MEASLES AND POLIO VACCINATION USING A MICRONEEDLE PATCH TO INCREASE VACCINATION COVERAGE IN THE DEVELOPING WORLD

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MEASLES AND POLIO VACCINATION USING A MICRONEEDLE PATCH TO INCREASE VACCINATION COVERAGE IN THE DEVELOPING WORLD

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To my parents, Mike and Martha Edens.

Without them I wouldn’t be here, literally.

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of variance
CDC	Centers for Disease Control and Prevention
CMC	Carboxymethyl cellulose
DI	De-ionized
DMEM	Dulbecco's Modified Eagle Media
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscular
IU	International unit
IPV	Inactivated polio vaccine
MeV	Measles virus
MN	Microneedle
OD	Optical density
OPV	Oral polio vaccine
P	A probability value to determine statistical significance
PBS	Phosphate-buffered saline
PRNT	Plaque reduction neutralization test
PVA	Poly-vinyl alcohol
SC	Subcutaneous
SD	Standard deviation
$TCID_{50}$	50% tissue culture infectivity dose
WHO	World Health Organization

SUMMARY

Needle and syringe injection has been used for decades to successfully deliver vaccines and other biologics. Despite the widespread adoption of this delivery method it presents a number of difficulties for mass vaccination campaigns in the developing world. Vaccine delivery using a needle and syringe increases the complexity and skill required to deliver vaccines, increases waste disposal costs due to sharps hazards and contributes to the high cost associated with cold chain storage and transport. A technology which could alleviate these issues could increase the reach and lower the cost of mass vaccination campaigns in the third world. A microneedle patch is a delivery vehicle that could meet this goal. Microneedles are micron-scale projections that can penetrate the skin and deliver proteins and other biologics in a targeted manner. They are simple to use, have the potential to reduce or eliminate the risk posed by contaminated sharps and could allow for the removal of some biologics from the cold chain. The goal of this project was to adapt exiting microneedle technologies to the currently approved live-attenuated measles and inactivated polio vaccines. This included stabilizing the vaccine components on both metal and dissolving microneedle patches. Following stabilization, the patches were used *in-vivo* to compare the immune response to traditional needle and syringe delivery.

The goal of the first project was to make the first assessment of the feasibility of using the live-attenuated measles vaccine with metal microneedle patches. Since coating these patches requires the vaccine to be dried, initial studies focused on maintaining viral infectivity in this state. Stock vaccine rapidly lost activity after drying. This loss could be mitigated with the addition of excipients. The sugar trehalose proved to have potent stabilizing activity after both drying and storage at a range of temperatures. Vaccine dried and stored at 4ºC and 25ºC lost less than 1 log unit of activity after 30 days of drying.

Storage at higher temperatures resulted in a much higher level of infectivity loss. Using this formulation, metal microneedles were coated with a full human dose of measles vaccine using a dip-coating system. These patches were inserted into the skin of cotton rats and the immune response was compared to the same dose delivered intramuscularly. Neutralizing antibody titers between the two delivery routes were not statistically different. This showed that microneedle can be made which maintain measles vaccine activity, the antigen successfully reconstitutes in the skin after insertion and induces a potent antibody response.

The second study focused on enhancing the stability of the measles vaccine after drying and storage. This was done using a screen of excipients. The traditional assay typically used to measure measles virus activity is not well-suited to this task so an alternative assay was developed. Utilizing a genetically-modified measles vaccine virus which has been altered to express green fluorescent protein, a simple, quick and scalable test was designed which could rapidly measure the titer of measles virus *in-viro*. Using this assay, more than 40 different excipients which have previously shown stabilizing activity in the literature were examined for their ability to minimize viral infectivity loss after drying and storage. It was discovered that the addition of a sugar and an amino acid resulted in a synergistic response. The combination of these two excipient types performed much better than either type individually. Using short-term screening studies at elevated temperatures it was found that a combination of threonine and sucrose performed the best. This combination was tested to longer times to investigate its full stabilizing potential. After 6 months in storage at 4^{o}C and 25^{o}C, more than 90% of the original activity was maintained. Although storage at higher temperatures resulted in faster degradation, this formulation did show a stark improvement over the trehalose formulation used in the initial experiment. A full log unit of infectivity was not lost until more than 8 weeks of storage at 40^{o}C using the stabilizers chosen in this screening study.

