the cooking period for vaccine potatoes so that adequate vaccine properties remain (Aryamvally et al., 2017).

The tomato-based vaccine is a recent development. Antigens for hepatitis, HIV, and rabies have been successfully developed in tomatoes (Han et al., 2006). Tomato was used for the development of the first edible rabies vaccine (Jelaska et al., 2014). Tomatoes have also been used to express the epitope for the respiratory syncytial virus, along with Hepatitis E virus, *Yersinia*, DPT endotoxin, and synthetic HBV-HIV antigen (Rybicki, 2010). Recently it has been found that tomatoes contain a seed-specific promoter that can produce large quantities of a target antigen in seeds, making the production of antigens in tomatoes feasible(Han et al., 2006). Tomatoes also have greater biomass yield and their growth can be effectively monitored in the greenhouse. They are also more appetizing than potatoes. (Jelaska et al., 2014) Another major development is the incorporation and expression of the antigen for the coronavirus that causes SARS in tomatoes. Furthermore, the Norwalk virus vaccine has also been developed in tomatoes and provided far better protection than potato vaccines. Tomatoes are also being used for the development of cholera vaccines. Another notable vaccine development using tomatoes is for Alzheimer's syndrome (Concha et al., 2017). Inoculation of mice with tomato-based edible vaccines gave positive outcomes. The vaccine was able to stimulate both IgG and IgA (Aryamvally et al., 2017).

Genetic engineering of carrot is highly developed since carrot is one of the first GE crops. Satisfactory levels of vaccine molecules have been expressed in carrot cells. The structural integrity of the vaccine is also preserved; as no cooking is required (Han et al., 2006). Carrots have been targeted as a candidate for edible vaccines for enteric pathogens. The genes for the antigens of *E.coli* and *Helicobacter pylori* are being expressed in carrots. The vaccine candidates are showing promising results in both rodent models. Carrots are also a target vaccine candidate for HIV and showed satisfactory results in rodents(Concha et al., 2017).

As for fruit that will serve as bioreactors and vehicles for edible vaccines, bananas and papaya pose promising results. Banana is a good choice for the edible vaccines since research on transgenic bananas has been going on for some time (Han et al., 2006). This is because bananas are eaten as raw fruit or puree by both adults and children. They are available in abundance in countries where the vaccine is most required.The MaExp1 promoter is important in the expression of vaccine protein during the ripening of fruit (Jelaska et al., 2014). Banana is still considered an ideal vaccine source because it is widely cultivated in both the tropics and the subtropics. Four types of expression cassettes have been used for vaccine development. The highest expression rates have been found in the leaves of the plant (Aryamvally et al., 2017). Another fruit that is recently considered an ideal option is the tropical fruit papaya. Synthetic antigens for two types of tapeworms have been transformed into papaya (Han et al., 2006).

Food crops have also been identified as good sources of edible vaccines. Some of the crops include- alfalfa, spinach, lettuce, legumes, maize, and rice.Leafy crops are also being favored as the potential for edible vaccines. Crops like alfalfa, spinach, legumes, and lettuce are good candidates. Hepatitis B antigen has been developed in both lupine beans and lettuce. They have been proven immunogenic in both human and animal test models. Expression of sunflower seed albumin in lupins also reduced the chances of allergenicity in vaccines. (Rybicki, 2010) Alfalfa is a good source because it is high in proteins and low in secondary metabolites. Apart from this, newer plants include celery, cabbage, and cauliflower (Han et al., 2006). The capsid protein of the Norwalk virus is also used in peas to produce a vaccine. The protein constituted 8% of the total plant protein in raw fruits. Apart from this, when expressed in pea seeds, the protein content was 20-40% and it allowed the storing and preservation of the vaccine molecule (Concha et al., 2017).

Lettuce has been targeted as a host and carrier for edible vaccines. Trials are taking place to develop an HBV vaccine in lettuce (Jelaska et al., 2014). One of the notable

developments is the expression of -MVH of the measles virus in lettuce. The vaccine was shown immunogenic in pre-clinical trials (Rybicki, 2010). Moreover, the heat-labile B-subunit of cholera toxin has been expressed in lettuce. The expressed protein accounted for 2% of the plant's total proteins(Concha et al., 2017).

Moreover, edible seeds that can be rapidly processed are also good vaccine candidates. This is because dehydrated seeds would be able to store the vaccines effectively for longer periods. Maize and corn are particularly used for edible vaccine production because of the highly refined milling and processing procedures and high yields (Rybicki, 2010). Maize is already being used as a transgenic protein source in many biotechnology companies for the trial and production of certain vaccine candidates. The rabies antigen has been expressed in maize; it is producing satisfactory levels of antibodies in both humans and animals. The vaccine content is 2.7% of the total phytoproteins and is stable to post-harvest processing. The vaccine stimulated both IgG and IgA antibodies (Concha et al., 2017). Maize kernels provide the benefits of expressing significantly higher levels of vaccine molecules in their grains (Aryamvally et al., 2017).

Cereals like rice are enriched in soluble proteins and can be easily separated from the plant, thus enhancing antigen concentrations. Rice is a promising vaccine candidate as well because it can be used to express certain target proteins at high levels using constitutive and endosperm-specific promoters (Han et al., 2006). Transgenic rice is self- pollinated to prevent the loss of vaccine molecules. Rice is being studied for the development of Japanese cedar allergy vaccines using Cry j 1 and Cry j 2 allergens. The vaccine successfully generated an immune response. A combined vaccine using roundworm antigen fused with cholera toxin is also being developed in rice(Rybicki, 2010). An edible vaccine against *E.coli* is also being developed in rice using the B-subunit epitope. The vaccine effectively induced an immune response. Soybeans are a recent edible vaccine candidate. The heat-labile toxin of ETEC has been successfully expressed in soybeans (Concha et al., 2017) Rice is another ideal option for edible vaccines due to it being available and widely grown in tropical regions. Trial developments for the zoonotic pathogen *Chlamydophila psittaci* antigen are being conducted using rice. Pre-clinical inoculation in mice produced both IgG and IgA antibodies (Aryamvally et al., 2017)

2.3 Selecting a candidate for the edible vaccine

Plants that would be employed for the production of edible vaccines need to be strong enough to survive under all environmental conditions and pleasant to eat. They would also need to be readily available and easily transformed (Athulya & Vethamoni, 2018). The choice of an appropriate plant candidate is an essential process for edible vaccine production. The edible plants can be terrestrial or aquatic. Terrestrial plants can easily be scaled up or down, whereas aquatic plants would offer confinement and a good response to growth conditions. For terrestrial plants, the next step is to decide whether to use food crops or not. Leafy food crops can be an option for land plants. The plants targeted should preferably have economically feasible biomass yield to commercially support vaccine production. However, a major drawback of most land plants is that the protein is synthesized in an aqueous medium. Apart from this, cereals have been also targeted as a vaccine source. They allow storing at room temperature and are essentially in a dehydrated state. Seeds and grains are also free of oxidizing compounds. Despite these, seeds can also be genetically variable (Twyman et al., 2005)

Table 3 - Different candidates for edible vaccines(Athulya & Vethamoni, 2018)(Twyman et al., 2005)

Candidates	Advantages	Disadvantages
Banana	reproductive sterility, consumed raw, affordable, contains Vitamin A	
Potato	affordable, long shelf-life	cooking could denature vaccine
Maize	cheap, no need to refrigerate	cooking could reduce vaccine action
Tomato	widely cultivated, fast-growing, contains vitamin A, lectin acts as an adjuvant, salad vegetable	low protein content (Aryamvally et al., 2017)
Alfalfa	could reduce the production cost, high biomass yield	cross-pollination occurs, crossing with wild relatives
Soybean	seed can be used as a vehicle, pharmaceutically-feasible, high biomass production, no wild species, self-pollinated	degradation of vaccine molecules
Lettuce	salad vegetable, self-pollinated, high biomass	
Carrot	taproot is storage as well as edible organ	
Maize	widely cultivated, greatest biomass production	cross-pollinated
Rice	the greatest yield per unit	

The above table presents information about the major food crops that can be employed as edible vaccines together with their benefits and drawbacks. The edible plants are mainly kept in focus for their use as administration vehicles as well as bioreactors. Therefore, an ideal edible vaccine-generating plant must be widely cultivated and available, robust, have high biomass yield, need to be eaten raw or semi-processed, and not be able to cross with wild relatives of the same species.

2.4 Advantages of Fruit-based Edible vaccines

According to (Khan et al., 2019), fruits can be a crucial tool for developing edible vaccines. Fruit tissues are derived from flower cells that are usually developed from a single ovum. Fruits can be consumed directly so there is no risk of antigen degradation due to cooking. Prototype edible vaccines were formulated using the two most common tropical fruits- papaya and banana. These plants are rapidly growing, easily cultivated, and rich in vitamin-A. Diarrhea is one of the first diseases for which edible vaccines have been designed. In this case, the fruits that can be eaten raw as salads- tomato and potato have been used. In both instances, the antigens for diarrhea have been expressed in plant tissues- the *E.coli* heat-labile toxin and Norwalk virus for potato, and Norwalk virus for tomato as well. Both vaccines produced satisfactory levels of antibodies. In addition to this, research is being carried out for incorporating rabies toxoid in spinach hepatitis B antigenic in lettuce and potato.

2.5 Can algae be used as Edible Vaccines?

In theoretical terms, algal systems can function as an edible vaccine as well. The method of transformation would be chloroplast transformation. The algae are capable of providing the eukaryotic machinations that are required for protein folding in vaccine formation. In addition to this, algal systems are not a host for human pathogens, so the threat of contamination is removed(Hempel et al., 2011).

In contrast to multicellular terrestrial plants, unicellular aquatic plants like those that green algae *Chlamydomonas* would provide an easy transformation. The algal systems of green algae could express the antigen at higher levels than land plants. The *Chlamydomonas* have all the benefits of the land-based plants along with its features like enhanced biomass accumulation. Their growth is independent of soil conditions and environmental factors. Green algae can be easily housed in bioreactors and harvested, eliminating the chances of cross-contamination. They can also be freeze-dried and stored under normal conditions for longer periods. Pre-clinical trials are being conducted for HPV and HBV(Criscuolo et al., 2019).

Moreover, another alga that is currently gaining interest to produce the edible vaccine is the *Spirulina.* The algae have high protein content resembling milk, all the vital amino acids, minerals like calcium, iron, and zinc, essential lipids as well as vitamins that are required for a healthy body. *Spirulina* would also work as a stimulating agent for cancers, infectious diseases, and neurodegenerative diseases. The algae would be a perfect vaccine production and delivery platform, which would be therapeutic and cost-effective(Dehghani et al., 2018).

Furthermore, *Dunaliella* microalgae having a high content of proteins, carbohydrates, and lipids, as well as many pigments like carotenes is being used in trials for a cost-effective HBV vaccine (Gong et al., 2011). In addition to this, the diatom *Phaeodacty lumtricornutum* has been used to express the HBV antigen and is shown to be more effective than terrestrial plants (Hempel et al., 2011).

Chapter 3

Production Process of Edible Vaccine

At first, the edible vaccine was developed only for study purposes. In this case, the vaccine molecule is inserted into non-food-crop plants like tobacco. Tobacco was the ideal plant for the production of such vaccines

Edible vaccines are developed using the same transformation techniques as transgenic plants. The first step of edible vaccine development involves the appropriate identification of antigenic compounds of pathogens. The antigenic protein must elicit satisfactory levels of immunogenic stimulation. The next step involves the transfer of the antigen sequence into a suitable vector. The transformation process is usually carried out using several conventional technologies(Concha et al., 2017).

The most suitable method is the use of plant viral vectors (Concha et al., 2017). When using viral vectors for transformation, involves modifying the viral vector to express the vaccine molecule. The modified virus is then transferred to the desired plants(Shah et al., 2011). This method is very promising because most of the soluble vaccine molecules could get integrated into the viral capsid, thus effectively multiplying and re-infecting surrounding tissues (Concha et al., 2017).

The next transformation process is non-viral methods. They involve classical methods like Agrobacterium-mediated methods and biolistic methods. For the *Agrobacterium*-mediated method, the Ti (tumor-inducing) plasmid is chosen as a vector. The plasmid is then disarmed by removing its t-DNA and oncogenes. The left border and right border are maintained as well as the origin of replication and virulence genes. The gene of interest and the marker genes (antibiotic resistance) is inserted in the place of t-DNA. The modified Ti plasmid is then capable of introducing the vaccine sequence into the plant cell. The plants are co-cultured with the vector and then transformed plants are selected using antibiotic markers. However, this method needs time and appropriate facilities.

A solution can be usedforthe transient expression of vaccine molecules using *Agrobacterium* or viral vectors. The process is easily controlled and financially feasible in terms of production.

Despite this, certain important vaccine plant candidates resist *Agrobacterium* infection. For such plants, particle bombardment methods can be used. The plasmid is coated around minute inert beads and explosively launched at plant tissues using high-pressure helium gas.

The method is robust but post-modification screening is required as the process is random as well. The technique can also be applied for chloroplast transformation (Concha et al., 2017). Another method is the use of electroporation, which uses high volts of electricity to form pores in the cell walls of plant cell cultures from where the plasmid can enter the plant. The cell wall can also be weakened using low concentration enzymes (Shah et al., 2011).

Chapter 4

The Feasiblity of Edible Vaccine – A Case Study

Edible vaccines can be used for a broad spectrum of diseases and conditions, ranging from therapeutic use to prophylactic use. Some of the diseases combated by such vaccines have existing vaccination regimes, while others are completely new scenarios. Furthermore, edible vaccines offer some protection against diseases that were not focused on by traditional vaccines. Edible vaccines are being developed and perfected for diseases like urinary infections caused by Enterotoxigenic*E.coli* (ETEC), tuberculosis (TB), HIV-1, rabies, cholera, dengue, hepatitis, rotavirus, tetanus, Norwalk virus, Alzheimer's disease, malaria, allergens, and cancer.

4.1 Enterotoxigenic *E.coli* (ETEC)

Enterotoxigenic *E.coli*, ETEC, is a pathogen responsible for causing diarrhea in children and travelers. The LTB (heat-labile enterotoxin B subunit) of ETEC is a powerful vaccine molecule in terms of pathogenicity and is an adjuvant. The LTB usually binds to the GM_1-gangliosides on the epithelial membranes of intestine cells. It usually has a pentameric structure.

LTB

One of the prominent developments in the field of edible vaccines occurred with the expression of heat-labile toxin, LTB, of ETEC in the potato.

(Haq et al., 1995) was among the pioneers who attempted to develop an edible vaccine for diarrheal diseases like cholera and ETEC. The research group studied edible vaccine development using both tobacco and potato. Two types of vectors were used for both plants-pLTB-110 (conventional vector) and pLTK-110 (SEKDEL— vector with a signal for protein retention in the endoplasmic reticulum). The team chose the potato, *Solanun tubersosum Frito- lay 1607* variety. ELISA assay results found that the SEKDEL potato contained more vaccine levels (110µg) than the conventional vector transformed potato (30µg).

The group then conducted feeding studies on Balb/c mice to determine whether the vaccine potato stimulates mucosal immunity or not with pLTK-110 lines. The mice, which are excellent respondents for immunization, were given 5g of tubers expressing 15-20µg of vaccine molecules. The mice consumed it for 2-6 hours. The study was conducted for 18 days at intervals of 4, 14, and 18 days. Mice developed both IgG and IgA antibodies.

After the initial research, subsequent studies were conducted by future groups (Mason et al., 1998) was one of the groups who decided to further study the results.

In his study in 1998, the research group (Mason et al., 1998), focused on developing an edible vaccine against ETEC using potato as a vector. The group used the *Solanun tubersosum Frito- lay 1607* variety potato with a modified version of the LTB gene containing a plant-optimized gene, sLT-B, which would favor potato codon usage. The second amino acid was changed from asparagine to valine and the synthetic gene was similar to the native gene. The vector, pTH110, for the transformation was prepared using the *E.coli* strain DH5α and was fused with *Agrobacterium tumefaciens* LBA4404 using electrophoresis.

Then southern blotting and ELISA were conducted to confirm the findings. The sequence was probed with sLT-B sequences and among all the transformed lines, the line TH110-51 was the best-performing line with the highest proportion of vaccine production. Antigen testing to determine the expressed protein level with ganglioside-dependent ELISA demonstrated that TH110-51 expressed the highest level of vaccine molecules.

Further studies were performed in mice for determining the immunogenicity of the two lines with the well-performing lines including TH110-8 and TH110-51. The studies included feeding Balb/c mice – either by gavages or normal feeding- with the transformed tubers (4µg of the former and 10 µg of the latter) or purified antigens at the 1-week interval for a fortnight. The results showed that even though all transformed lines effectively stimulated the immune system, TH110-51 was most effective in the stimulation of both IgA and IgG. However, gavage feeding was found to be more effective. Despite this, in most cases, the mice were partially immunized.

Two subsequent ETEC edible vaccine developments were conducted using corn plants.

(Streatfield et al., 2001) and his team conducted the prototype corn-based edible vaccine development using the LTB antigen. The chosen variety was Hi-II. A synthetic variant of the antigen was chosen and modified by the addition of the α-amylase signal molecule and enabled vaccine accumulation in the cell walls. The construct was introduced into the *Agrobacterium*Ti- plasmid along with the *pat* gene for resistance to the herbicide Basta. The serial transformation was conducted- the first during *Agrobacterium* infection and the next after collecting the transformed seed embryos.

Feeding studies were conducted on Balb/c mice for 30 days to determine immunogenicity. Mice were fed with either 5µg or 50 µg of the vaccine corn at 7 days intervals for 21 days; blood samples were drawn on 6, 13, 20, and 27 days, while fecal samples were collected before the feeding as well as on 4, 7, 11, 14, 18, 21, 25 and 28 days. 96-well immunoblot assay was used for both serum antibody (blocked with serum) and fecal antibody (blocked with BSA).

All the mice that were given the edible vaccine produced equivalent amounts of antibodies to that of recombinant antigens in both their sera and feces. The antibody production was more noticeable during day 13 in the serum and day 7 in the feces. It was also noted that 5μg doses were sufficient to induce an immune response, especially mucosal immunity.

In his next study, (Streatfield et al., 2002) and his team used two varieties of corn – one with α-amylase signal and another wild-type variety. The native form of LTB gene used in this study- one normal gene inserted into PGN8957 vectorand the α-amylase modification antigen in PGN7101 vector. The same corn variety was chosen as before. *Agrobacterium tumefaciens* strain EHA 101 was chosen as the vector. PCR analysis with antigen-specific primers and Northern blot analysis with LTB probe showed the presence of the vaccine gene in the plant genome. Other assays were as follows- immunoblotting with anti- LTB rabbit antibody for protein translation and GM1-ELISA to determine the conformation of the vaccine molecule. The PGN7101 variety was able to synthesize the vaccine of the native conformation. This variety was also highly specific in binding to the receptor. Vaccine level assay determined that the vaccine concentration in the soluble protein ranges from 0.013 to 1.8%. The normal variety was not as efficient. It was also noted that cross-breeding increased the vaccine content of both types significantly.

Furthermore, unique approaches and vehicles have been used for developing an ETEC edible vaccine.

(Kang et al., 2006) have tried to express the ETEC antigen in the Siberian ginseng (*E. senticosus* Maxim). They have used the synthetic LTB gene that was embedded in the pMY0111 vector. Somatic embryos of the Siberian ginseng were transformed using *Agrobacterium tumefaciens*LBA4404 strain. The method used the tri-paternal mating method to transfer the plasmid to the vehicle with the help of another plasmid, pRK2013. Kanamycin-resistance genes were used to select the transformed plants and cefotaxime was used as a marker for selecting plants that are free from persistent bacteria. The vector was mass-cultivated and then analyzed. PCR analysis revealed the presence of the 414bp vaccine gene. Northern blotting assay detected the positive signal of the vaccine gene. Western blotting illustrated that the expressed vaccine molecule is identical to the native antigen, forming a pentamer of 50kDa. Quantitative ELISA results demonstrated that the vaccine level is 0.36% of the total protein content. The edible vaccine was also shown to have a high affinity for GM_1-receptors during GM_1-ELISA.

Apart from this, the vaccine for ETEC has been further developed in other vegetables like carrot.

(Rosales-Mendoza et al., 2008) had worked with carrots in the development of an edible vaccine against ETEC. The team considered carrot as an option because the vegetable is usually eaten raw. Carrots are rich in Vitamin-A and Vitamin-A precursors, which are potent white blood cell boosters. They also contain lutein and cellulose fibers. Carrots have the potential of enhancing IgG and IgA. The team had used the carrot strain *Daucus carota* L. var. Nantes seeds and the Agrobacterium stain LBA4404. The transformation vector used was pBI121 in which the uidA gene was sliced out. In order to select the best-performing lines, PCR, RT-PCR and southern blotting was carried out. In order to check for serum antibodies, indirect ELISA with goat anti-mouse IgG, anti- IgA and goat anti-mouse IgG1 or IgG2a were added to the well.

Analysis of the vaccine content was conducted using GM_1–ganglioside revealed that each gram of tap root contain 3µg of vaccine; in lyophilized carrot powder, the vaccine content was estimated at 10µg in 430mg of powder. Feeding studies in mice with Balb/c mice was performed, where the mice were fed with gavages for 2 weeks at 7days interval, to find out if mucosal immune response will be elicited by vaccine carrot. The final volume of the vaccine was made 2 ml and administered during 10 hours at 2 hours interval with a dose of 0.2ml. Anti –LTB antibody response was present in the specimen from serum and intestine of the mice, with specific serum and intestinal IgG1 or IgG2a response.

Further studies were carried out in the development of edible vaccine using the LTB of ETEC in the tomato.

(Loc et al., 2014) has reported that the vaccine properties are retained even at very high pH. The researchers have developed an edible vaccine using tomato which expressed the LTB in the fruits of *Solanum lycopersicum L* - the tomato. Tomato was chosen as a bioreactor because of its dual-purpose; it can be eaten raw so the vaccine molecule would not degrade. The vector pMYO51 was chosen which contained a synthetic LTB portion and a signaling Kozak sequence that is controlled by the cauliflower mosaic virus-CaMV-35s promoter. The tomato was transformed using the *Agrobacterium tumefaciens* LLBA 4404. Then the transformed plantlets were assayed for the production of the vaccine at protein levels, DNA levels, and RNA levels. PCR examination showed the successful integration of the LTB gene in the genome of tomatoes. Southern blotting further confirmed the presence of the vaccine as well as highlighted that a single copy of the gene is expressed in the plantlets. Western blotting was used to determine the expressed protein in transgenic tomato by extracting the

soluble proteins from its leaves and fruits. Out of 5 transformed plantlets, 3 plants successfully expressed the pentameric protein in fruits while 2 plants produced oligomeric protein in leaves. From the results, it can be deduced that 1g of fresh tissue would contain 14-18µg of vaccine, while lyophilized tissues will contain 37.8µg of the vaccine.

SICL

In a recent study, a group of researchers has used a synthetic chimera ETEC vaccine to develop an edible multivalent vaccine for both ETEC and another related *E.coli*condition. (Shojaei Jeshvaghani et al., 2019) used two strains of pathogenic *E.coli*– the ETEC as well as the entero-hemorrhagic EHEC to develop a multi-potent vaccine. In this study, they have used the canola seeds (*Brassica napus* L.) Hyolacultivar. The team designed the vaccine molecule with the potent epitopes from the ETEC and EHEC. They enhanced the sequence with the KDEL signal and added the canola promoter FAE. This vaccine gene was called the SICL. It was added to the *Agrobacterium* with the vector pBI1400. All sequence designs were confirmed using PCR and restriction digestion. The transformed plants were identified using PCR with specific primers for SICL gene encoding the ETEC antigen. The total RNA content was analyzed using RT-PCR for detecting the vaccine mRNA. After the test, only the plants that provided positive vaccine signals were selected. The number of vaccine molecules in the chosen plants was determined using ELISA. It was revealed that the vaccine content is 0.4% of the total protein content.

For feeding studies, Balb/c mice were chosen. They were fed with either 5 doses of transgenic canola or 4 doses, of 20 µg vaccine molecules in 500µl and given a 5µg dermal booster. Then blood and fecal samples were taken. Then the antibody responses for each treatment group were determined. The IgG titers for the booster group soared without much lag time when compared to only edible vaccine response, where the levels rose gradually. In contrast, both the fecal and serum IgA titers had similar dramatic climbs. It was revealed that the serum IgA levels increased more uniformly compared to the fecal antibody levels. The responses were much higher in the booster group.

The binding-inhibition assay of the ETEC vaccine fragment with 100 Caco-2 cells and immune sera was conducted. It was found that the immunized sera were able to dislodge the attachment factors of the ETEC and ETEC formerly treated with the vaccine serum had decreased binding ability.

Moreover, a rabbit loop assay was conducted to check the toxin-neutralization effects of the vaccine-induced antibodies for LTB. The change in shape due to fluid accumulation

was observed. It was found that the loops had normal histology as well as the fluid accumulation was dramatically reduced in the immune sera.

It could be inferred that the edible vaccine for Enterotoxigenic *E.coli* is being developed and improved from as early as the 1990s and the work is still in motion to formulate a safe and effective edible vaccine.

4.2 Cholera

Cholera is a disease of the gastrointestinal tract that is caused by the Gram-negative bacterium *Vibrio cholera*. It has two common strains in the tropics and subtropics- O1 (Ogawa, Inaba, and Hikojima) and O139. It also has many different serotypes. It is mainly asymptomatic, however, if the patient is left untreated, it can cause severe acute diarrhea and dehydration, often leading to death. One of the main epitopes of *V.cholera*is the nontoxic CTB (cholera toxin B-subunit).

CTB

The CTB has been used to develop an edible cholera vaccine. Studies have been conducted since the 1990s and are continuing today.

The first study was conducted by (Arakawa et al., 1997). The group has fused the CTB with SEKDEL. The chosen potato variety was *Solanum tuberosun cultivar Binjte*. The vector chosen for the transformation is the pPCV701FM4 derived from the pPCV701 plasmid. The potato tissues were transformed using the *Agrobacterium*-mediated method.Luciferin and Kanamycin resistance gene were added as marker genes for rapid identification.

Potato plants that were both resistant to Kanamycin and had luciferase activity were selected and amplified. Detection of the CTB gene was conducted using the PCR method with the forward and reverse primers of the vector showed the successful incorporation of the 540bp antigen. Further confirmatory tests were done using immunoblot assay with anti-CTB antibodies confirmed a strong expression of the vaccine gene.An interesting observation was also noted, higher luciferase activity was detected in cells with more amplification products. This could be due to the fact that more than one copy of the vaccine gene was inherited by a cell.Quantification of the potato tissues was conducted using thequantitative chemiluminescent ELISA and immunoblot revealing the vaccine content to be 0.3-0.35% of the total protein concentration of the potato callus tissue- approximately 30-35μg by both methods. Furthermore, GM_1 –ELISA has conducted to assay the binding capacity of the

vaccine illustrated a high affinity for GM_1 –ganglioside and that the vaccine retained its native configuration.

The next study also focused on developing a cholera edible vaccine that would be expressed in tomatoes.

(Jani et al., 2002) selected the tomato (*Lycopersicon esculentum Mill var*iety Pusa Ruby) and transformed it with the pCAMBIACTB containing ctxB, gusA, nptII genes under 35S promoter. *Agrobacterium tumefaciens* LBA4404 vector was chosen. After transformation, four plant lines were transformed. PCR was conducted on these transformed plants and showed the characteristic 413bp band. Confirmatory tests were conducted on these plant lines by Southern blotting with ^{32}P probe. It was found that the transgene integrated into the host genome. It was all shown that lines A and B inherited more than 1 copy of the vaccine gene, while C and D have a single copy. Northern blotting was carried out to check the RNA transcripts with the same probe, mRNA signals were present for all lines; however, it was noted that higher signal levels were found for single gene copies. Western blotting was carried out using a blocking solution of non-fat milk and tris-buffer to check the vaccine protein in the plants. Immunoblotting with 15% SDS-PAGE analysis and incubation with anti-cholera toxin antibody from rabbit showed that the vaccine molecule from C and D were in oligomeric conformation. However, no protein was detected in A and B. The researchers deduced that since more copies of the gene were present there, gene silencing could occur. Densitometry studies revealed that the total vaccine level in the plant lines is 0.04%, meaning 100g tomato would contain approximately 440µg CTB. The binding ability of the vaccine was also tested using GM_1 –ELISA. This is done because the specific confirmation of CTB is required for specific binding. All the vaccine molecules were able to bind to the GM_1 ganglioside, proving that the vaccine retained their native form.

Efforts were also being made to express the CTB in the rice plant. Two studies were conducted using rice plant as vector

(Nochi et al., 2007) and his team decided to design an edible vaccine with rice. The selected the cultivars *Oryza sativa L.*cultivar *Kitaake* and *Hosetsu,* and transformed them using *Agrobacterium*-mediated transformation with the vector pGPTV-35S-HPT. Assay with PCR, Western blot and Northern blot demonstrated the transgene successfully integrated and was expressed in the plant. Analysis with densitometry and SDS-PAGE illustrated *Kiitake* variety had more vaccine levels (30µg/seed) compared to *Hosetsu* (5µg/seed). Western blotting revealed the vaccine molecule had proper configuration. The accumulated vaccine in rice endosperm was found to be approximately 75%.

Efficacy assay with mouse intestine cells using *Ulex europaeus* agglutinin (UEA-1) showed that the cells vaccine was effectively taking in the vaccine. For determination of binding inhibition, GM1-ELISA with GM_1-cells and treated sera was conducted.

Oral immunization was conducted using female Balb/c mice and C57BL/6J mice that have an nlrp12 mutation, which leads to reduced immune response in these mice. Six doses of 12.5, 25, 50, or 100 mg with about 10 µg vaccine at a weekly interval were administered. One week after the final dose, blood and feces were sampled and analyzed with inhibitory GM1- ELISA. The mice were also inoculated with 20µg CTB. The symptoms were observed.

It was found that the edible vaccine-treated mice prevented GM_1 binding assay in the samples, inferring the presence of protective antibodies. No clinical symptoms of diarrhea were observed. Intestinal water assay with CHO cells also illustrated a healthy morphology. The rice-based vaccine was also effectively stored in normal conditions and was able to retain its antigenicity for approximately 18 months. It was concluded that oral immunization successfully elicited antigen-specific IgG and IgA, at a dose as low as 75µg.

(Oszvald et al., 2008) has designed a vector pMYN317 together with the synthetic CTB gene, *hpt*gene providing hygromycin resistance,and SEKDEL retention sequence. However, in this case, the team transformed the rice cells using the biolistic method by particle bombardment method.It was found that seven plants were effectively transformed. Out of the seven putative lines, four lines were found fertile

PCR method with two CTB specific primers was employed to detect the transformed gene in the plant lines revealed 414bp band that might be specific for the CTB protein in the putative transgenic plants. Northern blot analysis with^{32}P probe conducted to detect the total RNA levels in the transformed lines reported positive signals for the transgene and 3 lines were producing high levels of RNA. The presence of the vaccine protein was determined by immunoblotting demonstrated strong patterns were present in the 40kDa region that corresponds to the CTB tetramer. GM_1-ELISA was also conducted for confirming the receptor binding capacity of the vaccine. GM_1-ELISA results also illustrated that the vaccine antigen was able to specifically interact with the GM_1 cells, thus retaining its native form. Quantitative ELISA showed that the amount of vaccine molecules in the plant is 1.5-2.1% of the total protein levels.

In addition to this, research has also been carried out to develop an edible cholera vaccine in fruits.

(Renuga et al., 2010) has attempted to develop an edible vaccine for cholera in the banana. The *Robusta*species banana was chosen as an expression and delivery vector. The gene for CTB was cloned into the pCAMBIA vector along with the 35S CaMV promoter and a 35S enhancer. The transformation was carried out using *Agrobacterium*-mediated process. To detect the presence of the vaccine gene in the banana calli, 12% SDS-PAGE separation of the vaccine molecules was carried out using coomassieblue stain, and then Western blotting was performed to analyze the protein content in the transformed plant. It was found that an 11.6kDA band was present that corresponds to the vaccine antigen. It was also discovered that boiling of the banana retained 50% of the vaccine molecule.

Subsequent research by the same team was conducted using a different approach. In the next study, the vaccine molecule from the transformed banana callus was isolated and analyzed in PCR. The results illustrated a 256bp band similar to that of the CTB antigen. Then the vaccine molecule was introduced into untransformed plants by the micro-syringe method. Western blot analysis revealed continued expression of the vaccine molecule in the new plants.

Apart from the conventional *Agrobacterium*-mediated transformation processes, a group of researchers has recently employed biolistic gene gun technology for the production of cholera edible vaccines.

(Suleiman et al., 2013) have used chloroplast transformation methods with the use of gene gun technology. They chose the lettuce, *Lactuca sativa*, as their candidate. The pUC based lettuce vector pLS-LF was chosen. An expression cassette with CTB gene fused to the BADH gene was designed. The BADH gene was used as a selectable marker conferring salt tolerance. PCR technique with forwarding primer 16SF and reverse primer BADH was used to detect the presence of the vaccine gene in the leaves; it was illustrated thatthe presence of bands that are more than 2000bp long, inferring it was due to the transformation cassette. The untransformed leaves were subjected to more rounds of transformation to fully transform them. Southern blotting was done to check the transformed DNA with the help of DIG chloroplast flanking sequence.It revealed that there was 4.5kb to 2.9kp fragments Then western blotting was done for assaying the protein content. It was found that the highest vaccine level was in those of old leaves (6.3%); followed by mature leaves (5.2%) than young leaves (2.6%).

Works have also started recently for developing a chimera edible vaccine containing the CTB antigen.

(Davod et al., 2018) designed a trivalent vaccine containing CTB, *IpaD* (*Shigella*) and PA20 (anthrax) antigens. The three epitopes were inserted into a single cassette and inserted into the pBI121 vector containing a protein retention signal. *Agrobacterium tumefaciens GV3103* strain was selected for the transformation. The heat-shock method followed by agroinfiltration of fruits and leaves was performed. PCR with antigen-specific antibody confirmed the presence of the 321bp fragment of IpaD antigen and 535bp PA20 fragment. Chromatographic assay done with his-tag column chromatography demonstrated the 50kDA vaccine molecule band. Immunoblotting assay with anti-IpaD polyclonal antibodies as well as goat anti-Rabbit HRP coded antibodies revealed that maximum precipitation occurred for the ripe green fruits followed by ripe red fruits. More research is underway for trials involving animals and humans.

So, it can be said that the development and improvement of an edible vaccine for cholera for medical use is going on from the early stages of agricultural biotechnology till today.

LTB - CTB

Furthermore, a group of researchers has tried to develop an edible vaccine for both ETEC and cholera.

(Chikwamba et al., 2002) and his group at first decided to work with the LTB. They have chosen maize as the vector. They used the maize hybrid line Hi II and the vector pRC4. They employed the plant optimized coding vector- sLTB that was isolated from the *E.coli* H10407 and transformed the plants using the particle bombardment method. Since an endosperm-specific promoter was used, it leads them to use an herbicide-resistance gene. PCR was performed to detect the presence of the transgene. Out of 60 samples, 47 lines were found to be transgenic. Three samples were chosen from 20 transgenic lines for re-enhancement. Out of the twenty samples, 19 lines were fertile, termed P77. These were then crossed with wild-type selfed commercial line B73 to produce a prototype edible vaccine. The majority of the mature kernels had less than 0.01% vaccine expression, a few had between 0.01-0.05percent while only 2 lines had more than 0.05% vaccine expression. Six lines were chosen for further enhancement. The lines were planted and then either self-pollinated or crossed with the commercial line and hybrid line. P77 from the first generation lines 2, 7, and 9 were chosen. Southern blotting showed they inherited more than one vaccine gene copy. Immunoblotting with the second generation kernels that were prepared from the lines P77-2, -7, -17, and -18 had revealed that they have the native pentamer conformation that retained its antigen capacity. Then feeding studies were conducted to analyze the stimulation of LTB antibodies and their cross-reactivity of CTB antibodies Feeding studies on Balb/c mice for 21 days on

days 3,7 and 21 with all the sampled kernels with 10µg dose revealed that the antibody levels increased at day 13. For transgenic vaccine maize, there was also a late-stage primary response due to antibody switchingthat is found to be specific IgG. The secondary response was observed at day 27, more rapid response with increasing IgG levels. Another important factor was the secretion of mucosal IgA, since the vaccine antigen will be in contact with the stomach. Fecal IgA response was found at increasing levels during both primary and secondary responses. The IgA levels reached their zenith on day 27. The same trend was also observed for serum IgA levels.

Noting valuable and conclusive data from this study, the group further analyzed a structural and functional antigen, the cholera CTB antigen. Cross-reactivity tests were conducted on the mice after immunization with CTB antigen. It was revealed that there was little initial primary response when compared with the anti-LTB response. The anti-CTB IgG response as well as both fecal IgA and serum IgA response boomed on day 27. Additionally, all the antibody levels were remarkably higher for the edible maize vaccine.

Another research group from Japan decided to carry on and refine the pilot study that (Nochi et al., 2007) started.

(Tokuhara et al., 2010) decided to prepare a bivalent vaccine for cholera and ETEC in rice. They coined the name MucoRice-CTB. The team aimed for stimulation of a cross-reacting antibody that will target both ETEC and CTB. With the aforementioned process, the team inoculated mice- Balb/c and Balb/c based polymeric Ig receptor (pIgR) KO – with 150µg MucoRice-CTB 3 to 4 times with a gap of about 2 weeks between doses. The booster dose was given 6 months after the initial immunization. Feces and blood were sampled on weeks 1, 4, 12, 16, and 24 and assayed by ELISA.

The pIgR cells of both types of mice were analyzed. The pIgR mice were developed in a way that made them lack secretory IgA. So these mice produced lower levels of mucosal antibodies. However, the Balb/c mice produced significantly greater levels of antigen-specific secretory IgA. The levels of IgG were comparable in both types. The AFC cells were analyzed using ELISPOT assay. Interestingly, it was revealed that the lacking mice produced more secretory IgA cells.

An antigen challenge was conducted using 20µg CTB or 30µg LTB. The mice were fasted for 12 hours, inoculated, and then after 9-12 hours, they were dissected and the intestine was removed to observe diarrheal symptoms, and the contents were analyzed to determine intestinal fluid.

The results illustrated that the immunized Balb/c mice had CTB-specific antibodies. The antibodies cross-reacted with LTB when it was introduced; the LTB antibodies were present in both sera and feces. The inoculated mice acquired immunity from ETEC.
An intestinal loop assay was performed next. The two groups of mice fasted for 3 days; a loop was made close to the stomach. Live pathogens- *Vibrio cholera* 01 Inaba and ETEC which only secretes LT were obtained from laboratories. The pathogens were delivered into the gut loops. After 12-18 hours, the mice were euthanized and the loops removed. The fluid buildup was measured. It was recorded the length should not be less than 30μL/cm.
The assay demonstrated the fact that the occurrence of diarrhea was 20-40% lower in the immunized mice; full immunity was attained for cholera and the mice developed greater resistance to the infection.
So, it was concluded that mucosal immunity was achieved by MucoRice-CTB and the immunity not only conferred to cholera but also extends to ETEC.

4.3 Rotavirus

Rotavirus is a pathogen that causes gastro-enteritis mainly in the bodies of children. The symptoms of the disease are diarrhea, vomiting, fever, and dehydration. Most children by the age of 5 around the globe are susceptible to getting infected by this virus. Infections mainly occur during winter-spring seasons. The virus is usually transmitted by close contact with a contaminated object or by ingestion of contaminated food or water. Currently, there are only two effective vaccines for this virus, so further development of child-friendly vaccines is required.

VP6 gene

The first step toward the development of the rotavirus edible vaccine began in the 21st century. The rotavirus VP6 antigen was used.
(Chung et al., 2000) and his team designed a simple edible vaccine for rotavirus using simple techniques. They chose tomato *Lycopersicon esculentum* Mill, as their vehicle. They used the vector plasmid pILTAB357-VP6 and inserted the VP6 gene inside it. Then they transformed the tomato with *Agrobacterium*-mediated transformation. They centrifuged the transformed tomato and carried out Western blot with guinea pig anti-V6 polyclonal antibody, and probed with rabbit anti-guinea pig IgG alkaline phosphatase conjugate to determine the presence of the vaccine molecule. It was found that the 45.5kDa vaccine molecule gave a band in the

intracellular fraction of the tomato blot with the addition of BM purple AP substrate solution. The vaccine content was determined to be 0.33mg per liter of fresh weight.

Another subsequent study was done on this transformed vaccine tomato for increasing its protein content.

(C. H. Kim et al., 2001) and this team decided to increase the vaccine content of the tomato using sodium butyrate solutions. They used sodium butyrate of varying concentrations for different durations. All results were analyzed using Western blot as the previous study by the group. At first, the optimum level of sodium butyrate was determined using 5-10mmol, where it was noticed that the tomato culture would slowly stop growing and eventually die off at higher concentrations. However, the content increased to 0.49 mg/l when 10mmol sodium butyrate was present. Next, the optimum duration of adding the solution was studied by the addition of the solution on the first, sixth, and twelfth days after incubation with *Agrobacterium*. It was noted that the levels reached 0.49mmol on day 6.