The third study used the advances made to develop a dissolving microneedle patch which could deliver a full dose of measles vaccine into the skin. Dissolving needles had previously been used to deliver a variety of vaccines, but never a live-attenuated viral one. Initial experiments focused on optimizing the various steps required to successfully cast dissolving needle patches reliably and reproducibly. It was found that the stabilizing solution developed in the previous experiment was applicable to the needle casting process. After successful creation, dissolving microneedle patches encapsulating measles vaccine were inserted in the skin of rhesus macaques. The immune response to vaccination was compared to the same full human dose delivered subcutaneously using a syringe injection. The neutralizing antibody titers between the two groups were not statistically different. Furthermore, after a subcutaneous boost of monovalent measles vaccine all animals demonstrated an increase in titer. Dissolving microneedle patches were also stored at a range of temperatures to investigate their stability. Patches stored at 4^{o}C and 25^{o}C showed no activity loss after 2 months of storage. Patches stored at 40^{o}C did show up to 1 log unit of activity loss at the 2 month time point.

Finally, the knowledge gained from the measles experiments was adapted to creating dissolving microneedle patches which could deliver a full human dose of the inactivated polio vaccine. It was found that the same basic patch creation process could be used for IPV. Patches were created and assayed to ensure they contained the correct dose. These patches were inserted into the skin of rhesus macaques and the immune response was compared to a full dose delivered by intramuscular injection. Following low neutralizing titers among both groups after a single dose of IPV, a full dose boost was delivered using the same route. For polio serotypes 1 and 2 the neutralizing response was statistically the same. The response to serotype 3 was inferior in the microneedle patch group. It was determined that the assay used was not specific for the immunogenic region of IPV type 3. After testing with a refined assay it was found that the microneedle

patches only contained about 30% of a full dose. This is a possible explanation for the poor response seen to serotype 3.

These studies show that microneedles were safe, simple to use and can produce a potent immune response in multiple well-studied animal models. We believe that microneedles have the potential to overcome many hurdles that currently exist on the way to polio eradication and measles elimination.

CHAPTER 1 INTRODUCTION

The morbidity and mortality of measles and poliomyelitis has been greatly lessened due to the introduction of cheap and effective vaccines for each disease. Poliomyelitis is approaching complete global eradication and endemic measles elimination is a standing goal for 4 of the 5 WHO regions [4, 5]. Both the live-attenuated measles vaccine and the inactivated polio vaccine are safe and effective. They are, however, both delivered using a traditional needle and syringe. While this method of delivery is by far the most prevalent way to deliver vaccines, it does bring with it a number of drawbacks.

Syringe injection is a medical procedure which necessarily requires trained medical personnel. While this is not a massive burden in first world countries such as the United States, this can greatly diminish the reach of large-scale mass vaccination campaigns in the third world. Trained professionals are scarce in this region of the world and the requirement that they give each dose of vaccine increases the cost and decreases the throughput of these campaigns. Needles also represent a hazard following even a successful delivery. They must be properly disposed of to prevent both malicious and therapeutic reuse. If proper precautions are not taken, used needles can result in the spread of blood-borne diseases such as HIV and hepatitis [6]. Finally, syringe injection requires the transport and storage of both the vaccine and the delivery vehicle increasing bulk and taking up valuable storage space in the developing world. Both the measles and inactivated polio vaccines are sensitive to temperature fluctuations and therefore must be kept refrigerated during the entire transport process [7, 8]. This system of temperature-controlled transport is known as the cold chain. Cold chain requirements vastly increase the financial and logistical challenges of large-scale vaccination campaigns. An integrated system which could maintain vaccine activity even after exposure to higher

temperatures could allow for house to house delivery. This technique has been used with the oral polio vaccine and has proven to be an effective way of maximizing vaccine coverage.

We believe that a new delivery system could solve many of the challenges presented by syringe injection of vaccines. A promising new development in this field has been the creation of microneedles. Microneedles are small, sub-millimeter sized needles which pierce the top layer of the skin and have the ability to deliver biologicals in a targeted manner [9-13]. This system is designed to deposit its payload in the upper dermal layers of the skin, rather than into the muscle or subcutaneous tissue. Previous work has shown that this method of antigen delivery is both safe and effective for a number of different vaccines. Different types of devices within the field of microneedles have also been developed. Solid needles made out of silicon were the first to be designed [14]. A second generation made out of stainless steel were created next. These metal patches were coated with the target vaccine which then re-dissolved into the skin after application. Many different biologics have been delivered to the skin using this microneedle design [1, 15-23]. While effective, this device did possess a slight biohazard sharps risk due to the fact that the needles, though small, remained on the patch after insertion.