Using the data collected, two-dose regimens of 10mmol sodium butyrate- one on day 6 and the other on day 9 – were used to boost the vaccine content. The vaccine content soared to 0.73mg per liter.

VP7 gene

Another potent rotavirus antigen is the VP7 antigen, which can lead to the production of powerful neutralizing antibodies. Another group of researchers conducted a study using this antigen.

(Wu et al., 2003) have decided to work with the human rotavirus serotype A VP7 gene. They used the potato,(*Solanum tuberosum* cv. taiwanhong) as a vehicle and used the whole VP7 gene along with the SEKDEL sequence was cloned into the potato genome using the HRV-VP7 expression cassette. The transformation was performed using *Agrobacterium*-mediated method. Twenty putative transgenic lines were found. Then the integration of the transgene was detected using Southern blot with 32P-labeled probe corresponding to the VP7 ORF. The results illustrated the presence of a 1074bp band for the transgene in all lines. Next, RT-PCR was performed for specific primers P1 and P2 to confirm the transcription levels. It was demonstrated in the results that the 1062bp was present in all plants. After that, the translation level of the vaccine molecule was analyzed using Western blot with primary goat anti-human rotavirus polyclonal antibody and secondary alkaline phosphatase labeled anti-goat Ig mouse antiserum. The 38kDa band for the vaccine protein was observed in the results. The quantity of the vaccine molecules was determined using ELISA with polyclonal antibody

antirotavirus protein and horseradish peroxidase IgG. The vaccine concentration was estimated to be 3.84µg/mg.

The immunogenicity of the vaccine was tested using feeding studies in mice to detect the immunogenicity of the edible vaccine. The dose was either 2g (84µg) of potato vaccine with 10µg CT, 1g (42µg) of vaccine with 10µg CT, 2g of vaccine with 10µg CTB and 1g of vaccine with 10µg CTB on days 0,7,14, and 42. Samples were collected on 0, 7, 14, 21, 35, 42, and 67 days and analyzed using the immunoperoxidase ELISA methodusing VP7 antigen. For the mice that showed a remarkable serum antibody response, 18 out of 19 CT-treated mice and 19 out of 20 CTB-treated mice also had very high specific intestinal IgA. However, they had lower urine and saliva IgA. Then neutralizing assay was conducted using mice sera or feces. Even though there were negligible neutralizing effects in the sera, neutralizing antibodies were detected in the feces.

The group then proceeded to conduct studies on this prepared edible rotavirus vaccine to determine if the vaccine will still be effective after 50 generations.

(Li et al., 2006) used the same potato vaccine's progeny that was grown to reach the 50th generation to determine the transgene inheritance and efficacy. RT-PCR with P1-P2 primers was performed to analyze the transgene inheritance. It was observed that all progeny inherited the vaccine gene. Next, Western blot analysis of the transcription levels was conducted using mouse monoclonal antibody against VP7 and horseradish peroxidase-conjugated rabbit antimouse polyclonal IgG antibody. The results illustrated that the immunogenic properties of the vaccine were retained. The vaccine content was assayed using ELISA by the same primary and secondary antibodies. The vaccine content was estimated to be 40µg per gram.

Feeding studies were conducted on highly responsive female Balb/c mice (on days 0,7,14, 21, and 42) with 2g of 50th generation vaccine with or without 10µg CTB and immunodeficient female C57BL/6 mice were fed with the only adjuvant vaccine in a similar fashion were injected with 20µL antigen. Moreover, they were observed for weight loss and other rotavirus symptoms. Sera, saliva, and feces were also collected similarly to before and analyzed with ELISA horseradish peroxidase (HRP) conjugated goatanti-mouse-IgG1. Furthermore, the IgG was analyzed using HRP conjugated rabbit anti-mouse-IgG antibody with TMB peroxidase substrate. A result to that of the former study was revealed, with little presence of antibodies in the sera and saliva and notable antibody presence in the feces. A similar neutralization was conducted. It was demonstrated that neutralizing antibodies were present in both sera and feces of both the edible vaccine and the adjuvant vaccine-fed mice.

Cytokine analysis of C57BL/6 mice was also carried out with splenocytes that were stimulated with inactive rotavirus 2 weeks after the booster. The interleukins levels showed little or no rise, the interferon-gamma, and TGF- beta levels are exceptionally higher. In the last stage, a specific cytotoxicity assay was performed with UV-inactive rotavirus cells. Effector cells were introduced to simulate cytotoxic T-cells for cell lysis. Cell lysis was noticed in the edible vaccine immunized mice.

CTB-NSP4 combined antigen

Combined vaccines using CTB antigens with other potent rotavirus antigens were also formulated.

The first attempt was performed using the CTB gene with the NSP4 gene. The genes were fused and inserted into the plasmid pPCV701CTB-NSP4 and used the Agrobacterium strain GV3101 pMP90RK for the transformation aided by electrophoresis. The chosen vehicle was potato- *Solanum tuberosum* cv. Bintje. PCR was performed using the primers 5' primer of CTB and the 3' primer of NSP4. In the six putative transformed plants, the results revealed 910kb band for the fusion protein. The expression level of the vaccine was determined using immunoblotting with primary anti-NSP4 antibody and secondary mouse anti-rabbit IgG conjugated with alkaline phosphatase. Two bands were present- the monomer protein of 33kDa and the pentamer structure of 165kDa. The vaccine level was determined using GM1-chemiluminescent ELISA with rabbit anti- NSP4 primary antibody and secondary alkaline phosphatase-conjugated anti-rabbit IgG together with a substrate. The light emitted was measured by the relative light units. One line was illustrated to have the most vaccine concentration- 0.026% (12.5–25µg) while another line demonstrated the least level-0.006%.

CTB – VP7 combined antigen

The development of the next combined vaccine used the CTB gene along with the simian rotavirus strain gene that can induce rotavirus in humans.

(Choi et al., 2005) used the gene for VP7 antigen and fused it with the CTB gene. They inserted the gene into the plasmid pPCV701CTB::VP7 and used *Agrobacterium*-mediated transformation using the strain GV3101. Their chosen vehicle was the potato- *Solanum tuberosum* cv. Bintje. The presence of the gene in the putative lines was analyzed using 5' primer of CTB and the 3' primer of VP7. The results illustrated the presence of a 1137bp band corresponding to the fusion gene. The expression of the vaccine molecule was checked usingthe immunoblotting method with rabbit antirotavirus antiserum and mouse anti-rabbit IgG conjugated with alkaline phosphatase. The monomer form of 42kDa was detected in the boiled samples and the 210kDa pentamer form was present in the raw samples. The level of

the vaccine molecule was studied using GM1-chemiluminescent ELISA with primary rabbit anti-cholera toxin anti-rabbit IgG conjugated with alkaline phosphate secondary antibody. The emitted light was measured in Relative Light Units. The vaccine content was determined 6.4mg per 100g of tissue.

4.4 Norwalk virus

The Norwalk virus or the Norovirus is another virus that causes gastroenteritis. The virus is commonly spread by contact with an infected person or by consuming contaminated food and water. The symptoms appear as early as 12-24 hours after infection and last for 1-2 days, but the illness lasts for as long as 2 weeks.

The research on producing an edible vaccine for the virus started as early as 1996. There have been two major works on this virus by the same group of scientists.

(Mason et al., 1996) tried to develop the edible vaccine for Norwalk virus using the Norwalk virus capsid protein gene. They used, which contained the patatin promoter. They transformed the plant, potato,in the potato- *Solanum tuberosum* "Frito-Lay (FL) 1607 and the vector pNV140 by *Agrobacterium*-mediated transformation. They were tested by ELISA with guinea pig anti-(i-rNV) serum and goat anti-guinea pig IgG-horseradish peroxidase conjugate for the presence and quantification of the antigen using Coomassie blue dye-binding assay. The confirmation of the vaccine molecules was assayed using a sucrose concentration gradient with increasing sucrose gradients - 10%, 20%, 30%, 40%, and 50% sucrose in PBS. The vaccine content was estimated to be 10-20µg per gram of tissue. In sedimentation studies using sucrose concentration, it was revealed that the particles were arranged similarly to the native Norwalk virus protein. However, about half of the vaccine molecules in the tubers regressed to the soluble protein form.

Next, feeding studies on mice to demonstrate the antigenic and stimulating effect of the potato edible vaccine with 40-80µg of tubers either with CTB or without it on days 1,2,11 and 28, and their feces were collected on days 6, 19, and 37 to be analyzed with anti-NVCP ELISA. It was illustrated that only 7 out of 10 mice showed serum antibodies with the adjuvant, while 4 out of 10 had antibodies without adjuvant. Their antibody titers were lower as well. Only 1 mouse had intestinal antibodies. It was deduced that it was due to the mice consuming it slowly and the gut cells absorbing the soluble forms more readily. It was concluded that the vaccine potatoes could stimulate specific serum antibodies.

The next study was conducted to compare the potato vaccine with another developing Norwalk virus edible vaccine after 10 years from the pilot study.

(X. Zhang et al., 2006) started to improve the Norwalk virus edible vaccine with another plant, the tomato (*L. esculentum* cv. TA234), and used another variety of potato (*Solanum tuberosum* L. cv. Desiree). They used different vectors- pNV110 and psNV110 and used electroporation along with *Agrobacterium*-mediated transformation.Seventy-four lines from the pNV110 and 24 lines from psNV110 were developed from the tomato and 57 putative pNV110 lines with 36 putative psNV110 lines from the potato.Five well-performing tomato lines and ten well-performing potato lines were selected. As with the original works, they used NVCP ELISA for quantification but for only the newly-developed tomato vaccine andthe vaccine level for the tomato is 8% while the potato is 0.4%. Next, Northern blot was conducted using DIG-labeled NVCP and sNVCP probes for both vaccines. Then sucrose gradient assay was conducted to study the conformation of the vaccines followed by ELISA analysis of translation or Western blotting with rabbit anti-rNV serum and goat anti-rabbit IgG-horseradish peroxidase conjugate. Sucrose gradient analysis illustrated that tomato and potato contained both subunits for the antigen but their proportions differed.It was deduced that the tomato vaccine was the best option.

Feeding studies were conducted on Balb/c mice to illustrate and compare the efficacy of the potato and tomato vaccine. Both vaccines were prepared of the same quantity of plant material at the same time intervals on days 1,4,17 and 20. The doses were 120, 144, and 240 μ g rNV/dose for potato and 192, 240, and 352μg rNV/dose for tomato. The mice could not consume the full plant samples. Sera and fecal samples were obtained on days 11, 27, 41, 55, and 69. Potato vaccine-fed mice showed negligible serum IgG but after an increment dose, the fecal IgA was noted. After the last two boosters, the serum antibody titers rose rapidly together with the IgG content. It was revealed that higher doses of potato vaccine got oxidized due to dilution and performed poorer. For the tomato vaccine, the mice produced noticeable amounts of serum and fecal antibodies after two doses, the serum antibody levels stayed high for the next six months. The IgG levels were also on the rise after the third and fourth boosters.

4.5 Human Immunodeficiency Virus (HIV)

HIV – Human Immunodeficiency Virus – is an RNA-virus or a retrovirus that infects and destroys the body's immune cells. Lack of treatment may lead the virus to develop the disease AIDS in the infected person. HIV has two forms- HIV 1 and HIV 2. Both of them have evolved from the primate virus, SIV (Simian Immunodeficiency Virus). Currently, there is no complete cure for HIV. However, treatment regimens focusing on anti-retroviral drugs can keep the virus at bay for a considerable time. Despite this, the body cannot properly fight off the virus as the immune cells have been affected. So, HIV usually becomes an infection that remains throughout life. Out of the two types of HIV, HIV 1 is relatively more common and highly infectious. HIV 1 is further classified into the M or main strain with further 9 substrains. Strain B is mostly responsible for widespread viral infections.

The SIV antigen

Certain research groups have attempted to develop an edible vaccine against HIV 1.

The earliest form of prototype HIV 1 edible vaccine was designed using the parental virus of HIV –SIV. The SIV virus is similar to HIV. In this case, the SIV of the chimpanzee which gave rise to HIV 1 in humans is used.

(Horn et al., 2003) aimed for the edible HIV vaccine in the early 21st century. The synthetic version of the antigen gene gp130 with a maize-optimized codon for α-amylase was used. Two different variants were used- a 3' constitutive promoter (pPGN9065) and a 3' maize embryo preferred globulin promoter (pPGN9066). The 3' proteinase inhibitor II transcription terminator was also used. *The Agrobacterium*-mediated method was employed for the transformation of the chosen vehicle *Zea mays*- corn. The vaccine corn was produced by crossing the engineered corn with a commercial variety. The ten best-performing lines were chosen and assayed with CD4- binding ELISA with anti-SIVgp130 sheep primary antibody and anti-CD4 rabbit polyclonal secondary antibody. The structure was determined using immunoblotting with Immobilon-P. It was revealed that 60% of the pPGN9065 lines and 30% of pPGN9066 were successfully transformed. The minimum levels of the vaccine were 0.007 % in both cases; the constitutive promoter line had the highest level of vaccine, 0.08 %; while the embryo promoter lines contained only 0.02 % of the vaccine. Immunoblotting illustrated that the vaccine showed the same signal as the standard gp130 gene with the vaccine molecule being in full form and properly glycosylated. It was deduced that the level of vaccine would be 10µg per seed, which could be used up to 100 µg for future mice feeding studies.

A year later, another group of researchers attempted to develop an edible vaccine for HIV using an HIV antigen gene.

(Karasev et al., 2005) have decided to develop an edible HIV1 prototype vaccine with the Tat gene because the Tat protein has a broad-spectrum non-specific action. A synthetic plant codon-optimized Tat gene was designed using pTMV125C vector containing GFP gene. The 125C vector is basically the transcribed DNA of the virus without the capsid. The plasmid was then linearized and transcribed. The capped RNA was then introduced into spinach, *Spinacea oleraceae*. Western blotting using goat anti-rabbit antibodies were conducted to analyze the inclusion of the vaccine gene into the genome. Feeding studies were conducted on mice with 1gm spinach for 3 weeks to analyze the toxicity and efficacy of the edible vaccine.

Immunoblotting provided evidence that the gene was highly expressed in spinach and the plant was able to tolerate the sideeffects of the transgene. The vaccine content was 100µg per 1 gram of spinach leaf. The vaccine also retained its conformation and antigenicity. Feeding studies demonstrated that the vaccine was well-tolerated in mice. However, their sera antibody titers were very low. Following a vaccination using plasmid DNA with a gene gun, significant levels of antibodies were induced in the mice fed with edible HIV vaccine. Even though the primary objective of stimulating the antibody by the edible vaccine was not as successful, it could be said that an edible HIV vaccine with the Tat gene could be used as a booster candidate.

HIV- CTB combination

Another group of researchers has tried to develop an edible HIV vaccine in tomatoes. They did a pilot study in the model plant – the tobacco – and identified the pros and cons of the transformation cassette, later they used the improvised version of the cassette to infect the tomato lines.

A certain team of researchers has decided to develop a fusion vaccine with the use HIV gene with CTB. They have used the HIV capsid glycoprotein gene gp20.

(T. G. Kim et al., 2004) was the pioneer of developing a chimera vaccine for HIV1 fused with CTB. The team chose the potato - *Solanum tuberosum* cv. Bintje as their vehicle. The plant vector pPCV701 and *Agrobacterium-* mediated transformation were used. Incorporation of the transgene was analyzed using PCR with the 5'primer of CTB and the 3'primer of gp120 and shown to contain the specific size band for the transgene in the putative transformed plant. Western blotting was carried out to determine vaccine expression levels. The sample was tested either in the raw state or boiled to check for any protein

denaturation. Then electro blotting was done. Specific signals for both the monomer and pentamer fusion proteins were detected in the raw plants. However, the chemical signals for monomers only was found in the boiled plants, suggesting possible vaccine denaturation. GM1-ELISA was used to detect the vaccine levels in the transformed plants which were revealed to be 0.0021 to 0.0041%. Rabbit anti-cholera antibody and mouse anti-rabbit IgG were used as primary and secondary antibodies respectively for both blotting and ELISA.Further trials are in process to improve vaccine development.

p24 –Nef combined antigen

(Zhou et al., 2008) and his team decided to combine two HIV antigens, p24 and Nef, into a single edible vaccine. They designed two types of constructs- single antigen: Nt-pZF5 (codon-optimized p24 construct) and Nt-pZF6 (the codon-optimized Nef expression construct) and double antigen: Nt-pZF7 (p24-Nef fusion) and Nt-pZF8 (Nef-p24 fusion). The transformation was carried out using the gene gun with the vector pRB95.

At first, the transcription levels were determined using Northern blotting, which illustrated the single antigen had shorter transcripts than the fusion antigens. Next, the translation levels were analyzed using Western blotting with specific anti-p24 antibodies; the results demonstrated that Nef-p24 construct was unstable. After studying the age-dependent expression, it was revealed that the vaccine expression was inversely proportional to leaf age. The highest level was revealed to be that from the freshly-growing leaves. The best performing line was decided to be p24-Nef. The vaccine content of the line was 4%.

From the results, tomato,*Solanum lycopersicum* cv. IPA-6, was chosen as the vehicle and transformed with the best construct p24-Nefusing biolistic method. Cre-Lox was employed to generate marker-free transgenic lines- Sl-pZF7lox. Both the transcription and translation levels were revealed to be higher than the first trial, with the vaccine level boosted to tenfold in green fruits.

Other groups have tried to develop the HIV edible vaccine using protein accumulation sequence SEKDEL.

(Lindh et al., 2009) attempted to develop an edible HIV 1 vaccine using the p24 gene which was enhanced by the SEKDEL signal. The team has used the carrot, *Daucus carota subspecies .sativus cultivar Napoli F1,* as the vehicle and transformed it with the vector pGreen0229 containing the p24 gene using *Agrobacterium*-mediated method. The insert was verified using PCR with 35S_F/G35_Modified primers revealing carrots 8 out of the 17 revealed that putative plants inherited the vaccine gene. The number of insert copies was determined using Southern blotting using p24 cDNA labeled with 32P-dCTP and the results

illustratedthat only 1 carrot line contained 2 transgene copies. The presence of vaccine protein was determined using Western immunoblotting technique which gave evidence that out of the well-performing 5 lines out of 8, only 3 lines were expressing the vaccine in both their taproots and leaves Then monoclonal anti-HIV-1-p24 antibodies were used as primary antibody and anti-mouse-IgG secondary antibody. The accumulation of the vaccine was checked using ELISA with HIV-1 subtype B anti-p24 antibodies. The vaccine content was found to be 62 ng/g of fresh weight.

Further analysis was conducted using sera of HIV patients and healthy candidates. It was shown that the vaccine antigen was completely neutralized by the patient serum, it was deduced that the vaccine retained its antigenicity that confirmed the findings of ELISA.

TBI-HBS

A group of researchers developed a two-step study in order to design an edible vaccine for both HIV and hepatitis B virus. The group at first designed a preliminary feeding study on mice with the fusion gene edible vaccine, and then conducted a full *in vivo* study.

(Shchelkunov et al., 2005) developed an edible vaccine against both HIV 1 and hepatitis B virus using the chimera gene TBI-HBS. They used the tomato plant, *Solanum lycopersicum* cultivar Ventura. They designed the plasmid pBINp35sTBI-HBS#15 containing the fusion gene TBI-HBS. For better preservation purposes, the tomato was lyophilized. Using solid-phase enzyme immunoassay, the total vaccine content was determined to be 0.3 ng/mg of powder. After that, feeding studies were conducted on Balb/c mice to analyze the duration and dynamics of antibody synthesis. The mice were fasted for 12 hours before feeding. The mice were orally immunized for 28 days at 2 weeks interval; at the 42nd day, some of the mice received hybrid DNA vaccination. Humeral and systemic responses were observed in the serum and fecal samples. Enzyme immunoassay was used for the analysis. It was revealed that both HBV and HIV antibodies were stimulated by the vaccine. Serum HIV antibodies were induced during the second feeding, while the fecal antibodies were induced much earlier. The same was observed in the case of HBV antibodies. The introduction of the plasmid vaccine boosted the antibody titers of HIV vaccine but did not affect HBV antibodies.

In the next study, the duration of antibody production was examined. F_4–generation tomatoes with fusion vaccine were selected. The process was carried out similarly to the previous study.