Other microneedle systems have been created in which the entire needle is made of a bio-absorbable polymer [2, 24-26]. These patches encapsulate the vaccine within the needle matrix which then dissolves after introduction into the skin. After removal, the needles are no longer present on the patch which eliminates the risk of reuse and decreases the danger to both the patient and the administrator. Both types of microneedle patch were used during the course of this study. The final patch design was based on the dissolving needle concept primarily due to the increased safety profile following insertion and removal.

To study microneedle delivery of both the live-attenuated measles and inactivated polio vaccines, this project covered four major areas:

1. To design a microneedle system which can deliver the measles vaccine into the skin and then determine the immune response to vaccination in a small animal model.
2. To perform an excipient screen to create a temperature-stable formulation of measles vaccine using a novel, high-throughput screen.
3. To develop a dissolving microneedle patch which can maintain measles vaccine activity and then examine the immune response to skin-delivery in a non-human primate model.
4. To develop a dissolving microneedle patch which can deliver the inactivated polio vaccine into the skin and then examine the response to vaccination in a non-human primate model.

This represents the first set of studies which examine vaccination against measles and polio using microneedles. It also examines the response of microneedle vaccination in non-human primates for the first time.

CHAPTER 2 LITERATURE REVIEW

2.1 Measles

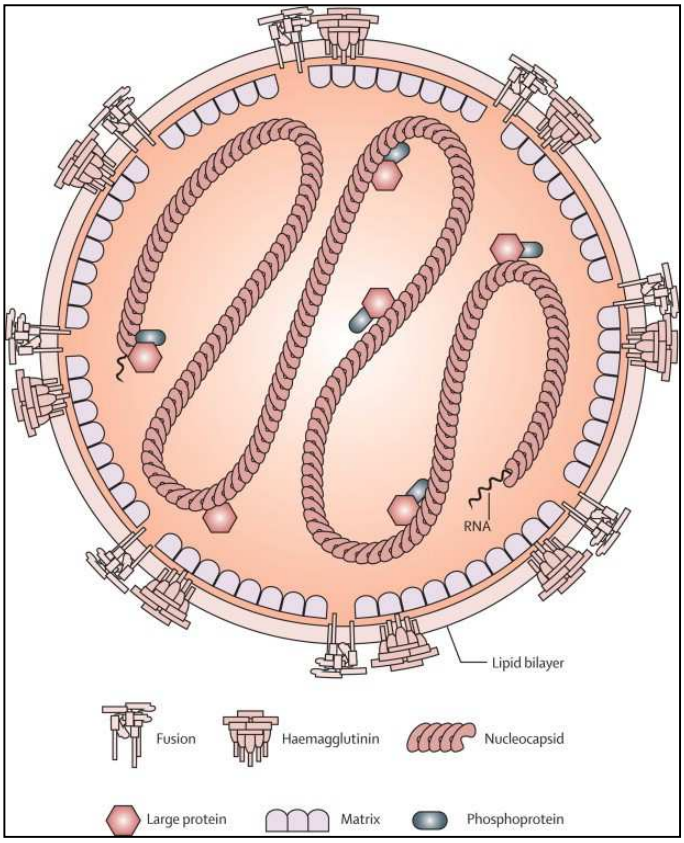

Figure 1: Measles virus structure [3].