(Salyaev et al., 2009) used the p24 antigen primer using the GENSCREEN PLUS kit for HIV antigen and the enzyme immunoassay with HBsAg antigen for antibody detection. 1.8– 2.1 ng/mg HIV content was used for HIV and 50–70 ng/mg and HBV content.
Antibodies for HIV 1 were detected using Western blotting with p18 marker. Routine sample collections were taken on days 27, 29, 35, and 43. It was noted that antibody production resumed through days 27 to 35 and gave the distinct p18 band. However, it fell on day 43.
For determining hepatitis B antibody duration, samples were collected on 10, 30, 180, 270, 330, and 570 days. Antibodies were observed on as early as day 10; high titer levels were seen even after 180 days and 330 days, but at the 330 days mark it was slightly lower. The levels slurped after 570 days.
In short, it can be found that the HIV edible vaccine is also being developed and tested for as long as the 2000s.

4.6 Hepatitis B Virus (HBV)

Hepatitis B virus is an agent that infects billions of people worldwide. The identification of the surface antigen from the cells of infected patients revealed VLPs or virus-like particles, which are envelope proteins. The VLPs consist of HBsAg epitope and lipid membrane. These molecules can be recovered and used for the production of vaccines that can be targeted HBV specific antibodies.

HBsAg antigen

The first works on developing an edible vaccine for HBV began in the late 1990s.
(Richter et al., 2000) and his team decided to develop the HBV vaccine in the potato tubers. They chose the Hepatitis B surface antigen and inserted it into the pPAT-HB plasmid containing tuber-specific promoter –patatin. The vaccine content was found to be 1.1µg for 1 gram of fresh tubers. Feeding studies on mice to demonstrate if oral vaccination with potato vaccine stimulated serum antibodies. Three doses were administered with 5.5 µg dose vaccine with CTB adjuvant per week demonstrating that high titers of primary specific antibody lasted for three weeks. Reinoculation with commercial antigen elicited immediate remarkably higher titers of antibody. It was concluded that the edible vaccine successfully produced immunogenic memory.
A group of researchers has conducted a threefold experiment with transgenic potato-based HBV edible vaccine. They attempted to study the immunogenicity of the raw edible vaccine, booster edible vaccine, and denaturation of edible vaccine and its effects.

(Kong et al., 2001) and his team have also used HBV epitope for the development of an edible vaccine in potatoes. They used the potato variety *Solanum tuberosum* L. cv. FL1607 and transformed it with the vector pHB114 through *Agrobacterium*-mediated method. To determine the expression of the hepatitis gene in the transgenic potatoes, transmission electron microscopy was used. The putative lines FL1607 HB114-16 were selected and examined using ELISA. The vaccine content was estimated at 8.35μg in 1 gram of fresh tuber. BALB/c mice were fasted overnight and then fed with 5g tuber once every week for 3 weeks together with CTB adjuvant. ELISA was conducted on collected sera samples and treated with rabbit antibiotin with horseradish peroxidase to produce a yellow color. To determine the booster effect of the vaccine, the mice were injected with 0.5μg recombinant antigen followed by the transgenic vaccine.

The observation illustrated that the VLP accumulated as grainy particles in vesicles bound to the endoplasmic reticulum. ELISA assay showed that the antibody titers gradually increase at 1 week after the first two doses, reaches its peak in the 4th week after the three doses are completed then slowly decline. When the mice were re-injected with the antigen to check memory response, a strong and rapid primary response was detected. For booster studies, the recombinant vaccine produced negligible antibodies for the first 5 weeks, however after three weekly feedings of the edible vaccine; the antibody titers rocketed after the second dose and peaked at 3 weeks after the third dose.

The edible vaccine was also boiled for 10 minutes and fed to mice, no immune response was observed. However, after inoculation with recombinant antigen, immune stimulation was observed. It was concluded that heat-denatured the vaccine but its antigen properties retained.

S-pre S2 combined antigen

The next group of researchers decided to use different antigens for the production of edible vaccines. They targeted the mid-portion of the HBV gene containing the antigens surface S and pre S2. Like the other fragments, this fragment can also elicit specific antibodies. The pre-S antigen groups are an important binding factor for the hepatocyte receptors.

(Joung et al., 2004) and his team decided to design the HBV edible vaccine with the middle portion of the HBV gene. They selected potatoes of the Desiree variety and employed the *Agrobacterium*-mediated method with the vector pB1121. They employed three promoters-the CMV promoter linepMHBV, double 35SS promoter line pDHBV, and the potato-specific promoter patatin line pPHBV. Twenty putative lines were produced.

PCR analysis with HBV –specific primers revealed about 20 putative transgenic plants. Southern blot with digoxigenin label revealed that some plants have a range of one to three copies transgene. Only 3 transgenic lines expressed high transcription levels by Northern blot analysis with HBV S Ag-specific mouse monoclonal antibody H67 and anti-mouse Immunoglobulin horseradish peroxidase and were revealed to be from the CMV line. The patatin line had the least transcription level and the double promoter line was most active compared to the other two lines. Apart from these, several lines produced high levels of the vaccine as shown by ELISA and Western blot. However, surprisingly, the vaccine production was of the transgenic plants was inverse to that of the mRNA production. The patatin line had one plant with a threefold increase in vaccine production, while another two (P5 and P8) with more than fourteen-fold increases in vaccine secretion. The CMV line two plants with only two to three-fold (M1 and M11) increase in vaccine levels while only one line with containing lesser quantities. One plant (D12) from the double promoter line showed slightly increased vaccine content while the others had very low vaccine production. The vaccine content was as follows- P5 and P8 with 0.09% expression level with 0.008% and 0.012% mid fraction levels, M11 with 0.04% vaccine content with 0.004% middle fraction and D12 with 0.03% content with 0.003% mid fraction portions by ELISA.

Feeding studies with Balb/c mice were conducted using a5g potato with a dose per week for three weeks to illustrate whether memory cells were activated or not. The patatin lines were chosen for the feeding studies. The mice had little immune stimulation to the infection after the feeding was done for 5 weeks, however after a booster dose of the recombinant shot was given, the rapid immune response was observed, mainly for P8 lines. It shed light on the fact that immunogenic memory was triggered by the edible vaccine.

Apart from using potatoes, another group of researchers also attempted to express the HBsAg's' gene in the banana.

(Kumar et al., 2005) and his group have tried to produce an edible vaccine for HBV in the banana. They used the banana cultivar Rasthali and designed four constructs with different features- a retention signal HER and without signal HBS and cloned intopBR322 together with *ubq3* promoter. They further bifurcated the cassettes with the Ethylene-forming enzyme (EFE) promoter from banana to produce 4 separate cassettes- HBS, HER, EFEHBS, and EFEHER. The promoter enhanced groups were cloned into pEFEGUS. The *Agrobacterium* strain EHA 105 was cultivated with the banana. The putative lines were analyzed using PCR and Southern blot with 681bp's' primers and probes. RT-PCR was conducted to analyze the RNA transcripts. The vaccine content was analyzed using ELISA.

PCR analysis detected the 681bp region in the putative lines and Southern blotting analysis demonstrated the binding of the s-gene probe to the 681bp region. RT-PCR analysis with cDNA formation and reverse transcription highlighted the transcription of the transgene. It was noted that the vaccine expression was the highest for the pEFEHBS line *in vitro* while higher for the HER line in the greenhouse. It was decided that both EFE promoter and retention signal could be used inbanana for the production of HBV edible vaccines.

In addition to this, another team of researchers tried to develop an edible HBV vaccine in the tomato. They have also used a different portion of HBV to design a novel vaccine.

(Lou et al., 2007) have used the large surface antigen, termed the S antigen, containing the pre-s1, pre-s2, and S genes together with SEKDEL signal. They selected the tomato as their vehicle and enriched and used the plasmid pYF9616 encoding the fruit-specific promoter 2A11. *Agrobacterium*-mediated transformation using electroporation was employed.

The incorporation of the transgene was determined by PCR. The PCR showed the presence of the PRS-S1S2S large surface antigen gene. 13 putative transgenic lines were found. Then RT-PCR was conducted to determine the transcription level of the transgene. The partial fragment of the transgene S1S2S portion was revealed to be actively transcribed in all lines. ELISA assay was performed to analyze and quantify the vaccine levels. It was demonstrated that the vaccine expression was higher in the mature fruits compared to the leaves. The total vaccine content was estimated at 400ng per gram of fresh weight. To observe the conformation of the vaccine molecules in plants, transmission electron microscopy with monoclonal antibody to BALB/c mouse HBsAg and secondary gold-labeled 5 nm anti-mouse antibodies were used. It was illustrated that the gold particles were observed close to the ER, assembling into capsid-like and virus-like particles.

Moreover, two researchers managed to design an edible HBV vaccine in the Brazilian lettuce variety for the first time.

(Marcondes & Hansen, 2008) have decided to use the S fragment of the hepatitis antigen to design an edible vaccine. They have chosen the lettuce variety, *Lactuca sativa* L. "Vitória de Verão" as their vehicle. They designed basic lines of transformation cassettes – one with the SEKDEL sequence and the other without the signal, forming plasmids pG35SHBsAg and pG35SHBsAg[ER]. They have employed the bacteria *Agrobacterium tumefaciens* C58C1Rif in this case. Analysis of the plants post-transformation with PCR has located the transgene have successfully been integrated into the genome of putative transgenic plants.

preS1 – S fragment

In the same year, another team of scientists decided to design an edible vaccine for HBV in rice. They have used a unique approach to fuse two antigens of HBV to design a vaccine.

(Qian et al., 2008) have designed an edible HBV vaccine based on rice. They designed a chimera from the pre-S1 and the S portions of the antigen, terming it the SS1 gene. They chose the rice variety rice *Oryza sativa* L. cv. Zhonghua No.11, using the vector p1300GSS1 and *Agrobacterium* strain EHA105 aided by electroporation for the transformation. For detecting the vaccine gene integration, PCR using SS1f and SS1R primers revealed that out of the 461 events, SS1 gene in 164 lines. Out of the 164 lines, only 1 line expressed higher amounts of SS1. Southern blot with ^{32}P-labeled probe for the chimera was carried out. For finding the copy numbers of the transgene, real-time PCR was performed with SYB Green I dye. Both Southern blot and real-time PCR confirmed the presence of two copies of vaccine genes. RNA blot was conducted to confirm transcription of the vaccine gene. Quantitative ELISA was used to estimate the vaccine levels and the antigenic capacity was determined using Western blot with mouse anti-S peptide monoclonal antibody-preS1 epitope monoclonal antibody as primary antibody and goat anti-mouse IgG as the secondary antibody.Quantitative ELISA results determined that the SS1 content was the highest in this line as well- 31.5 ± 1.4ng. Other lines had varying levels of vaccine content. Western blot assay illustrated that both pre-s1 and S antibodies were able to detect the fusion antigen. Microscopic analysis was also conducted to observe the formation and structure of the antigen. Observation by solid-phase immune electron microscopy revealed that the SS1 can form virus-like particles and Western blot assay illustrated that both pre-s1 and S antibodies were able to detect the fusion antigen.

Next, studies were carried out on Balb/c mice for analyzing the immunogenicity of the vaccine. The mice were inoculated with 3 doses of 0.5µg vaccine at 2 weeks interval. Their sera were collected weekly for 12 weeks and examined with indirect ELISA with mouse anti-S peptide monoclonal antibody-preS1 epitope monoclonal antibody as primary antibody and goat anti-mouse IgG as the secondary antibody. Immunological analysis on mice demonstrated that anti-pre s1 antibodies were elicited 4 weeks after the first dose and plummeted 3 weeks after the last dose. The anti-S antibody was stimulated 5 weeks after the first dose and reached its apex in the 8th week. Both antibodies were remarkably high during the whole experiment.

To sum up, it is seen that the Hepatitis B virus edible vaccine is being developed and improved for a long time

4.7 Tuberculosis (TB)

Tuberculosis is one of the leading causes of death worldwide. It is caused by the bacterium *Mycobacterium tuberculosis*. Formerly it was only a disease of the lungs. Now TB can also infect other organs like the brain, spinal cord, kidneys, and skin. The disease is mainly spread by droplet transmission. TB can be the latent or inactive type that is not contagious and asymptomatic. The disease can also be active TB which can affect the body and can infect others.

Generally, antibiotic combinations and the BCG vaccine were used to combat the disease. However, now many multidrug-resistant strains are evolving which makes it difficult to control the infection. The vaccine is also only effective in childhood, which makes the body susceptible to the disease in adulthood.

It has been agreed upon that in order to develop a vaccine that can provide protection against TB in adults and children, newer approaches and antigens should be employed. The favorability of ESAT6 gene and the Ag85B gene is very positive in developing an alternative vaccine. Promising results could be obtained in developing a fusion antigen with these two genes and inserting it into transgenic plants with edible parts. Moreover, another important immunogenic gene is the CFP10 gene.

ESAT6 and CFP10 genes

The pilot works on the development of an edible vaccine for TB were performed using the antigens ESAT6 and CFP10.

(Zeng et al., 2008) and his team attempted to design simple vaccines with individual antigens.They used the potato virus X (PVX) expression vector. The team designed two sets of studies – one with the antigens fused with the GFP reporter gene (PVX-ESAT6/CFP10: GFP) and another with native genes (PXV-ESAT6/CFP10). The vehicle was tomato- (*Solanum lycopersicum* 'AilsaCraig'). The transformation efficacy was determined visually. b. Then RT-PCR was performed to check the transcription of the transgenesby three different pairs of primers: PP82 and PP83 for transgenes, PP269 and PP270 for PVX RNA, andPP271 and PP272 for plant 18S rRNA. The expressed antigens were determined by the illumination of the GFP with long-wavelength UV light. Western blotting was conducted to check the translation levels using anti-H His6-Gly tagged antibody, conjugated to alkalinephosphatase and 5-bromo-4-chloro-3-indolylphosphate- nitroblue tetrazolium substrates.

Visual detection of the putative plants visualized that the infected plants produced local symptoms-lesions and systemic symptoms – chlorosis and mosaic patterns. RT –PCR

analysis demonstrated that all the components- PVX DNA, transgenes, and plant rRNA were effectively integrated into the tomato vehicle. UV illumination easily illustrated the GFP – antigen molecules by emitting strong green fluorescence. Western blotting revealed the 38kDa and 39kDa bands of ESAT6 and CFP10 vaccine molecules.

ESAT6 and Ag85B genes

The next study on edible vaccines on TB was conducted using the antigens ESAT6 and Ag85B gene chimera.

(Matvieieva et al., 2009) and his group worked on developing an edible TB vaccine using the ESAT6-Ag85B gene combination. They chose the lettuce, *Lactuca sativa* L. of the cultivars Eralash, Snezhinka, and Rubinovoe Kruzhevo; and used *Agrobacterium* strain GV3101. They used two approaches – one with only the ESAT6 gene (pCB063)- only for the cultivar Eralash and the other with the fusion gene (pCB064) for all three cultivars.

PCR analysis with ESAT6, Ag85b, and fusion gene and marker gene-specific primers detected the ESAT6 transgene in pCB063 line as well as the fusion gene in the pCB064 line of the Eralash cultivars. The fusion transgenes were also detected in the cultivars Snezhinka. Except for one plant, all the other plants of the cultivar Rubinovoe Kruzhevo had the fusion transgene.

In the analysis with RT-PCR, some astonishing data were revealed. Some of the plant lines of the Snezhinka cultivar lacked the ESAT6 gene transcriptions. It was deduced this could be due to gene silencing. Despite this, all the plants of the other two cultivars and other plant lines of the Snezhinka cultivar were transcribing the vaccine antigen.

ESAT6 gene

The same team conducted another research to develop an edible TB vaccine with another plant using only the ESAT6 gene.

(Matvieieva et al., 2011) and his group tried to formulate a second tuberculosis edible vaccine with the transcription regulator esxA gene of the ESAT6 antigen. They have chosen the chicory *Cichorium intybus L.*cv. Pala rossa and used the pCB063 vector to transform the chicory using *Agrobacterium* tumefaciens strain GV3101.

The putatively transformed plants were analyzed by PCR using esxA specific primers. All transformants revealed the 299bp band. Eight random samples were selected for further analysis by RT-PCR. Three plants were shown to lack the transgene transcription, similarlyto the former lettuce transformation result. The transformation rate was estimated to be 4-5 plants per explant.

The latest research on an edible TB vaccine focused on using the ESAT6 antigen.

(Saba et al., 2020) and her team have used the ESAT6 antigen in order to develop an edible vaccine for TB. They used a unique vehicle, the broccoli- *Brassica oleracea* var. italica. The Agrobacterium strain C58C1 and the plasmid pEXP-ESAT-6agr for the transformation.

PCR analysis was conducted using the primers Esat-6 Gfp F and Esat-6 Gfp R to detect the transgene in the putative transgenic lines. It was revealed that all the lines contained the 767bp band for ESAT6. Quantitative RT-PCR was performed to determine the gene copies of the transgenic lines sense primer Esat-6-I F and anti-sense primer Esat-6 I-R. The results illustrated that except for one line that contained two copies of transgene, the other lines contained one copy. For detection of translation levels, Western blot was carried out using primary anti-ESAT-6 antibody and secondary HRP-conjugated goat anti-mouse IgG. The results demonstrated that all lines possessed two bands- one of 10.74 kDa for the monomer structure and another of 43 kDa for the pentamer structure. Quantification was done using ELISA with primary Anti-ESAT-6 antibody and HRP-conjugated goat anti-mouse IgG secondary antibody. The vaccine content was estimated to be 0.5% of the total protein content. Purification with nickel-affinity purifier and silver staining was also done; it was observed that both monomer and slight traces of oligomer were present.

Feeding and inoculation studies were also performed with Balb/c mice. Mice were divided into oral group and subcutaneous group. The oral group was fed with 3g in 500µl PBS of broccoli-transgenic, wildtype, and only PBS for each subgroup, and the subcutaneous group was inoculated with either 50µg antigen from transgenic line in 100µl PBS or just pure PBS for 21 days at 7 days interval. ELISA assay of the sera withHRP-conjugated goat anti-mouse IgG secondary antibody showed that IgG response was present in both oral and subcutaneous groups.

ESTA6, Ag85b, MPT64, and MPT83

TB – specific antigens are more immunogenic once combined. Apart from ESTA6 and Ag85b, other antigens include MPT64 and MPT83. The four antigens are crucial for stimulating antigen-specific immunity against tuberculosis. A group of scientists has designed a tetravalent TB edible vaccine.

(Y. Zhang et al., 2012) and his team have conducted a detailed study on the development and effects of a tetravalent edible TB vaccine. They made a combined vaccine using the epitomes ESAT6, Ag85b, MPT64, and MPT83. They inserted the fusion antigens into the plasmid pCAMBIA 2300-Patation. They chose the potato of the Desiree variety as their vehicle. Agrobacterium-mediated process was used for the transformation.

PCR analysis with antigen-specific primers detected the specific bands for individual antigens- Ag85b-996bp, MPT83-681bp, MPT64-687bp, and ESAT6-333bp. The translation levels of the vaccine were determined using Western blot with primary monoclonal antibodies to the fusion gene produced by mice and secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG. Specific protein bands were observed - Ag85b-35kDa, MPT83-22kDa, MPT64-25kDa, and ESAT6-10kDa.

Feeding studies were conducted to analyze the humoral immune response of the tetravalent vaccine using C57BL/6 female mice, which have dominant Th1 response with INFɣ production. The mice were fasted for 8 hours and then inoculated with edible potato vaccine for 1 month at 1 week intervals. The final dose was given as a booster of BCG vaccine in 100µl saline. The spleens of the mice were collected and analyzed for antigen-specific cytokines. The cells were exposed to the different antigens and observed using indirect ELISA with either fusion molecule or the BCG protein. It was illustrated that the levels of cytokines were remarkably higher for the fusion vaccine. Next, PMBC assay using flow cytometry was conducted to check the T-cell based immune response. The four antigens were added to the sample. It was demonstrated that antigen- stimulated cultures showed exceptionally higher T-cell response with the fusion vaccine. Finally, the memory response of the fusion vaccine was tested and it was observed that memory cell concentrations were higher even after 2 weeks after infection.

ESAT6 and CFT10 fusion gene

A second team of researchers have decided to study the antigenicity of these the antigens ESAT6 and CFT10 in another fusion edible vaccine.

(Uvarova et al., 2013) and her team combined these two antigens and added them to the pBI121 plasmid. They planned to produce an edible conjugated vaccine with the carrot- (*Daucus carota* L. cultivar Nantskaya). They used the *Agrobacterium* strain DH10B for the transformation. In order to check the integration of the transgene, PCR was performed using ESAT6 and CFP10 specific primers. The results illustrated the 288bp ESAT6 and 303bp CFT10 fragments. The expression of the vaccine molecule was determined using dot-blot for CFP10 with polyclonal antibodies and Western blot for ESAT6. The dot-blot results demonstrated the specific signal for the CFP10 antigen and Western blotting analysis highlighted the four fragments of the ESAT6 (98, 50, 30, and 20kDa) that can bind to specific antibodies. The vaccine levels were determined to be 0.056% TSP for rESAT6 and 0.024% TSP for rCFP10.

Immunological studies to assay the cell-mediated immune response was performed with female ICR mice (used in toxicity and drug efficacy studies) that were either fed with the carrot vaccine with 5g tissue at 2 weeksinterval for 28 days or injected with recombinant antigens separately. b more immunogenic than CFP10 antigen. In spite of this, specific IgG for both antigens were found in the inoculated mice.

GroES gene

A short study was conducted using another TB gene – GroES which is a very immunogenic antigen.

(Jose et al., 2014) and her team tried to develop a prototype TB edible vaccine with the antigen GroES. They did a pilot study on the model plant and tobacco and decided to design a putative edible vaccine with potato -*Solanum tuberosum L.*, variety Kufribahar. They used the plasmid pTBSJ2 together with the antibiotic-resistance Hpt gene and the Agrobacterium mediated transformation. For checking the presence of the transgene, PCR with primers TBSJ1F and TBSJ1R and Hpt –specific primers were used. The putative transgenic lines illustrated the 400bp transgene and the 1kb antibiotic marker gene. Next Western blot analysis was done with primary monoclonalantibody SA-12 and secondary anti- mouse IgG raised in sheep conjugated to HRP enzyme to detect the vaccine protein levels. It was demonstrated that there were no 10.7kDa vaccine molecule band in the potato. Next, immuno-dot blot assay was carried out with primary anti- body, SA-12 and the secondary anti-mouse IgG anti- body conjugated to HRP to develop a red-brown precipitate. The results revealed that the potato samples emitted weak signals. Quantification was done using ELISA with primary antibody SA-12 and secondary antibody, Antimouse IgG linked to HRP whole antibody from sheep with TMB to form a blue precipitate. The potato vaccine level was estimated to be 0.33µg for 1ml of protein.

The prototype vaccine was not as much successful as its other vaccine counterparts but it provided valuable data and scopes for improvement.

4.8 Dengue

Dengue is a viral fever that affects people of all ages. It causes dengue fever that can lead to hemorrhagic fever and dengue shock syndrome if left untreated. The disease is predominant in the tropics and subtropics. It affects millions of people worldwide every year. There are 4 major serotypes of dengue – DENV1, DENV2, DENV3, and DENV4. Among all these

serotypes, DENV1 is very unusual in its characteristics. The virus is transmitted by the bite of the female *Aedes* mosquito.

Among the key structures that are effective at generating immune response in the host are the pre-membrane proteins (prM), virus-like particles (VLPs), and envelope proteins (E). The VLP is non-contagious but immunogenic, however, they cannot replicate. The pre-membrane protein and envelope proteins can self-assemble to form VLPs. The envelope proteins have 3 distinct parts – domain II has cross-reactive antigens while domains I and III have specific antigens.

The prM/E fusion gene

One of the earliest researches on the development of edible dengue vaccine began in the 2010s. (Kanagaraj et al., 2011) have attempted to express the fusion gene for the prM/E for dengue 3 serotype in the lettuce- *Lactuca sativa.* The fusion gene was cloned using specifically designed primers. Then it was ligated into the DENV3prM/E vector. Gene gun method with gold particles was employed to transform lettuce chloroplasts. A streptomycin resistance marker was used. After a few rounds of selection, a further assay was conducted.

In the primary assay, PCR was performed using 16SF/3M primers to detect the integration of the transgene. This resulted in the formation of a 2.8kb band only in the putatively transformed lines. Then in the secondary PCR, 5P/2M primer pair was used to locate the integration of the transformation cassette. It demonstrated the formation of a 3.8 kb band where the cassette joined the *aadA* gene. Next, to confirm the PCR findings, a Southern blot was conducted with α-32P probe. The results illustrated a 7.4kb fragment for the transformed lines. Out of the 7 lines that were generated, 5 lines were fully transformed. Then Western blot was carried out with polyclonal anti-dengue primary antibody and HRP- conjugated goat anti-rabbit secondary antibody to determine the protein expressionusing the young, mature, and old leaves. The results revealed the presence of a 65kDa band that is expected for the fusion protein. It was also noted that more protein was expressed in the older leaves compared to the young ones. In the final step, transmission electron microscopic analysis was conducted of the transgenic lettuce. Structures resembling the VLPs were observed in the chloroplasts.

Synthetic cEDIII gene

The next group decided to focus on all of the strains of dengue to develop an edible vaccine.

(M. Y. Kim et al., 2012) and his group aligned the genome of all the serotypes and detected the consensus sequence for the envelope protein to design a tetravalent vaccine, synthetic cEDIII gene (scEDIII). They also attached the retention sequence for the endoplasmic

reticulum to the synthesized antigen. The inserted the gene into the plasmid pMYV657. They also added the HPT gene as a marker. Their chosen vehicle was the rice plant - Rice callus (*Oryza sativa* L. cv. Dongin). They used the particle bombardment method for the transformation. Analysis of the transformation events was conducted using PCR with the envelope protein-specific primers, followed by electrophoresis with ethidium bromide. It was found that the synthetic gene-specific band of 490bp was present in the putative lines. Transcription levels were determined using Northern blot with 32P-labeled cEDIII probe. 13 lines were found to actively transcribing the vaccine gene and 3 best-performing lines were selected amongst them. Translation levels were checked with Western blot analysis with SDS-PAGE separation and blotting by primary mouse anti-dengue virus monoclonal antibody and secondary goat anti-mouse IgG conjugated with alkaline phosphatase. Two different-sized bands of around 14kDa were observed for transgenic lines. The level of vaccine content was also estimated using the same method. It was found that the vaccine expression levels were 0.45 mg/gram.

Domain III envelope protein

Another group of researchers have designed an edible vaccine for dengue with the domain III envelope protein and also tried to improve its efficiency.

(M. Y. Kim et al., 2016) have used the Domain III gene for dengue and fortified the antigenic properties with the CTB gene. They chose the tomato as their vehicle. The transformation was carried out using *Agrobacterium*-mediated process. Incorporation of the transgene was confirmed using PCR with the fusion gene-specific primers. The results illustrated the 734 nucleotides band specific for the fusion gene. Transcription of the transgene was determined using Northern blot by 32P-labeled CTB–EDIII probe. 5 out of 7 plants gave positive signals for transcription.

The translation of the vaccine molecule was determined using two processes- anti-cholera anti-sera with alkaline phosphatase-conjugated anti-rabbit IgG and anti-dengue monoclonal with anti-mouse antibodies. A protein band of 130kDa was demonstrated using both methods of immunoblotting. The protein was deduced to be in the pentamer form. The 60kDa band for the CTB was also noted. Next, GM1-ELISA was performed to check the immunogenicity and estimate the vaccine content. The vaccine molecule was found to have an affinity for the GM1 cells and bind to them. Both types of antibodies were also able to bind to the antigen. The vaccine content was estimated at 0.015% of the total soluble protein.

EDIII-1-4 domain protein

Recently, another group of researchers decided to work with the tetravalent antigen of the dengue virus. They conducted a complete assay for determining the different parameters in their pre-clinical studies.

(van Eerde et al., 2019) and his team chose the fusion dengue antigen envelope protein 1-4 to cover all the serotypes of dengue. They selected the lettuce as their vehicle. They transformed the lettuce using plasmid pEXP-PN-ediii-1-4-L through the particle bombardment method. Confirmatory studies with PCR analysis illustrated that 1841bp long in the putative transgenic line S12-PN-EDIII-1-4. This line was then analyzed using Southern blot, which revealed the presence of the 6533bp fusion gene in the transformed line. Immunoblotting with diluted polyclonal anti-dengue primary antibody and anti-rabbit-IgG-AP secondary antibody produced in rabbits demonstrated the 47 kDa fusion antigen expression. The tetravalent protein gave a single full-length band. The vaccine content was estimated to be around 3.45% of the total protein content with Western blot with anti-dengue primary antibody and anti-rabbit-IgG-HRP secondary antibody.

Immunogenicity of the lettuce vaccines was conducted with rabbit that responds to a wide variety of antigens with higher antibody production. The rabbit was inoculated with one dose of 0.1mg vaccine followed by 3 booster doses at 2 weeks intervals. The first booster was given 2 months after the inoculation. The sera were sampled and analyzed with ELISA usinganti-dengue mouse monoclonal primary antibody and a goat anti-rabbit polyclonal antibody conjugated to HRP. The antibodies were observed to be stimulated after 9 weeks of vaccination. It was also noted that the antibodies could identify the chimera antigen.

In addition to this, a simulated gastrointestinal study was also performed. It was shown that the vaccine was intact during the oral and gastric routes, however, it degraded in the intestine. A similar detailed study was conducted in the same year by another group of scientists using the same tetravalent antigen but with new modifications.

(B. Y. Kim & Kim, 2019) have also focused on a tetravalent vaccine that will work against all serotypes of dengue. They used the synthetic version of the tetravalent envelope protein gene and fused with it ER retention signal and M-cell ligand peptide- Co1. They formed two versions of the construct- one with the retention signal and the other without it. They inserted it in the plasmids pMYV804 and pMYV690 respectively. They used the gene gun method to transform rice cells of the cultivar Dongin. The putative lines were analyzed using PCR with specific primers and the 1.3kb was present in both variants of the constructs. A northern blot with a 32P probe was performed and four lines with the strongest signals were selected.

These lines were analyzed using Western blot with anti-dengue monoclonal antibodies. The results highlighted the 47kDa protein band for the fusion protein.

Next, the same method was used for the vaccine content determination using mouse anti-dengue primary antibody and anti-mouse IgG conjugated with alkaline phosphatase. It was revealed that line pMYV804 produced higher vaccine content than line pMYV690. The vaccine content of pMYV804 was estimated to be 316.7 µg per gram of dry weight. After that feeding studies were conducted on female Balb/c mice with 100µg vaccine at 2 weeks intervals for 4 weeks. Then feces and sera samples were analyzed. Both samples illustrated specific IgG and IgA antibody reactions. Spleen cells were also collected and an ELISPOT assay was conducted to check the presence of IgG withalkaline phosphatase (AP) conjugated anti-IgG antibody. The results highlighted the presence of specific T-cells and B-cells. A booster subcutaneous dose of antigen was administered on week 5 to check the effect of the vaccine. The results demonstrated that the vaccine-induced antibodies could singularly react with native antigens.

So, it can be said that, even though the dengue edible vaccine was designed relatively later than the other vaccines, the perfection of the vaccine is going on at a remarkable rate compared to the others.

4.9 Rabies

Rabies is a deadly disease that is transmitted by the bite of an animal, in most situations by domestic dogs. Rabies is known as Neglected Tropical Disease which is nearly present in nearly all the countries of the world. The disease is fatal once the symptoms appear. Nearly half of the patients include young children and teens. However, the disease can be prevented using the vaccine.

The first attempt to develop an edible rabies vaccine took place in the mid-1990s.

(McGarvey et al., 1995) and his team tried to develop an edible vaccine for rabies using the outer capsid glycoprotein, G protein in the tomato- *Lycopersicon esculentum* Mill var. UC82b. The RGRgpis construct with the G protein. *Agrobacterium* –mediated transformation was used. Four putative lines were found using PCR with G protein-specific primers, out of which two lines had the vaccine gene shown by Southern blot. These lines were also shown to have detectable transcript levels with Northern blot with 32P probe for G-protein. One line was selected and self-pollinated. 18 out of 22 plants showed positive results. Western blot analysis with mouse monoclonal antibody 6-15C4 revealed that two bands correspond to the

protein fractions of the antigen. The location of the vaccine in the plant was detected with immunogold labeling with anti-rabbit IgG with gold particles illustrated the molecules were present in the Golgi body, vesicles, and cell wall, with most particles in the vesicles and cell walls.

The next study also used the G protein antigen in a unique vector. The team conducted a pilot study on tobacco (Vidadi Yusibov et al., 1997) to test the transformation ability of the expression cassette. The team then proceeded to develop an edible vaccine with the result of the study.

(Modelska et al., 1998) and her team developed a construct according to the pilot study by (Vidadi Yusibov et al., 1997). The team constructed an expression vector using the coat protein of the alfalfa mosaic virus. The antigen peptide of the B cell G5-24 and T cell 31D of the rabies G protein - Drg24 was chosen. Then they inserted it into the vector pBRzCPDrg24. Their chosen vehicle was the spinach- *Spinacia oleracea*. Immunological studies were conducted to assay whether the antibody produced by the edible vaccine could counter live virus with Swiss–Webster mice and different strains of the rabies virus. The mice were given three treatments- 3 doses of 50μg injection, 4 doses gavages feeding of 250μg of purified protein, and feeding of 1g dose of spinach leaves. The former two were ongoing for 2 weeks while the latter one was for 1 week. The sera and feces were collected at predetermined times and analyzed with ELISA with peroxidase- conjugated goat anti-mouse IgG and IgA.

The titer patterns demonstrated that negligible antibody was present after the first dose of the injection, only a slight rise after the second dose. However, after the last dose, there was a noticeable spike in the titers. Only 40% of the mice survived and stayed healthy. For the gavages group, the pattern was similar but was twofold higher.

Feeding studies illustrated that the rabies-specific titer was nearly three times higher compared to the gavages group. Even though the dose from the spinach that the mice consumed was only 25μg and relatively lower systemic antibodies were present, it elicited a remarkable specific immune response. It was deduced that the antigen-stimulated the mucosal sites.

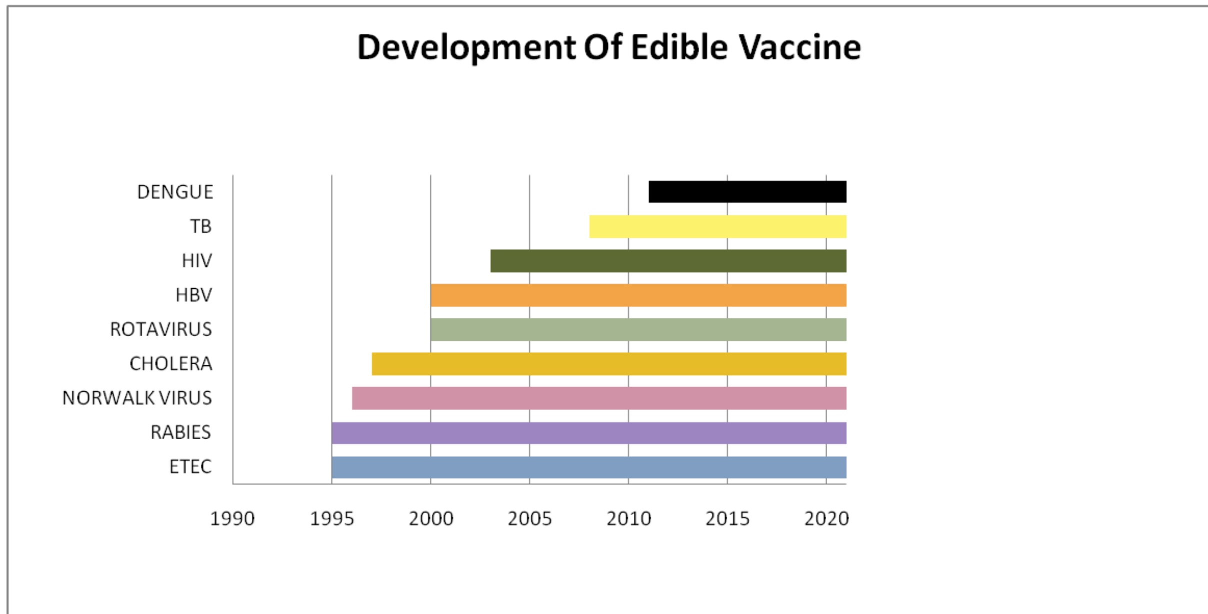


Figure 1: Timeline for the development of an edible vaccine

The bar graph illustrates the fact that the development of edible vaccine started as early as the late 20th century. The timeline is on the y-axis and the category of vaccine on the x-axis.

The earliest edible vaccine development was for the diseases ETEC and rabies; the most recent edible vaccine development was for dengue.

It can be inferred from the chart that the development of different types of edible vaccines is gradually being carried out for a long time. The earliest form of edible vaccine was for enteric pathogens and animal-borne diseases. The later developed edible vaccines were for diseases with current treatment regimens or which can be easily managed.

Table 4: Progress on the Development of Edible Vaccine

Type of Edible Vaccine	Development – *in vitro* assay	Pre-clinical studies – *in vivo* assay
ETEC	yes	yes
CHOLERA	yes	yes
HIV	yes	yes
HBV	yes	yes
TB	yes	no (trials for combined antigen only performed)
DENGUE	yes	yes
ROTAVIRUS	yes	yes
NORWALK VIRUS	yes	yes
RABIES	yes	yes

The table above depicts the scenario of the progress on edible vaccine development. It also demonstrates the urgency of edible vaccines according to the health conditions.

It can be inferred that most of the edible vaccines have undergone *in-vivo* feeding studies for analysis of safety and efficacy.

The table also illustrated that there are minimum pre-clinical studies conducted for two of the conditions for which current treatment regimens are present- TB and cholera and one ailment which drastically affects the body –HIV. Most feeding studies were performed for gastrointestinal pathogens and infectious diseases.

Chapter 5

Edible vaccines for Novel Conditions

Apart from commonplace infectious diseases, edible vaccines have also been targeted for the treatment or prevention of conditions ranging from allergic reactions to cancers. Studies have been conducted to engineer the epitope and introduce it to the body so that the body can prepare and defend itself if such a situation arises. Such conditions include various allergies, some types of cancer, and Alzheimer's disease.

5.1 Allergens

Allergens are foreign particles that are usually not dangerous to humans. However, in some sensitive individuals, the body reacts vigorously to the presence of these particles in a way that can be life-threatening to the individual. Allergens come in a wide variety of forms. They are introduced into the body using inhalation of foreign particles, ingestion of certain proteins in foods and beverages, and injection via the insect bite. Allergens can also affect the individual through close contact with contaminated places and they can even be present in household settings. Usually over-the-counter medications like anti-histamines and prescriptions drugs like decongestants and corticosteroids. Most allergens usually induce a very high IgE response.

The parameters for the analysis of allergic reactions in experimental settings are as follows-

1. Allergen-specific IgE, IgG1, and IgG2a: The IgE specific to the antigen binds with its receptors, this leads to a domino effect in the cells and causes the symptoms of the disease. Allergen-specific IgG is responsible for the secondary Th2-mediated immune response. Allergen-specific IgG is present in the airways together with allergen-specific IgE. The IgG have crucial stimulant as well as inhibitory roles
2. Delayed type hypersensitivity: The DTH response is stimulated after re-exposure to the allergen. Mainly memory T-cells are involved in this case. It occurs 1-2 days later.
3. allergen-specific CD4+ T-cell: They activate the innate immune response, B-cells, cytotoxic cells in addition to suppressing the immune response and secreting cytokines
4. Histological study using microscopy
5. AHR reaction analysis – airway hyper responsiveness
6. Observation of clinical symptoms
7. Cytokine analysis

Oral Tolerance

In case of allergen edible vaccines, the vaccine molecule is required to develop an oral tolerance to the immunogen.

According to (Tordesillas & Berin, 2018) oral tolerance to food antigen can be induced in a variety of ways. Immunogens can be untaken by gut phagocytes, or they may pass through the goblet cell or vesicles. The mucus produced by goblet cells could make the immunogen tolerable to the body. Activities of regulatory T cells could also aid in the development of oral tolerance. The cytokine IL10 is also of importance. Apart from this, the anergy or lack of T cells in the gut can also contribute to oral tolerance.

I. The found-bound antigens would be largely digested by the gut but a small proportion may reach the antigen-presenting cells the M-cells of the Peyer's patches. The antigens could get neutralized by the secretion of IgA, which could lead to oral tolerance.
II. Soluble antigens are transported through the vesicles in the lumen of the intestine, and lysozymic actions could degrade it.
III. Soluble antigens also get transported to the goblet cells of the mucosal linings of the intestines, and travel through the immunogen passages associated with goblet cells which are involved in tolerance development.
IV. Regulatory T cells with functioning Foxp3 gene also contributes to oral tolerance. Usually the regulatory T cells that are found in the peripheral systems are attributed to tolerance development. Other specific regulatory T cells include the Th3 cells and the Tr1 cells. The Th3 cells can stimulate the functions of the Foxp3 cells by the signal TGFβ. These cells are activated during oral antigen delivery. The Tr1 cells induce oral tolerance by the production of the cytokine IL10. The IL10 also co-stimulates the Foxp3 cells in developing oral tolerance.
V. One of the main sites of oral tolerance development is the intestinal lymph nodes along with Peyer's patches. Any antigen transported through the mucous lining will activate naive T cells into regulatory T cells by releasing TGFβ and retinoic acid, both of which induce Foxp3 cell activation. They also stimulate the migration the regulatory T cells to the mucous linings.