2.1.1 Virus and vaccine

Despite the widespread availability of a cheap and effective vaccine, the measles virus remains one of the leading causes of vaccine-preventable morbidity and mortality in the world [27]. The measles virus is an enveloped, spherical, single-stranded RNA virus (Figure 1). It binds to cells using a hemmaglutinin receptor, which also mediates

membrane fusion and viral entry into the target cell. Antibodies to this protein provide the strongest neutralizing response and can confer lifetime protection against the virus [28]. The measles virus is spread primarily through the air. It was originally thought that upon inhalation, the virus began to infect tissues lining the respiratory tract in the throat and lungs [29]. Recent work has shown this may not be the case. The discovery of nectin-4 as a measles virus receptor, along with previously established receptors SLAM (CD-150) and CD-46 suggests an alternate pathway [30]. It is now thought that the virus first infects the myeloid and lymphatic tissues and then crosses into the respiratory epithelium in the later stages of infection [31]. This occurs through viral transmission from CD-150 positive lymphocytes into respiratory epithelial cells using the nectin-4 receptor. This late stage infection is what ultimately results in a patient becoming contagious. Symptoms of a measles infection begin to show roughly 10 days after contact, presenting first with a fever and progressing 4 days later to a characteristic rash. Doctors who work in areas of high measles incidence can quickly diagnose the disease once the rash presents. In areas where measles transmission has been interrupted, the disease is sometimes misdiagnosed because of the relative ambiguity of the initial symptoms [32]. Treatment for a measles infection is mostly palliative, though some antiviral compounds such as ribavirin and interferon alpha have been used in cases of severe measles [33]. The sole preventative measure is vaccination, which is over 90% effective when the recommended dose is delivered [34]. As with many vaccine-preventable diseases, high levels of herd immunity are important for interruption of transmission. The measles virus is extremely infectious and can successfully spread in a population with a herd immunity of more than 95% [35]. As a result, near universal vaccination coverage is required to combat the disease.

2.1.2 Measles elimination efforts

The World Health Organization (WHO) has a standing goal of eliminating endemic measles through the use of mass vaccination campaigns [36]. In 2003, the organization set a goal of reducing measles mortality by 50% from 1999 levels. In that year, more than 850,000 people worldwide died of the disease [37]. Following a concerted effort to increase vaccination coverage in developing countries, measles deaths fell to approximately 164,000 worldwide by 2008 [38]. Further reductions in morbidity and mortality have been less successful. Despite a goal of complete endemic measles elimination in Europe by 2010, infections have actually been on the rise in the region. This is thought to be a result of decreased vaccination coverage among school-age children [39]. Fears about the measles, mumps, and rubella (MMR) combination vaccine have circulated in the press and in a small number of peer reviewed journals [40]. A proposed link to autism has since been widely discredited and the original article making this claim has since been retracted by the journal [41]. Nevertheless, vaccination rates in developed countries such as the United State have fallen to the point that during the first 19 weeks of 2011, there have already been more measles cases than any year since 1996 [42]. Besides negative publicity, there are a number of factors associated with mass vaccination against measles which need to be addressed. Foremost is the requirement of the cold chain for proper vaccine storage. The live-attenuated measles vaccine rapidly loses activity after reconstitution from a lyophilized cake. Multi-dose vials must be fully used within 2 hours or they have to be discarded [43]. This time can be even shorter in regions where ambient temperatures can routinely top 40^{o}C. A key link in the cold chain is the final trip away from major population centers into surrounding town and villages [44]. If the vaccine could be made stable for this period of time, a significant amount of vaccine which would otherwise be disposed of could be properly utilized.

2.1.3 Alternative delivery strategies for measles vaccine

Due to the issues presented by syringe injection, other avenues for delivery of the live-attenuated measles vaccine have been attempted. Delivery into the skin was attempted using a variety of methods. The first attempt utilized a jet injector and attempted to deliver the same dose (1000 $TCID_{50}$) both intradermally and subcutaneously [45]. This experiment found that both delivery methods produced similar results. No dose sparing was seen but all vaccinated children produced potent antibody titers following measles vaccine administration. Other studies utilizing jet injection produced different results. A study in Zaire which attempted to deliver a 1/5 human dose intradermally found lower levels of both antibody production and seroconversion when compared to a full dose delivered subcutaneously using a needle and syringe [46]. The inferior response to low-dose measles vaccine delivered intradermally using a jet injector has been shown in other studies as well [47]. Other investigators have attempted to induce an immune response with even lower doses of measles vaccine. Transcutaneous delivery was attempted with negative results [48]. Here, skin on the arm was tape stripped and a 1/5 dose was applied to the skin surface. The area was occluded with sterile gauze for 24 hours. The exact dose that entered the skin was not determined. Following vaccination, the transcutaneous group did not produce neutralizing antibodies to measles. Further studies utilizing an extremely low-dose of measles vaccine delivered using a needle roller or bifurcated needle also showed insufficient neutralizing titers after delivery [49]. Finally, aerosol delivery of measles vaccine has been recently attempted in the rhesus macaque animal model. The investigators showed that delivery to the lungs was well tolerated and produced a potent humoral and cellular response [50]. This study did however utilize a dose 5x greater than the standard human dose. Studies have not been done comparing similar doses delivered by aerosol device versus a subcutaneous

injection. To date, no previous work has been done examining measles vaccine delivered using a microneedle patch.

2.2 Poliomyelitis

2.2.1 Virus and vaccine

Unlike the measles virus, poliovirus was not a massive killer throughout human history. The fear of polio resided not in a fear of death, but in the images of children who had been crippled as a direct effect of infection. The vast majority of sufferers of polio recover completely, but in a small percentage of cases the virus enters the nervous system [51]. This can lead to permanent paralysis of limbs, and in extreme cases, paralysis of the respiratory system as well. Poliovirus is a small member of the enterovirus family which is known to follow the fecal-oral route of transmission [52]. The initial susceptible cells in a poliovirus infection are not currently known, but virus has been recovered in the gut-associated lymph tissues including the tonsils, Peyer's patch and feces prior to the onset of symptoms [52]. This indicates a latency period when the individual is infectious but displays none of the common outward symptoms of the disease. In the vast majority of infected individuals (95%) the disease progresses asymptomatically [53]. This makes diagnosis virtually impossible until a more serious case develops. The remaining 5% of infections proceed in a similar manner to many gastrointestinal illnesses. Common symptoms include sore throat, fever, diarrhea, headache and a general lack of energy. Diagnosis of polio infection can be confirmed via real-time PCR of an individual's stool. In a minute portion of infected individuals, the virus invades the central nervous system [54]. This can lead to the disease's most well-known complication, paralytic poliomyelitis. This results in flaccid paralysis of the infected region, which can be fatal depending on the location and degree of paralysis.

Two different but similarly effective vaccines currently exist for the prevention of polio infection. A live-attenuated vaccine developed in the 1960's by Albert Sabin rapidly became the predominant vaccine worldwide [55]. This was because of its simple

administration and inexpensive cost to produce. The vaccine is given orally where the attenuated virus can infect the lymph tissues of the gut. This mimics the natural route of infection and creates potent intestinal immunity to subsequent infection [56]. Following vaccination, individuals excrete the attenuated vaccine strain for a number of days. This provides for secondary vaccination opportunities from a single immunized individual [55]. These reasons led to the selection of the oral polio vaccine (OPV) as the leading candidate for use in mass vaccination campaigns. A second vaccine based on formalin inactivation of wild-type polio vaccine was developed by Jonas Salk during the same time period. This vaccine is given using an intramuscular injection and current formulations require multiple doses to achieve potent seroconversion [57]. Even though it was the first polio vaccine created, it was not used in large quantities until large areas of the world were certified polio-free. Along with the drawbacks associated with syringe injection, this vaccine also costs much more to produce than OPV [58].

2.2.2 Polio eradication efforts

The Global Polio Eradication Initiative was established by the World Health Organization in 1989 [5]. It initially called for zero incidence of worldwide polio cases by the year 2000. Progress towards this goal proceeded rapidly during the initial ramp up in global mass vaccinations. Following the delivery of millions of oral polio vaccine administrations, polio transmission was interrupted in the Americas with the last endemic case occurring in Peru in 1991 [59]. Despite some setbacks, numerous milestones have been achieved on the road to eradication of polio. For the first time in recorded human history, India has seen no new cases of polio over a 27 month period. Additionally, wild polio serotype 2 is thought to have been completed eradicated with no new cases detected

in over 2 years. As global eradication draws closer, a shift must occur in the polio vaccine used worldwide.

In a very small portion of individuals who receive OPV, viral recombination occurs, which can result in the attenuated strain reverting to the wild type virus [60]. This disease is known as vaccine-derived poliomyelitis. The risk of reintroducing endemic polio to the Americas via OPV led to a phase-out which culminated in 2000 with the elimination of the oral polio vaccine from the childhood immunization schedule [61]. Replacing it was a vaccine developed by Jonas Salk in the 1950's which consists of wild-type poliovirus that has been inactivated using formalin. This vaccine is given via a traditional syringe injection and does not confer the intestinal immunity provided by the oral vaccine. Because no live virus existed in the vaccine it also does not carry with it the risk of vaccine-derived poliomyelitis. The switch to the inactivated polio vaccine (IPV) is planned to occur worldwide following the complete interruption of endemic polio [61]. This brings with it a host of new complications.