Inhalation Allergen- Sunflower seed Albumin

The sunflower seed albumin-SSA has been recently identified as a potential antigen that can bind to IgE and can stimulate an allergic response. It also has a considerable amount of sequence similarity with other potent antigens from the Brazil nut. This allergen also can cross-react with other potential allergens of its species. The most widespread plant allergen group- lipid transfer protein, LTP, is present in the sunflower seeds as well. The symptoms include increasing allergic response in the oral area, asthma attacks inflammation of the mucous membranes, lesions, and puffy eyes(Ukleja-Sokołowska et al., 2016).

A group of researchers has decided to develop an edible vaccine for this allergen.

(Smart et al., 2003) , in their unpublished study, have expressed the SSA gene successfully in the narrow-leaf lupin-(*Lupinus angustifolius* L.). In their present study, they plan to evaluate the efficacy of the SSA allergy edible vaccine in suppressing allergic responses. They have used Balb/c mice. The mice were given powders of either plain lupin or SSA-lupin in a dose of 250µl two times a week for 1 month. In the next step, they inoculated the mice with SSA in alum after one week of feeding, followed by another exposure of SSA. The asthma indices were then measured.

At first, the SSA-specific IgE response along with IgG1 and IgG2a response wasmeasured.. The IgG was detected using ELISA with biotin-conjugatedgoat anti-mouse IgG1and IgG2a. The IgE was analyzed using biotin-conjugated SSA. It was revealed that only IgG2a was stimulated with vaccine-fed exposed mice, rather than IgE or IgG1, which means cellular mediated Th1 response and not humeral Th2 response was noticed. The AHR was nearly inhibited in the vaccinated mice as well.

Then the delayed-type hypersensitivity response-DTH- was observed by the footpad size. In this case, no inflammatory response was noticed.

Next, allergen-specific CD4+ T-cell response was analyzed using cytokine kits. The histology of the airways and lungs of vaccinated mice illustrated the presence of mononuclear cells in place of eosinophils. The cytokine profiles by PBLN T-cells demonstrated that both allergic and protective response was elicited.

Inhalation Allergen - Multiple Epitope Allergen, including Japanese cedar pollen

The next study combines different types of allergen in a fusion gene model. The focal point of this fusion gene was the allergen from the Japanese cedar pollen- *Cry j I and II*.

The Japanese cedar allergy is one of the main allergic conditions and public health concerns of Japan. The allergens involved are the pollen allergens Cry *j I and II*. The *Cry j I* is a

glycoprotein that is found in the cell walls of pollen. The *Cry j II* is another glycoprotein found in the starch granules of the pollen. It has been found that these allergens show sequence homology with another powerful allergen- the Japanese cypress allergen. Most patients have an IgE reaction specific to the Japanese cedar allergen.

(Takagi et al., 2005) have decided to develop an edible vaccine with the fusion gene that contains the Japanese cedar allergen. They selected rice *Oryza sativa L.* cv Kitaake as their vehicle and transformed it with *Agrobacterium*-mediated transformation using vector pGPTV-35S-HPT. Southern blot analysis was used to check the integration and copy number of the fusion gene. 30 transgenic lines were obtained with about 7μg per seed. Then Northern blot analysis was used to check the transcript levels. The RNA transcripts were only present in the seeds. Next, Western blot analysis was done to detect the presence of vaccine molecule using rabbit anti-crp1 and anti-crp2 primary antibodies and goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase.The translation levels analysis showed three expected bands matching the allergen molecules.

After that, Balb/c mice were fed with 70μg fusion protein one time daily for four weeks. After seven weeks, the blood was sampled. This was performed to study the effect of the vaccine rice grains on the allergen-specific T-cell mediated response.

Allergen-specific ELISA was performed with anti-mouse IgE or anti-mouse IgG antibody and pollen grains. It was revealed that both the serum IgG and IgE as well as IgG1, IgG2a and IgG2b were significantly reduced in the edible vaccine-fed mice. Then, allergen-specific CD4+ T-cell analysis was conducted withan anti-mouse CD4 Antibody-conjugated magnetic bead. It was illustrated by the results that the immunized mice were able to suppress the allergenic T-cell response. The levels of both Th1 and Th2 cytokines were also remarkably lowered.

Then the mice were then re-inoculated with the 0.1 mg allergen at weeks 4-5. At weeks 7-8, the mice were again subjected to pollen extract. The clinical symptoms were determined using sneezes in 5 minutes. The sneezing was reduced in the vaccinated group.

Inhalation and Contact Allergen- House dust mite.

The house dust mite is the most common source of indoor allergen that affects people. Allergic proteins in the droppings of the insects are the cause of allergic reactions. The symptoms of mite allergy are similar to other allergy types- asthma, eczema and rhinitis. A scenario of exposure to these allergens is via the bed. The dominant allergen is *Der p* 1, a powerful allergen that binds to the IgE receptors and vigorously stimulates IgE response. It

has cysteine protease activity. A general treatment of the patient includes introducing the allergen into the patient. However, there are adverse effects of this procedure like anaphylactic shock and most patients fail to complete the regimens despite the success rate.
A group of researchers started working with *Der p* 1 allergen to design an edible vaccine.
(L. Yang et al., 2008) and his team attempted to introduce the *Der p* 1 gene into the rice *Oryza sativa* cv.Kitaake to produce a safe and effective edible vaccine for this allergy. They used Agrobacterium-mediated transformation using the vector pGPTV-35S-HPT. The amount of vaccine produced was determined by quantitative immunoblotting using anti-Der p 1 antibody and by a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase. Thirty putative lines were generated from this event, and the vaccine level was determined to be 58µg per grain of seed.

Inhalation Allergen-American Cockroach Allergen

The American cockroach is another source of household allergen that is present all year round. The allergenicity of this insect is similar to that of the house mite. The American cockroach is prevalent in the humid tropics and subtropics. The major allergens are called frass protein. It is present in the dropping of the cockroach. The main group of allergens that is present is *Per a* 1-12. Of these allergens, *Per a* 2 is the most potent. Treatment for the condition is not convenient and requires a long time. Usually, anti-histamines and corticosteroids are used, together with synthetic allergen extract inoculation. Sothe method for more effective prophylaxis is required.
A group of researchers has planned to develop an edible vaccine for the American cockroach allergen.
(M. F. Lee et al., 2018) decided to express the *Per a* 2 gene in the Chinese cabbage- *Brassica campestris* L. var. chinensis. They designed it in 2 ways – full-length gene- Per a 2 996 and the other with the hypo-allergic form- Per a 2 372. The vector pTuMV was used. The transgene expression and quantification were done using immunoblotting. Bands of expected sizes were found.
Then feeding studies were conducted on Balb/c mice with placebo, full-length, and hypo-allergic plants to analyze the efficacy of the edible vaccine on the prevention of the onset of allergy by oral delivery. The mice were fed with 1µg of allergen at 1 dose per day for 3 weeks. Then, after one week, the mice were subjected to the allergen on days 28, 35, 42, and 49 four times, followed by nasal inoculation on days 56, 59, and 61.

The serum allergen-specific antibodies were observed using biotin-conjugated rat anti-mouse IgE and horseradish peroxidase-conjugated rabbit anti-mouse IgG1 or IgG2a. It was revealed that the IgE levels were significantly lower, compared to the IgG titers that were affected negligibly. The AHR reaction was also detected by noting the broncho-constriction. It was illustrated that compared to the full gene, the hypo-allergic gene remarkably lowered the IgE titers and relaxed the airways. The cytokine types were also determined by splenocyte culture. Then cytokine profile demonstrated that there was a noticeable lowering of the Th2 cytokine response in the hypo-allergic group as well as the heightening of the allergic mediator cytokines. Next slides were made from the mice lungs and studied under the microscope for inflammatory response. It was seen that only the hypo-allergic mice lungs showed the subsiding of inflammation.

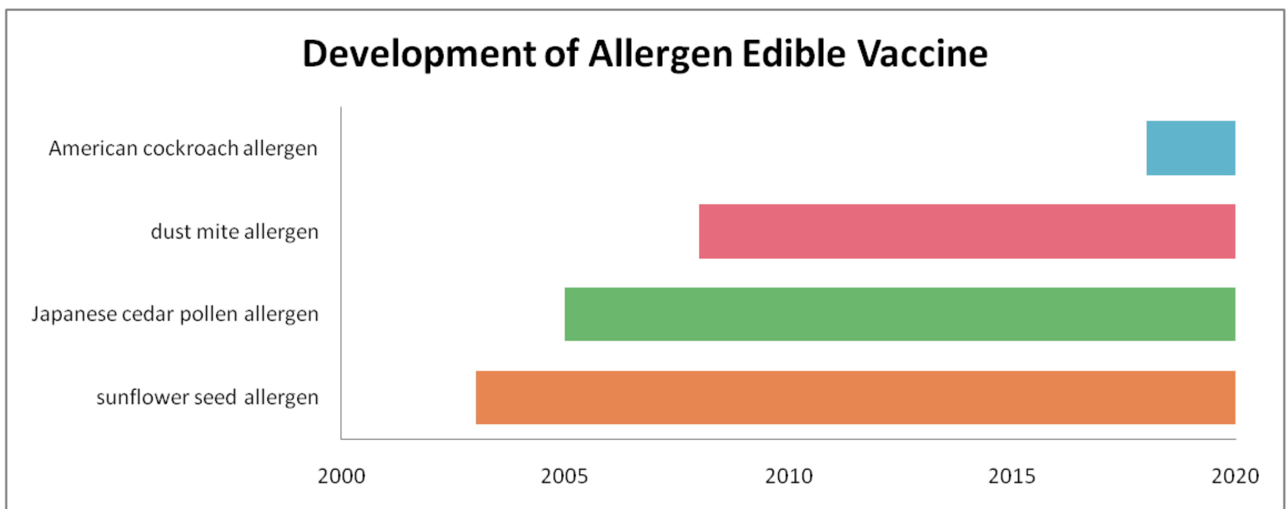


Figure 2: Timeline for the development of edible allergen vaccine

5.2 Cancer

Cancer is a generic term for a wide variety of diseases of almost 200 categories that can affect almost all parts of the body and affect normal cell growth and health. About 1 in every 2 men and 1 in every 3 women suffer from any of the types of cancer. Many factors play an important role in cancer development- environment, genetics, carcinogen, and infections. Some factors like radiation or carcinogen damage the DNA directly, while others like malnutrition or an unhealthy lifestyle pose a risk of getting cancer. A normal cell becomes a cancerous cell when it evades the crucial checkpoints for halting its growth by growth regulators, cellular mediators, and adjacent cells as well as losing the anchoring adhesins. The bodies of healthy people contain genes that have key roles in modulating the growth and developmental controls of a cell, called proto-oncogenes. When these important genes are mutated or amplified, the proto-oncogenes become oncogenes.

Currently, there is no permanent cure for cancer. The main treatment regimens include radiotherapy, chemotherapy, surgery, and immunotherapy. There are some anticancer drugs available, but they are toxic to natural cells as well. There are two available cancer vaccines- preventive vaccines for oncoviruses and therapeutic vaccines that combat cancer cells.

One of the first types of preventive cancer vaccine is that of the HPV- Human Papillomavirus. This virus can cause cancers of the head, neck, throat, cervix, vagina, penis, and anus. The virus has two types of strains- low risk: HPV 6 and HPV11; and high risk: HPV16, HPV 18, HPV 31, HPV 33 , HPV 35 , HPV 45 , HPV 52 , HPV 58 and HPV 59. Among these strains, HPV 16 and 18 are more life-threatening and HPV 16 itself is responsible for most of the cases of cervical cancer.

Cervical cancer

Cervical cancer occurs when the HPV bypasses the cell regulatory mechanisms and breaks through the normal genetic makeup of the cell. The cells divide uncontrollably and reject the signals from the tumor suppressor proteins. There are various risk factors associated with cervical cancer like lifestyle, genetics, and environment.

The search for an effective edible vaccine for cervical cancer started in the early 21st century. As with the other cervical cancer vaccines, the chosen immunogen was the human papillomavirus.

(Biemelt et al., 2003) have decided to develop an edible cervical cancer preventive vaccine in the potato. They selected the epitope HPV16 L1 and expressed it in potato, *Solanum tuberosum* cv. Solara using the vector *Agrobacterium tumefaciens* strain CV58C1.

Then Western blot was performed for purification and for the analysis of the protein expression with rabbit anti- L1 VLP antiserum.The event produced 48 lines out of which 15 were estimated to be putative transgenic lines.The vaccine content was revealed to be 0.2% of the total protein content. However, their levels were relatively lower (3-5 µg/0.35g of tuber). The antigen-specific antibody was analyzed using ELISA with peroxidase-conjugated goat anti-mouse antibodies.The different conformations were isolated using a sucrose gradient. The purification process illustrated that the vaccine molecules were pentamer in form.

To test whether the L1 plants could be used as an edible vaccine, five groups of C65/BL6 mice were prepared- the wildtype group, 4 and 5 were fed normal potatoes, the other group 1-3 was given oral potato doses of 5g on days 1, 14, 32, and 46. Samples of blood were drawn on days 0, 12, 30, 44, and 58. Additionally, one sub-group was fed plasmid DNA, 3-5, and another sub-group, 2, was given a CTB adjuvant. The antibody titers were also determined using ELISA with polyclonal rabbit antiserum and goat-anti-rabbit peroxidase conjugate.

ELISA assay was conducted It was demonstrated that some mice from groups 2 and 3 produced antigen reactions after the third feeding. Despite that, the response disappeared after the fourth feeding. The sera from group 1 were elevated at a minimum. Since only weak and temporary antibodies were stimulated, it was decided that a booster dose is required. All the mice were inoculated with 20µg VLP and it was estimated that 800µg antigen was required, as revealed by the results that there was a significant immune response only after two doses.
In the same year, another team of scientists decided to work with another strain of HPV-strain 11.
(Warzecha et al., 2003) and his team have used the L1 gene from the HPV11 and tried to develop an edible vaccine in the Desiree variety of the potato. They used two forms of the gene- the full form and the truncated form. *Agrobacterium*-mediated transformation was used. 100 putative plants were grown from each event, but only a few plants were viable from each line. It was revealed that the truncated line was more suitable for vaccine development. Out of the 7 truncated lines, only 1 line had double copies of the transgene as illustrated by Southern blotting. Transcript levels were found in all truncated lines as revealed by Northern blotting. The lines were allowed to grow and only 3 lines formed tubers. ELISA analysis by HPV11 or HPV16 virion-neutralizing polyclonal antibodies (N-PAb) and anti-rabbit immunoglobulin G [IgG] polyclonal antibody-enzyme conjugate of the tubers demonstrated that the vaccine molecules could rearrange into VLPs in the plants. This was also confirmed by microscopy.

Female Balb/c mice were given either normal tuber by injection or 5g transformed tubers with or without adjuvant for 4 weeks, then their blood was collected on weeks 2 and 4. Next they were either fed vaccine potato or the VLP by gavages with adjuvant at 6 and 9 weeks. Their blood was sampled at 8 and 11 weeks. VLP ELISA demonstrated that there were negligible changes in the antibody levels after the first inoculation. In contrast, when a booster with adjuvant was given, the results illustrated a significant rise in antibody levels.

After a few years, another team of researchers attempted to develop an edible vaccine against HPV strain 16 but with a different gene.

(Nandhini & Sivanandham, 2014) tried to develop an edible HPV vaccine in the carrot, *Daucus carota*, using the E7 gene of the HPV16. They used Agrobacterium-mediated transformation for the study. The team has used a GUS reporter gene in their construct pCAMBIA 1305.1 for easy detection of the transgene. X –Gluc was added to the GUS assay and the blue color development revealed the successful incorporation of the transgene. The transgene expression was determined using SDS-PAGE; the results illustrated the presence of the 21kDa vaccine molecule band. The translated product was highly expressed in one line-no 2. Quantification was carried out using sandwich ELISA primary HPV16 E7 mouse monoclonal antibody followed by different concentrations of antigens and anti-mouse IgG peroxidase conjugate (labeled antibody). Then the blue precipitate was formed using TMB substrate and it changed color to yellow with the stop solution. It was demonstrated that 0.3g of carrot had the most vaccine molecules- 231.84ng/ml.

Breast cancer

Breast cancer is a condition that is caused by abnormal and uncontrolled changes in the breast tissues. It can grow and spread to the entire breast, its lymph nodes, and even to surrounding tissues. The malignancy can develop in either the ducts or the lobules of the breast. About one-fifth of all breast cancers are HER-2 positive. The Human Epidermal growth factor Receptor -2, HER-2, is a signaling molecule that is present in the breast, which stimulated its growth. HER-2 signaling proteins are sometimes present in excessive numbers in the breast, and it may result in the uncontrollable division of breast cells. The MUC-1 gene is also related to the tumor gene. It sends a signal for the synthesis of a protein called mucin that makes up the protective mucus linings of the stomach, lungs, pancreas, and breasts. The MUC-1 protein is highly expressed in 7 out of 10 cases of all breast cancers.

A group of researchers has devised an edible vaccine for breast cancer.

(Mehrab Mohseni et al., 2019) have designed a chimera gene of the HER2-MUC1 and used it to develop an edible vaccine in the canola - *B.napus*L.cultivar PF 7045-91. Two plasmids were designed- pBI1400-hm and pBI121-hm and *Agrobacterium*-mediated transformation was used. The two constructs developed into six putative lines- hm lines, her2 lines, and muc1 lines. PCR was performed using specific primers to check the integration and copy numbers of the transgene. ELISA was used to determine the vaccine levels in the transgenic lines. It was revealed that all the plants contained 0.015% vaccine levels.

Feeding studies for 5 weeks were conducted on Balb/c mice with the 10µg vaccine seeds from all three lines mixed with mouse feed were given at a 1-week interval. Similarly, another group was prepared, with the exception that it was given a 10µg booster of the raw fusion gene instead of the edible vaccine in their last dose.

Blood samples were collected after 1 week and tested for specific IgG and IgA antibodies against the antigens. The results illustrated that the antibody levels of the inoculated mice were considerably higher. Moreover, the combined epitope group had even more elevated levels of antibodies than single antigen groups. Four weeks after the immunization, splenectomy was carried out to determine the presence of IFNγ and IL–4 by ELISA as well as the lymphocyte assay by MTT assay. The cytokine assay demonstrated that the production of cytokines IFNγ and IL–4 was significantly enhanced. The MTT assay results revealed that the immunized mice had increased levels of lymphocyte proliferation.

Colorectal cancer

The colon and rectum are parts of the large intestine. Colorectal cancer is the third leading cause of cancer deaths in many countries and the fourth most occurring cancer. Cancers in the intestine are usually developed from polyps in the inner lining of the intestine. Not all polyps are malignant in nature; some polyps called adenomas can turn cancerous. Having larger or more than one polyp or polyps with abnormal cells can also increase the risk of colorectal cancer.

The EPCAM is a tumorigenic gene that produces the protein for epithelial cell adhesion molecules. This protein is found in the membrane surrounding the epithelial cells and in the epithelial cells. The gene has a cancer-related antigen that is expressed in both normal and GI carcinomas.

A team of researchers has decided to develop an edible vaccine for colorectal cancer using the EPCAM gene.

(Y. R. Lee et al., 2020) have attempted to develop an edible vaccine for colorectal cancer in a three-step process. They chose the Chinese cabbage, *Brassica rapa L. ssp. pekinensis,* as their vehicle. They designed two expression constructs – one with EPCAM gene that is fused with IgM and the other with an ER-retention signal. They inserted them into the vector pRCV2. The plant was transformed using *Agrobacterium*-mediated process.

The presence of the two types of the transgene was analyzed using PCR with specific primers for EPCAM, ER signal chain, and a marker gene. All putative lines illustrated the specific 837bp (EpCAM), 1053bp (IgM), and 1023bp (marker) and 543bp (ER signal). Then Western blot analysis was performed to check the translation levels of the gene using either primary mouse anti-human EpCAM antibody or oat anti-human IgM Fc μ-chain conjugated to horseradish peroxidase (HRP) and secondary antibody goat anti-mouse IgG 2a heavy chain conjugated to horseradish peroxidase. Except for a few lines, the other lines expressed the IgM proteins, and some sampled lines expressed either high or low antigen protein, as revealed in the results.

In the next step, the well-performing lines were self-pollinated and their seeds were crossed with the lines with the signal peptide. Similar assay procedures were conducted and it was demonstrated that most lines contained the vaccine gene with the signal in PCR and all progeny expressed the vaccine molecule.