Unlike OPV, IPV is delivered using a traditional needle and syringe [62]. This presents many problems for a mass vaccination setting. Injections require trained personnel to deliver the dose to prevent improper administration. This could vastly increase the cost of delivering IPV on a large scale by increasing the cost of campaign personnel. It could also slow throughput and reach of a vaccination campaign. While administration of OPV can be done on a door to door basis, IPV requires trained medical personnel to ensure proper storage of the vaccine and elimination of the medical sharps waste created after delivery. Medical waste and the safe disposal of sharps presents a problem for large-scale vaccination campaigns [63]. This burden is in addition to the increased risk of needle reuse or misuse created by delivery using syringe injection. An alternative delivery system for IPV which could mitigate many of these issues be beneficial to the continued campaign to eradicate polio worldwide.

2.2.3 Alternative IPV delivery methods

Due to the rapid rise in use of OPV, alternative methods of delivering IPV have not been well studied until more recently. The original examination of IPV intradermal delivery was performed by Jonas Salk using a jet injection system [64]. He found that a fractional (1/6, 1/10) dose delivered using this method produced an inferior immune response when compared to a full dose delivered intramuscularly. Jet injection of fractional IPV was not examined again until more recently. A study in Oman found that a fractional (1/5) dose of IPV induced similar seroconversion rates to IPV type 1 and type 3 when compared to traditional subcutaneous injection [65]. The study did, however, show significantly lower antibody titers following ID administration. This phenomena was also seen in a study performed in Cuba. The investigators found that a fractional (1/5) dose delivered using a jet injector produced both inferior antibody titers and lower seroconversion rates [66]. These results show that there still exists a need for a cheap, effective, and simple method of delivering IPV which can induce a comparable immune response to syringe injection.

2.3 Vaccine delivery

2.3.1 Intradermal delivery

The vast majority of vaccines are currently delivered to muscle or subcutaneous regions of the skin. This is due primarily to ease of access to the location and the simplicity required administering injections to these regions [67]. In recent years, more and more research has concentrated on evaluating other areas of the skin as possible candidates for a more effective vaccine delivery location. The skin has become an active area of interest because of its immunological makeup. Owing to its barrier function for the body, the dermal and epidermal layers of the skin contain numerous resident dendritic cells [68]. These cells function as an immune surveillance system, sampling the extracellular environment and trafficking foreign substances to the draining lymph nodes. Here portions of the offending proteins are presented to immune effector cells which begin the process of the adaptive immune response. In addition, these cells secrete cytokines which can help ramp up the innate immune system, which is the body's first defense against foreign pathogens. These cells, along with macrophages, invade the muscle tissue after an injury such as a syringe injection. However, in the skin, they already exist, waiting for an activating interaction. It is thought that delivering vaccine antigens directly into the intradermal layer of the skin may result in a more rapid and more potent immune response [69]. This could serve to provide dose-sparing activity or might be beneficial in overcoming the maternal antibodies which can prevent seroconversion in young infants [70]. Both of these benefits would be useful in a third world setting.

Historically the most well-known example of an intradermal vaccine was the smallpox vaccine. This was traditionally delivered to the skin via a small, two-pronged device known as a bifurcated needle. A very small amount of liquid vaccine was trapped

between the prongs using simple surface tension. The device was then repeatedly inserted into the skin, delivering the payload [71]. This proved to be a relatively inefficient and unreliable method of delivery. This problem was mitigated to some extent with the creation of jet injectors. A jet injector is a device which aerosolizes a liquid vaccine and then rapidly propels that mist across the barrier layers of the skin [72]. By altering the nozzle dimensions, these devices can direct their payload to the intradermal region. The velocity required to reliably penetrate the outer layer of the skin did create some drawbacks. It was found that a small quantity of biological material could be propelled back into the jet injector, creating an avenue for cross contamination between patients [73-75]. This led to a sharp drop in their use in mass vaccination campaigns in the 1990's. Another popular method for administering substances to the intradermal layer of the skin is known as the Mantoux method [76]. This is widely used in the tuberculin (TB) skin test. In this procedure a high-gage needle is inserted into the skin at a very small angle. The contents are then deposited into the skin forming a distinctive "bleb". This procedure requires substantial training to confer expertise [11]. In addition, the dose is delivered across many layers of skin because of the large size of the needle bore compared to the relative thickness of the skin. A promising technology which overcomes many of these limitations is known as a microneedle.

2.3.2 Microneedles

Microneedles are micron-sized needles made of stainless steel or a variety of polymers which have the ability to penetrate the barrier layers of the skin [13]. The basic premise of microneedle-mediated drug delivery is based on the fact that the barrier layer of the skin only comprises the upper most layers of the skin. This region is roughly 20 microns thick [77]. Studies have shown that once this layer has been breached, drug

delivery into the skin increases by orders of magnitude [10]. This makes it unnecessary to penetrate deeply into the skin to efficiently deliver a payload. A depth of between 100 µm and 1 mm is the ideal location for the distribution of drugs targeted to the intradermal layer of the skin. Microneedles patches have been created out of both metal and dissolvable polymer.

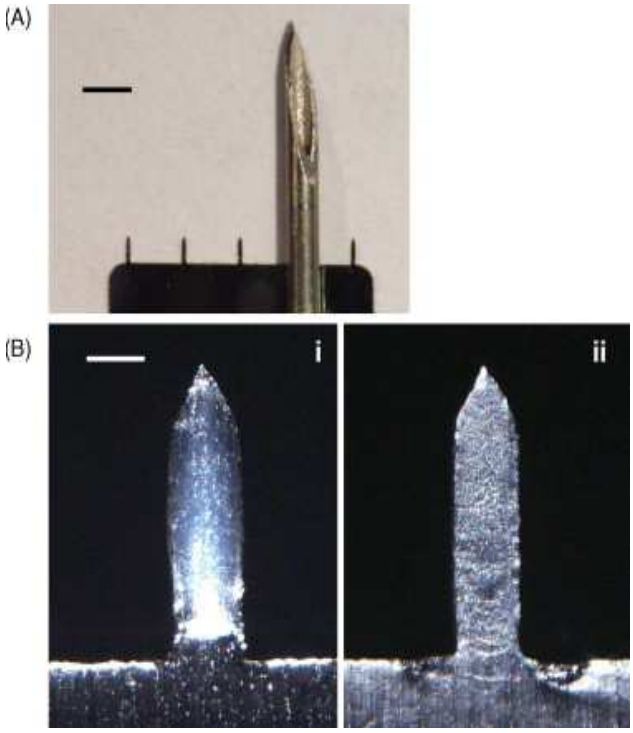

Figure 2: Metal microneedles at high magnification [1].A: Metal microneedle patch compared to a traditional hypodermic needle. B-i: Single metal microneedle coated with a vaccine solution. B-ii: Single metal microneedle after successful insertion into the skin.

2.3.2.1 Solid, metal microneedles

A number of different drugs and antigens have been successfully delivered via microneedle systems. An early system composed of solid, silicon needles created by adapting common microelectronics processes showed that after insertion of the needle array the permeability of the skin increased by four orders of magnitude to the small

molecule calcein [10]. Subsequent research confirmed this result with larger molecules such as human growth hormone and bovine serum albumin [25, 78, 79]. This work led to the idea of skin vaccination using the microneedle system.

Vaccines typically consist of either whole virus particles or selective antigenic proteins. These molecules or supramolecular assemblies are much too large to passively diffuse through the skin, so the microneedle system seemed like a logical vector for delivery. It was shown that after coating the inactivated influenza vaccine on the surface of a metal needle array, mice could be immunized by simply inserting the needles into bare skin [80]. After vaccination it was shown that the groups receiving the microneedle patch had superior protection against a subsequent challenge with live influenza virus. Following challenge, microneedle group mice had lower levels of lung viral titer when compared with mice receiving the same dose via a traditional intramuscular injection [1]. It is theorized that this improved response is due in part to increased levels of immune system cells secreted IFN-y and IL-4, two key mediators of both the innate and adaptive immune responses [1]. This supports the theory that the skin is a potent reservoir of primed immune surveillance cells and a superior target for vaccine delivery. Further studies examined the ability of microneedles to confer dose-sparing.