Then binding affinity of the vaccine was performed using rabbit anti-human IgG Fcγ antibody conjugated to HRP and goat anti-human IgM Fc μ-chain antibody conjugated to HRP along with TMB substrate and stop solution. The assay results revealed that the IgM group emitted signals while the IgG group did not.

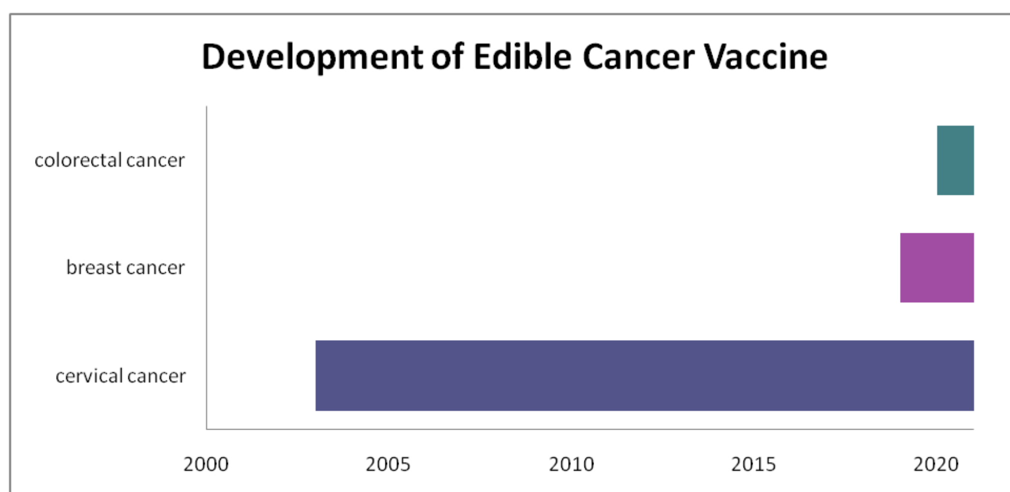


Figure 3: Timeline for the development of cancer edible vaccine

5.3 Alzheimer's Disease

Alzheimer's disease is a condition that gradually develops and slowly impairs the functions of the brain. This can also cause dementia as it progresses. There is no permanent cure for Alzheimer's disease but the symptoms can be treated.

The prognosis of the condition is due to the accumulation of proteins in the brain. The aggregates are found in two forms- plaques and tangles. The plagues are composed of beta-amyloids, Aβ, and the tangles are from Tau protein. Mutations that occur in the beta-amyloid processing enzyme that uses amyloidal precursors are causes of the early-onset form of the condition. For the late-onset form, the main cause is the presence of the E4 isoform of apolipoprotein E. or ApoE.

An agent to prevent plaque formation is one of the main targets of the Alzheimer's vaccine. The early trials showed promising results by stimulating antibody production for the Aβ protein, even though the trials were not successful for the sideeffects (Ishiura et al., 2019).

An edible vaccine for this condition would be beneficial for the elderly because the vaccine can be readily consumed and be not expensive. The first edible vaccine development for Alzheimer's started in the 2010s.

(Ishii-Katsuno et al., 2010) pioneered in the development of an edible Alzheimer vaccine. Since the direction of the vaccine is to reduce deposits of plaque, the Aβ gene was used. The vehicle was the *Capiscum annuum* var. angulosum leaves. The vector was the plant virus, TocJ. The antigen was attached to the GFP reporter gene. The vaccine quantity was determined using Western blot by mouse monoclonal antibody 6E10 and horseradish peroxide (HRP)-conjugated secondary antibodies. The vaccine content was 100-600μg per gram of leaf.

Two sets of mice were prepared- healthy B6 mice that had fully-developed immune systems and mutant Alzheimer model Tg2576 mice. The healthy mice were prepared to analyze the antibody production and determine the process of the immune response, while the model was prepared to assay whether the edible vaccine was effective or not.They were divided into 3 subsets-controls, subcutaneous and oral. Both the healthy mice and the mutant mice were inoculated two weeks a month; the healthy set from three to eleven months of age and the model mice from five and a half to eighteen months of age. After the regimen, both sets were given a booster dose of antigen after two weeks. Then blood was sampled from the heart. ELISA was performed with monoclonal anti-Ab antibody 6E10 and an HRP-conjugated secondary antibody followed by TMB substrate to determine antibody levels.

Analysis was conducted on the antibody stimulation and the IgG types of both sets of mice. The B6 mice analysis results demonstrated increased production of antibodies. It was also found that the oral group had lower levels of inflammatory IgG2a antibodies than the other groups. The Tg2576 model group results illustrated that both inoculated groups had a rise in antibody levels. Similarly, the oral model group had less level of inflammatory antibodies than the injection group.

The model mouse brains were homogenized and sandwiched ELISA with a monoclonal antibody against the human Ab42 N-terminus and HRP-conjugated antibody raised against the Ab42 C-terminus for antibody titers. The levels of Aβ plaque were revealed to be significantly reduced in the oral group. The outer brain parts were stained using anti-Ab antibody 6E10. It was observed that there were fewer plaque developments in the outer hemisphere as well.

Another follow-up study was carried out by another team of researchers for Alzheimer's disease.

(Yoshida et al., 2011) attempted to develop an edible vaccine for the disease using the same gene construct. They have decided to use rice - *Oryza sativa* L. cultivar Hayayuki- as the vehicle and employed the plasmid pIG121-Hm. *Agrobacterium*-mediated transformation was used. The integration of the transgene in the grains of individual lines was detected using Southern blot with the GFP marker carrying the complete antigen sequence probe. Strong luminescence was found in the aleuronic layer of raw rice but weaker signals were found in processed rice. PCR was also carried out with sGFP-5'-XbaI and Aβ-3'-SacII primers for the leaves, the transgene was shown to be successfully incorporated in the genome. Western blot with anti-Aβ antibody 6E10 and HRP-conjugated secondary antibody was used to check the antibody levels. Eight microns of vaccine molecule were estimated in a single grain.

Doses of 10μg of vaccine rice with CTB were prepared. Three groups of C57BL/6J mice were prepared- control, vaccine, and boiled. The mice received a weekly dose of vaccine from eight to eleven weeks and a booster dose of 0.5μg antigen to all groups at 14th week. Blood was sampled on weeks 8, 12, 14, and 16. The presence and levels of antibodies were determined using Western blot and ELISA using anti-Aβ antibody and HRP-conjugated secondary antibody followed by TMB substrate. It was shown that the antibody titers rose from week 12 but became undetectable during week 14. An increment of a booster antigen-stimulated the second wave of stimulated antibody.

So, the case studies on edible vaccine development for many ailments reveal that the edible vaccine for such conditions has undergone successful pre-clinical trials on laboratory

animals. Therefore it can be inferred certain health conditions that were never before treated with vaccines have shown promising prospects by treatment with edible vaccines. It was highlighted by the fact that the feeding studies were able to elicit antigen-specific antibodies.

It can be said that edible vaccines can be beneficial for both the treatment and prevention of infectious diseases and for the prevention of health issues that were not thought to be tackled by vaccines of ant type

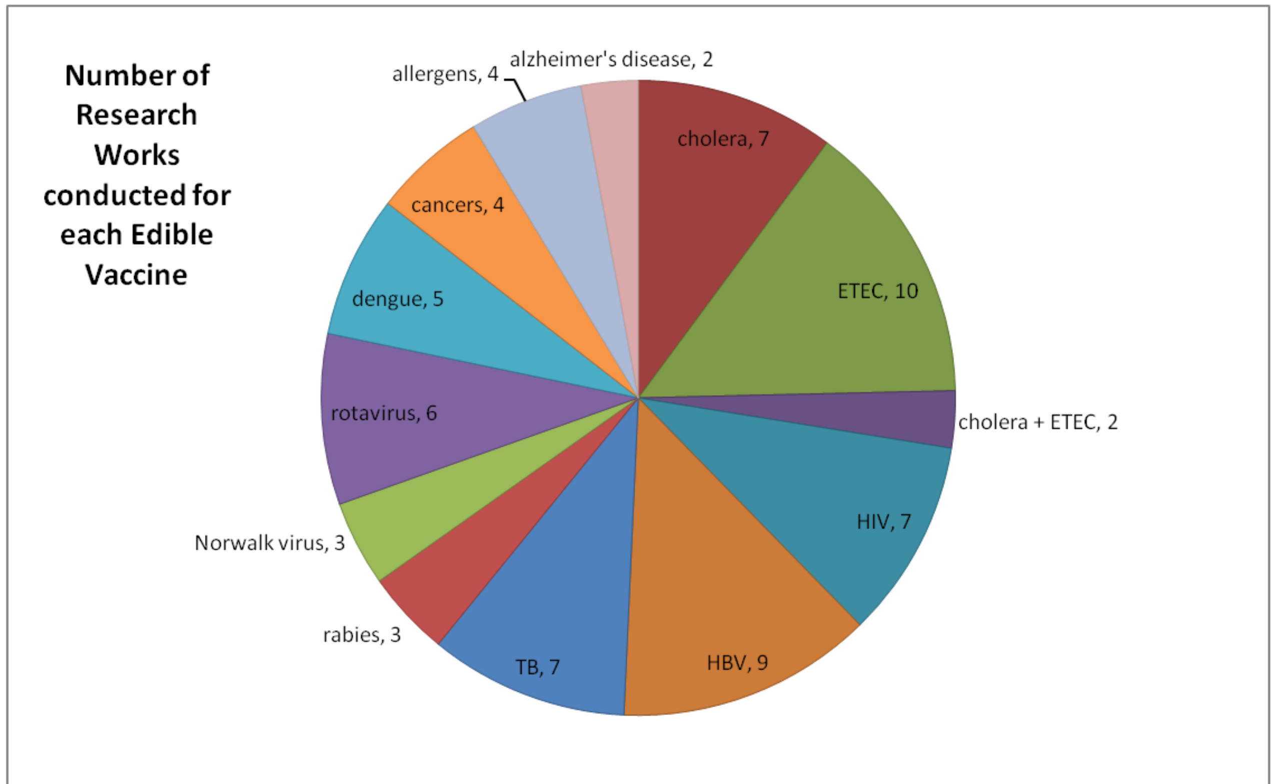


Figure 4: Number of published works conducted for each edible vaccine.

Chapter 6

Current Status of Edible Vaccines

6.1 Clinical trials

In every new medicine and treatment regimen, clinical trials are an important part of drug safety and efficacy procedures. The clinical trials involve the help of volunteers that guide the research team in their crucial decision-making process. They are used to test different parameters of new drugs and dosage regimens. Clinical trials are usually preceded by preclinical studies on cultures of cells of human and animal models.

The clinical trials of edible vaccines are being carried out for a long time. They basically start from Phase 0 since this is a novel form of vaccination produced using modern biotechnology techniques. This level of trial is mainly for safety assessment but it could also predict the efficacy of the vaccine. The trial is carried out using less than 15 participants.

Phase 0 – ETEC edible vaccine

(Tacket et al., 1998) conducted the very first clinical trial on the edible vaccine. The potato vaccine was of the variety Frito Lay 1607, transformed using pTH- 110. The team recruited 14 volunteers and divided them into three groups. All of the participants had their blood tested for anti-CTB antibodies; only one participant showed a slightly positive result. Eleven participants ate the vaccine potato. The first group (6 members) was given 100 gm vaccine potato, the next group (5 members) had 50 gm vaccine potato and the last was a negative control. The second and third dose was given on days 7 and 21. The participants were told to keep a record of any health symptoms that could arise from the vaccine. Two volunteers of the first group suffered from nausea- one after the third dose and the other three days after the second dose. Four out of the eleven volunteers suffered from diarrhea.

Then blood was sampled for 28 days at 7 days intervals. The antibody-secreting cells, ASC, producing anti-LTB antibody was determined using ELISPOT method. The ASC were detected in the blood after 7 days of immunization and it was inferred that the GI cells had contact with the antigen, leading to antigen presentation. They were not detected after 14 days of vaccine ingestion due to migration to mucosal locations. Then the peripheral blood mononuclear cells, PBMC, were isolated and their antigen secretion against the vaccine was analyzed with goat anti-human IgA alkaline phosphatase conjugate or IgG conjugate. Specific IgA antibodies were found 7 days after the first dose, the titers decreased on seven days after the second dose. The titers reached their apex after 7 days from the third dose. Whole blood analysis using ELISA with goat ant-human IgG and anti-human IgA on days 7,

14, 21, 28, and of the 59 days illustrated that 10 out of 11 volunteers had four times increase in IgG and 6 out of the 11 volunteers had specific IgA. The titers of the volunteers with IgG also gradually rose on 7 days after the first dose. The stools were collected similarly and analyzed with ELISA. 5 out of those 10 volunteers also expressed fourfold secretary IgA. Neutralization assay with Y-1 mouse adrenal cells assay demonstrated that 8 out of 11 volunteers had neutralizing antibodies.

It was found that a transgenic plant-based edible LTB vaccine showed remarkable antibody stimulation in participants.

Even though the results of the clinical trial were satisfactory, further supplementary studies are usually done to analyze and rectify any issues that could hamper the effectiveness of the vaccine as well as to make it safer for the patients.

The team then proceeded to conduct a clinical trial on another edible vaccine. They targeted the Norwalk virus edible vaccine in their next clinical study. The team decided to conduct a Phase I trial. A Phase I trial usually includes about 20-80 healthy participants. The trials evaluate the maximum dose and the different dosage forms of the medication. They also monitor the long-term effects of the drug on the body. It is reported that about 70% of Phase I medications move on to Phase II.

Phase I – Norwalk virus edible vaccine

(Tacket et al., 2000) had designed the Norwalk virus edible vaccine with the same variety of potato, Frito Lay 1607 with pNV140 to form the well-performing line NV140-13 that inherited four vaccine genes. They planned a double-blind trial with 24 volunteers who were divided into three groups- 10 participants who would receive 3 doses of vaccine on days 0,7 and 21,another 10 participants received 2 doses of potatoes on the first and last days, and 4 participants who were negative control. 150 g doses were prepared from the skinned potato, containing 215 -751 µg of the vaccine. The volunteers were told to record any physical discomfort they felt for three days after consumption of the vaccine.

Serum was isolated on days 0, 7, 14, 21, 28, and 61 and bothIgG the IgM were analyzed. Blood was sampled on days 0,7,14, 21, 28, and 30; it was assayed using ELISPOT. PBMC, as well as ASC, were analyzed. The stool was collected on days 0,7,14, 21, 28, and 61 and studied for secretory IgA and antigen-specific IgA. The blood specimens were collected after the first dose. It was revealed that 19 out of 20 volunteers stimulated ASC that produced IgA. Moreover, 13 out of 19 volunteers responded after the first dose. 6 volunteers had ASC IgG response and 4 volunteers with no IgG response had serum IgM. The IgG titers increased by 12 times while IgM titers by 7 times. The stool antigen-specific IgA also rose by 17 times.

Antibody responses were also revealed after receiving the second dose in 6 out of 8 of the previous responders for both IgG and IgM. The IgG response stayed for 2 months while the IgM disappeared before 1 month.

It was decided that the antibody-specific serum antibody response was not as strong as it was expected. It could be because not all of the vaccine molecules assembled into VLPs and immunogenicity are elicited from the assembled particles.

The next group of scientists decided to conduct a clinical trial using an edible vaccine with a different vehicle. They started performing a Phase 0 trial.

Phase 0 – HBV edible vaccine

(Kapusta et al., 2001) used the lettuce as a vehicle for HBV edible vaccine. They transformed the lettuce using Agrobacterium-mediated transformation. They recruited 12 healthy volunteers and tested their blood for hepatitis antigen and antibodies. Seven volunteers were administered with 3 doses of vaccine lettuce- the first two doses at the one-week interval for two weeks and the last dose four weeks after the second dose. They gradually increased the vaccine level for each dose. Blood was sampled at two to four weeks after the second dose and two weeks after the third dose and the antibodies were analyzed using ELISA.

Antibodies began to appear two weeks after the second dose in three of the volunteers, after another week, all the volunteers developed antibodies. Even though the serum titers were quite low in the beginning and they started to disappear four weeks after the second dose, the titers soared up after the third dose.

It was concluded that the trial results were satisfactory. The low levels of antibody were possibly due to lower vaccine doses as well as not using adjuvant.

Phase 0 – Rabies edible vaccine

(V. Yusibov et al., 2002) attempted to develop an edible vaccine in the spinach using the vector Av/A4. They transformed the spinach using a mechanical method. The team decided to conduct a clinical trial in two groups- in rabies vaccinated participants and unvaccinated participants.

The five vaccinated volunteers received 3 doses of 84µg vaccine at 2 weeks intervals. Then blood was sampled on days 0, 14, 28, and 42 and analyzed using ELISA with biotin-conjugated goat anti-human IgG for human IgG and biotin-conjugated mouse monoclonal anti-human IgA1/IgA2. The 3 volunteers' blood assay revealed high levels of rabies-specific antibodies and the rabies-specific IgG rose after each dose.

The nine unvaccinated volunteers were provided with a similar vaccination regimen but with 700µg doses. One week after the third dose, the volunteers received the booster dose of the conventional rabies vaccine. Blood was sampled on days 0, 35, and 42. ELISA was performed as before. 6 of the volunteers responded with elevated antibody levels. The blood assay results illustrated that 4 volunteers had rabies-specific IgG, and 2 volunteers from these 4 and another volunteer expressed antigen-specific IgA. Only 3 volunteers stimulated rabies neutralizing antibodies after the booster.

The results of the trial highlighted the fact that the edible rabies spinach vaccine can be effectively used as a booster.

Another team of researchers decided to improve the LTB edible vaccine development. They switched to a relatively new vehicle for vaccine delivery. The team decided to hold another Phase 0 trial.

Phase 0 – ETEC edible vaccine

(Tacket et al., 2004) have used corn as a vehicle for the ETEC edible vaccine. They decided to concentrate the LTB antigen by separating the germ layer and removing the fat layer through processing. Thirteen healthy volunteers were chosen from an outpatient facility. Nine volunteers were given 2.1gm of corn meal on days 1, 7, and 21. The volunteers were told to keep a record of the side effects for 7 days after the first dose. The vaccine was tolerated in the body, only one patient had diarrhea and two suffered from cramps. Blood and stool were collected on days 7, 14, 21, 28, and 60 after the first dose. Similarly, the ASC were assayed on days 7, 14, 21, and 28.

The blood samples were tested with ELISA using horseradish peroxidase (HRP)-labeled goat serum anti-human IgG and IgA and TMB substrate. Seven participants developed serum IgG specific for the antigen and four participants developed serum IgA antigen-specific antibodies. The ASC was determined using ELISPOT assay with HRP-labeled goat anti-human IgG and IgA. Seven volunteers developed antigen-specific ASC for both IgG and IgA. The stool was assayed using ELISA with HRP-labeled goat anti-human IgA and TMB. Four participants had secretory antigen-specific IgA, and two among them were from after the first dose.

It was decided that the corn ETEC edible vaccine was both well-tolerated and immunogenic in humans.

After the Phase 0 clinical trials of the HBV edible vaccine, the second group of researchers decided to conduct a Phase I trial for HBV edible vaccine

Phase I – HBV edible vaccine

(Thanavala et al., 2005) developed an edible vaccine in the potato variety Frito-Lay 1607 and employed Agrobacterium-mediated transformation using the plasmid vector pHB114. They selected forty-two healthcare workers who previously received the full course of hepatitis B conventional vaccine together with sufficient HBV IgG response. The participants were asked to keep records of any systemic, gastrointestinal, and fever responses from each vaccine dose for three days. The volunteers were divided into 3 groups: placebo (9 volunteers), double dose (17 volunteers), and triple dose (16 volunteers). The participants were fed either 100-110 g vaccine potato double dose on days 0 and 28 or the same doses of potato on days 0, 14, and 28. Blood was sampled on days 0, 7, 14, 21, 28, 35, 42, 56, and 70 and assayed using biotin-tagged HBsAg and rabbit anti-biotin conjugated with horseradish peroxidase.

Ten volunteers who took the triple dose had a noticeable rise in antigen-specific antibodies; one amongst them had a doubling effect, while another four of them had a four-fold riseonly after one dose. Moreover, nine of the volunteers who took the double dose also showed a similar increase in antibody titers; five participants had twice as much antibody and two participants showed a four-times increase in the titers only after the first dose.

The trial was considered a success because the vaccine did not contain any adjuvant, the viral antigen is not an enteric pathogen but it stimulated a response when given orally and the viral protein coat is non-replicating. 19 out of 33 immunized volunteers showed a remarkable boom in antibody production and titer levels; inferring that it could be used for booster doses.

Phase II trials usually involve hundreds of volunteers who are affected by the health conditions that are being researched. So, the researchers need to be extremely cautious when applying the new medication to the volunteering patients. The volunteers receive the same dose and dosage forms as Phase I trials. The researchers usually closely monitor the patients and thoroughly record the effects of the developing treatment on the patients' bodies as well as their side effects. The process usually takes several months to years. The data collected from Phase II usually acts as a reference for Phase III trials. Approximately 33% of Phase II candidates move over to Phase III.

According to (T. G. Kim & Yang, 2010) a Phase II clinical trial is being conducted by Arizona State University on the edible vaccine for HBV. There are also unpublished reports concerning the commencement of Phase III trials of some edible vaccines. Therefore, it can be said that the future of edible vaccines is promising indeed and it is a matter of time and regulatory approval processes before edible vaccines will indeed become a reality.

Until now, two of the edible vaccines that have published documentation are undergoing optimization processes at industrial levels for the Phase II trial. They have registered trade names and are undergoing developmental evaluation to assess their efficacy for the proposed ailments.

6.2 Progress of Edible Vaccine Approval

Norwalk virus edible vaccine - noroVAXX®

The edible vaccine that was developed by (Tacket et al., 2000) completed the Phase I trial successfully with the most promising results. The vaccine had registered and had been undergoing improvements as an edible vaccine candidate by Arizona State University and has been allowed a Good Manufacturing Practice (GMP) facility for the optimization of the production process through Kentucky BioProcessing, LLC (Fischer et al., 2012). The facility allows process optimization using the model tobacco plant. The KPB has state-of-the-art technology for the expression, extraction, and purification of recombinant proteins for commercial use. Investments have been made to refine the Norwalk virus edible vaccine for further clinical trials(Vidadi Yusibov et al., 2011).

MucoRice-CTB

Another team of researchers went on to refine the promising cross-reacting antibody vaccine MucoRice-CTB.

(Kurokawa et al., 2013) aimed to process the rice vehicle by removing the allergens in rice and optimizing the vaccine content fit for a commercial-scale vaccine. They changed the rice variety to a japonica variety of rice, Nipponbare, and transformed it with an Agrobacterium-mediated process using the vector pZ2028. The group planned to decrease the content of the storage proteins glutelin A and prolamin using the RNAi silencing method.

The MucoRice line was the line with the highest vaccine content. They self-pollinated the line. SDS-PAGE and Western blotting with monoclonal Ab to glutelin A and anti-13kDa-prolamin antiserum were utilized to determine the levels of allergens. It was revealed that the allergen content was decreased significantly, thus proving space for vaccine production. The vaccine molecules were analyzed similarly with rabbit polyclonal anti-CTB. Next IgE immunoblotting was performed to check the expression levels of the antigen with patient sera and horseradish-peroxidase (HRP)-linked antihuman IgE. It was observed that the intensity of precipitate of the allergen was lower in MucoRice. 2G- DIGE analysis was conducted to analyze the salt-soluble proteins with low-intensity dyes. The CTB spots were detected in

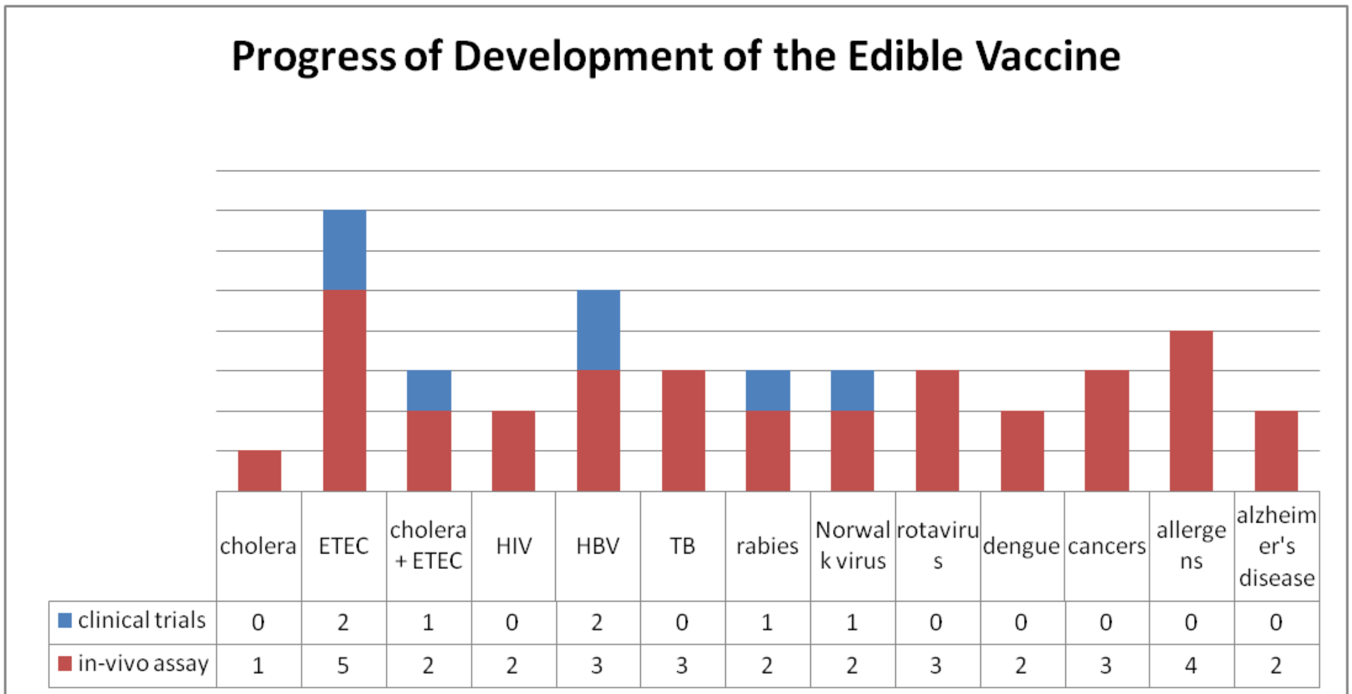
both single and double unit structures. The allergen content was determined using anti-RAG2 globulin IgG and marked with peroxidase-labeled antimouse IgG or peroxidase-labeled antirabbit IgG. The results demonstrated lower expression of the allergens. Quantitative real-time PCR was conducted with allergen and vaccine-specific primers to assay the RNA expression levels. The results illustrated that the allergen expression was successfully downregulated in the MucoRice and the monomers were slightly reduced in content.

Recently, a Phase I clinical trial was conducted using MucoRice-CTB.

(Yuki et al., 2021) conducted a random, double-blind, dose-escalated placebo trial in Tokyo. Sixty volunteers were tightly screened for the absence of unwanted criteria as desired by the researchers. Three dose regimen with doses of 1g, 3g, and 6g with the volunteers receiving the vaccine for 8 weeks with a single dose every two weeks. The sera and feces were analyzed at weeks 1, 2, 4, 6, 8, and 16 after the first dose. Primarily, the safety and tolerability of MucoRice were assayed. Secondarily, the serum antigen-specific antibodies, fecal secretory IgA, and APC were analyzed. The cross-reactivity of the vaccine for LTB was also assayed.

It was revealed that most of the volunteers developed some form of adverse effects. The results illustrated that the sera antigen-specific antibody IgG and IgA were elevated to about fourfold in the volunteers depending on both dose and time. The serum IgG and IgA gradually rose after the first vaccine; the titers escalated and reached their apex after 8 weeks for IgA and 16 weeks for IgG. Participants with the minimal dose showed a weak immune response, however, the responses became stronger with increasing dose. The sera were sampled and GM1-binding ELISA was performed with LTB. The study demonstrated that the CTB antibodies prevented the receptor-binding of the LTB.

The team concluded that MucoRice-CTB was highly effective in eliciting an immune response for both LTB and CTB and estimated that a 6g dose was required for stimulating an adequate immune response.



	cholera	ETEC	cholera + ETEC	HIV	HBV	TB	rabies	Norwalk virus	rotavirus	dengue	cancers	allergens	alzheimer's disease
clinical trials	0	2	1	0	2	0	1	1	0	0	0	0	0
in-vivo assay	1	5	2	2	3	3	2	2	3	2	3	4	2

Figure 5: Summary of the progress of edible vaccine development

Chapter 7

Biosafety Concerns and Regulatory Affairs

Edible vaccines are transgenic plants that are designed to confer immunity to the health conditions of the human body. The plant systems produce the antigen that would be directly consumed by the public. Unlike conventional transgenic plants which are only modified to introduce a desired trait in the plant, edible vaccines do not restrain the transgene to the plant. One of the most fundamental concerns of edible vaccines is the potential adverse effects on human and animal health. A transgene that has the potential to enter the food chain would be under rigorous observation by the regulatory agencies. Toxicity is a major factor concerning the biosafety of edible vaccines. Since antigens of potentially powerful pathogens are employed, they could trigger adverse reactions in the body of the consumers. Another risk factor is that of allergenicity. As it is already described how edible vaccines can be prophylactic for allergy, however, a potent allergen may cause allergenicity in the consumer before imparting immunity to it.

Furthermore, edible vaccines may induce either allergenicity or oral tolerance. In some schools of thought, the post-translational modifications and the presence of oral adjuvant in the edible vaccine cassette may elicit an anaphylactic response, which could extend to even daily foods. In contrast, frequent intake of edible vaccine and adjuvant may elicit the presence of antibody-secreting cells at all times, which could reduce the potency of the vaccine by producing oral tolerance(Takeyama et al., 2015).

Even though the environmental issues are not as diverse as traditional engineered crops, there are still lingering fears concerning edible vaccines. One such concern is the fate of the antigen after the lifespan of the plant. If the antigen, which is highly purified, comes into contact with a non-pathogenic microbe and induces pathogenicity in them. Another concern is the leaching and contamination of the antigen in the environment.

The transgene can be carried using insect or animal vectors or be accidentally crossed with wild-type species thus decreasing the gene pool of the vaccine and consumed by unsuspecting people. They can be transmitted via pollen as well. (Takeyama et al., 2015)

Edible vaccines can be termed both LMOs and pharmaceutical agents. This duality of nature of edible vaccine is difficult to fit into the Cartagena Protocol on Biosafety. Even though LMO-based pharmaceuticals are exempt from the Protocol, the transformation process as well as the presence of the transgene in the final products turns the edible vaccine into an LMO.

The first meeting regarding the edible vaccines was conducted in 2005 by World Health Organization. It was agreed upon to use the current guidelines for developing and evaluating such vaccines. It was also decided that the clinical trials of edible vaccines would be carried out by applying to the US Investigational New Drug application procedures. All the processes should follow the regulatory and Good Manufacturing Practices, GMPs. Moreover, necessary steps would be needed to prevent the contamination of the environment by the antigen (Sahoo et al., 2020). The first guideline for plant-based pharmaceuticals including edible vaccines was developed by the FDA and USDA. The whole platform was remarkable development as it involved all sorts of plants and alga that can be used for transformation. The Food and Drug Administration, FDA, oversees all the processes of plant-based compounds including edible vaccines. The organization makes sure to certify the edible vaccines are free of other transgene and resistant marker genes. Good Agricultural and Collection Practices, GACP, which are applicable for herbal medications, are also to be applied to edible vaccines derived from plants. Quality control assessment reports would have to include the identity, purity, and content of transgenic products along with their assay methods. The United States Department of Agriculture, USDA, plays an important role in determining the nature of the donor and acceptor plants, their genetic constitution and transformation and the chance of cross-contamination. The USDA also plans and implements risk management strategies. (Takeyama et al., 2015)

However, the scenario in Europe is more complicated. The development of transgenic proteins is regulated under Pharmaceutical products- Regulation 2309/93; stricter guidelines are needed for plant-based medical products like edible vaccines. According to EMA, the development and early stages of clinical trials are observed by the local regulatory agencies, whereas the commercialization and the steps before it are overseen by EMA. (Sahoo et al., 2020)

The European Union has the strictest guidelines for the assay of any transgenic product. The European Food Safety Authority, ESFA, performs the entire assay in their reference laboratory in addition to risk assessment, risk management, and advisory tasks. The ESFA is a precautionary and systemic body for biosafety regulation of transgenic plant products and vaccines that is performed on a case-by-case basis. The European Medicines Agency, EMA, controls the examination of all biopharmaceuticals including vaccines. Recently the European Commission made the safety assessment more rigorous for all transgenic plants by implementing a mandatory 3 months feeding assay; the safety assessment would be made more stringent by extending the duration to 2 years. (Howard & Hood, 2014)

The GMPs of edible vaccines have to address a plethora of issues from development to commercialization. Despite this complicated process, four key points have been identified that would lead to the development and implication of GMP-based regulatory guidelines.

1. Standardization – Edible vaccine production for mass vaccination campaigns would usually be developed in the industry. So, it is necessary to select an ideal plant candidate which could comply with the mass-production processes of the industry. Standardization would offer many economic and regulatory advantages, but the process in itself is very complex due to the diversity of the vegetation present. Standardization should be done by analyzing the CHO ratio of the plants. Many edible vaccines are patented, therefore necessary negotiation with the creators is required to develop an industry-grade edible vaccine.
2. Production platform- All the platforms need to be reviewed and the most appropriate platform in terms of cost and regulatory procedures should be selected.
3. Plant expression system – the benefits and drawbacks of stable and transient transformation processes need to be assayed to determine the most cost-effective, safe, efficient, and regulatory compliant transgene expression system.
4. Downstream processing – It can be said that the downstream processing of bacteria and mammalian cells is completely different from that is required by the plant systems. Even if the plants are handled in the most advanced greenhouse, there will be a lag period during its transfer to a GMP- based harvesting facility. A high level of hygiene is required for the handling and transfer of raw inputs. (Fischer et al., 2012)

   If the plants are cultivated in closed systems, then they will fall under the conventional regulatory guidelines of USDA, FDA, and any other national regulatory systems. However, when open field cultivations and trials are conducted, the proceedings of the Animal and Plant Health Inspection Service of the US Department of Agriculture or their equivalents in other countries would be followed. (Twyman et al., 2005)

GMPs are required for the commercial processing of edible vaccines. The facilities developing the edible vaccine also need to comply with the GMP. The GMP additionally oversees any unwanted modifications and their effects that can arise in the vaccine as well as maintains the required purity and quality. (Park & Wi, 2016)

Chapter 8

Edible Vaccines and Bangladesh

Bangladesh is steadily progressing in the field of Agricultural Biotechnology. With the successful development of Bt-crops and Golden Rice in the pipeline for release, the first edible vaccine was also developed and tested *in vivo* in Bangladesh.

(Akter, 2019) pioneered in the development of a pneumonia edible vaccine in the tomato. She engineered the pspA antigen of the *Streptococcus pneumoniae serotype 7F* in the tomato,*Lycopersicon esculentum*, using the *Agrobacterium*-mediated method. She designed 3 separate vectors- alpha, gamma, and fusion Ds-red zein. Epitope 1 was used in alpha-zein, epitope 2-4 in gamma-zein and Ds-red zein contained a combination of alpha and gamma. PCR analysis was conducted to detect the integration of the transgenes. The expected band lengths of the vectors were illustrated in the results.

The immunogenicity of the edible vaccine was demonstrated using Balb/c mice feeding studies. The 1g vaccine leaves were dried and lyophilized, then formulated into tablets with mouse feed. Mice were vaccinated with 6 doses at a 1-week interval for 6 weeks. Then the mice were subjected to a dose of the *S.pneumoniae* that is 10 times more potent than the lethal dose.The procedure was repeated a second time. The death of the mice was recorded for one month.

During the first trial, it was observed that out of the 12 mice for each construct group, 3 out of 12 mice died for the alpha-zein group, 2 mice dies in the gamma-zein group and 2 mice were also dead in the fusion group. On the contrary, in the second trial, it was noticed that out of the 14 alpha-zein mice, only 2 mice were found dead. Furthermore, out of the 12 mice of the gamma-zein group and 10 mice of the fusion group, none of the mice died.

It was concluded that the mortality rate was reduced significantly after the final trial. The survival rate is the same for alpha-zein, but the rate increased for gamma-zein and Ds- red zein.Therefore, the edible vaccine was effectively able to elicit a protective immune response

Chapter 9

Edible vaccines for SARS-COVID2

The SARS –COV2 is responsible for the ongoing streak of the Covid-19 pandemic. The virus usually consists of 4 types of structural proteins- spike (S), envelope (E), membrane (M), and nucleocapsid. The spike protein forms the outer part of the virus and forms a crown-like structure, hence the term ‘coronavirus’. The viral S protein binds to Angiotensin-converting enzyme 2 (ACE2) using the domain-binding receptor (DBR). The DBR is a part of the S1 subunit. The S protein cleaves into S1 and S2 subunits.

In general, the viruses from this family have 99% sequence similarity with the S2 protein and 70% similarity with the S1 protein. (Venkataraman et al., 2021)

It can be inferred that there is still hope for the development of an edible vaccine for Covid-19.

The positive news started with the development of an edible vaccine against another strain of coronavirus, Severe Acquired Respiratory Syndrome (SARS). Researchers were successfully able to clone the S1 fragment of the SARS virus, SARS-COV, in the tomato cultivar Money Maker along with a low-nicotine tobacco variety *Nicotiana tabacum* cv. LAMD-609 and Wisconsin. They used *Agrobacterium*-mediated transformation using the plasmid pE1801-$79S_{HDEL.}$ Western blot analysis was conducted on the transformed lines, revealing the expression of the spike protein fragment of low molecular weight-50kDa- in some green fruits and most red fruits. The tobacco expressed the truncated 79kDa protein as well as other protein fragments.

For immunological studies to detect the efficacy of the spike protein orally, Balb/c mice were fed with 500mg of 3 doses of freeze-dried tomatoes or 50mg tobacco. Their sera and feces were collected after 10 days of the last immunization. The results illustrated the secretion of mucosal IgA in the feces of mice fed with low molecular weight vaccine tomato. However, negligible serum IgG was present.

Another follow-up assessment was conducted where the mice were primed with a 50mg subcutaneous injection of transgenic tobacco thrice at 2 weeks interval. No immune response was detected this time. Despite this, when administered with a 5µg S-protein booster, a significant serum IgG response was elicited.

It was concluded that the smaller fractions of the S1- fragment were more immunogenic and its expression in transgenic plants was in sufficient amounts to induce noticeable immunity levels. (Pogrebnyak et al., 2005)

Recently, scientists from all parts of the globe are trying to design an economic and easily scalable Covid-19 vaccine. The most favorable option for such a vaccine development is considered that of using transgenic plants.

In recent times, two groups of researchers have successfully attempted in developing the SARS-COVID2 edible vaccine.

A group of scientists from the Center for Genomics and Bioinformatics of the Academy of Sciences in Uzbekistan has expressed the spike protein of the SARS-COVID2 in tomatoes. A press representative from the research informed the media that the vaccine is deemed to be effective. It works by having the tomato digested in the stomach, protecting and effectively releasing the vaccine. ("Uzbekistan Developing " Edible " Vaccine against Coronavirus," 2022)

Another team of researchers had been developing an experimental Covid-19 edible vaccine in the lettuce. Researchers and interns at the Faculty of Science in the Ottawa University's Department of Biology expressed the virus in the lettuce to develop an edible vaccine. The experimental vaccine is now undergoing Phase I clinical trial in the Ottawa Hospital. The group is currently awaiting results. (*From Green Thumbs to Edible Vaccines – A Journey Not like Any Other*, n.d.)

Chapter 10

Discussion and Future Directions

Edible vaccines targeting specific health conditions have been developing for quite a considerable time; researchers are testing out different transformation procedures and platforms to engineer the safest and most effective edible vaccine. Edible vaccines could be used to prevent various gastrointestinal conditions, as well as ailments like TB, rabies, HIV, and HBV. Moreover, edible vaccines are showing promising results in the field of allergy treatment. Furthermore, certain diseases like cancer and Alzheimer's disease, which are not currently treated by vaccines, could be dealt with edible vaccines. The edible vaccine is also being developed and tested for the current Covid-19 pandemic. Six of the edible vaccines have already reached the end of Phase I clinical trials. Among them, two of the vaccines have been documented to enter the industrial level processing to undergo Phase II trials. They have registered trade names in their own countries.

Researchers need to focus on the health and environmental issues related to the introduction of the immunogen in food crops to devise an ideal edible vaccine. They also need to consider the industrial factors related to the edible vaccine, mainly planning a way to properly regulate the vaccine levels in all the plants of the same batch in addition to following regulatory guidelines. An alternative option could be to process the vaccine vehicle in a way to formulate a chewable tablet or capsule.

If all the conditions could be addressed, edible vaccines that are in the pipeline have high hopes of getting approved in the near future.

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