Microneedle delivery of an influenza virus-like particle vaccine showed superior protective ability at lower antigen doses compared to the same vaccine delivered via intramuscular injection. At 1/3 of the full antigen dose, the microneedle groups had full protection against a lethal challenge while the intramuscular group had only 40% of the animal survive the challenge [21]. Other vaccines have also been successfully delivered using solid, metal microneedles such as hepatitis C and Bacillus anthracis [17, 81-83]. Further development of the microneedle technology itself has been undertaken.

2.3.2.2 Dissolving microneedles

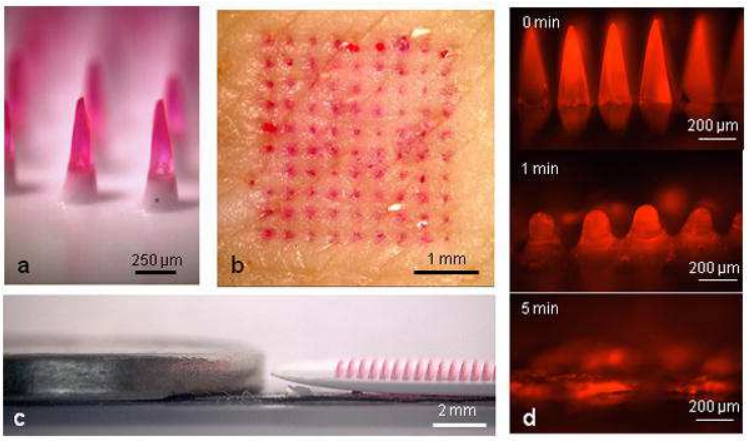

Figure 3: Dissolving microneedle patches before and after insertion [2].
A: Dissolving microneedle patch at high magnification. B: Pig skin after insertion and dissolution of a microneedle patch containing a dye. C: Dissolving microneedle patch compared to a US nickel coin. D: Dissolving microneedle patch before and after insertion.

Polymer needles have been created which have the ability to encapsulate various antigens and disperse them once they have entered the skin. These needles have been made with a variety of polymers such as polylactic-co-glycolic acid (PLGA), polyvinylpyrrolidone (PVP) and maltose [2, 26, 84]. Each of these polymers has unique characteristics including dissolution time, strength and the ability to impact the stability of the encapsulated antigen. Polymer needles have some features which make them an ideal candidate for vaccination campaigns in the third world. Following delivery of the payload into the skin, the needle patch is rendered harmless by the dissolution of the polymer needles. Unlike stainless steel needles, after use a polymer-based needle system has none of the dangers commonly associated with sharps. The patches are water soluble and can be easily and cheaply disposed.

Dissolving microneedles have also been previously used to successfully deliver vaccines [22, 85-87]. In one study influenza vaccine was encapsulated in a PVP microneedle patch and subsequently deposited into the skin [2]. This produced a robust

immune response that fully protected mice against a lethal challenge. It was found that the microneedle patch induced a more potent cellular immune response that resulted in decreased lung viral titers following challenge. Various geometries and needle designs have also been investigated. A dissolving needle with an arrowhead designed was created to facility rapid delivery of the microneedle into the skin [84]. This unique geometry allowed for nearly complete microneedle delivery in seconds. Rapid needle deposition in the skin combined with a slow-dissolving polymeric needle has the potential to achieve controlled release-style kinetics.

2.4 Vaccine Stability

2.4.1 Vaccine wastage

Product wastage presents a major problem during mass-vaccination campaigns. It is estimated that up to 44% of the deliverable vaccine does not make it to a patient due to spoilage caused by temperature fluctuations or improper handling [88]. This loss adds to the cost of the campaign and lowers the eventual number of doses delivered. The current attempts to prevent vaccine spoilage revolve primarily around maintenance of the cold chain and increasing stability during storage. Virtually all currently licensed vaccines have a recommended storage temperature of under 8^{o}C [89]. This becomes increasingly expensive as the vaccine is moved from first to third world countries. Transport, shipping and storage at the site of vaccination constitutes a major portion of the cost to deliver vaccines on a mass-scale [44]. Minimizing the need for low-temperature storage could greatly reduce this cost. However, simply increasing the stability at an elevated temperature for a transient period of time could lessen vaccine spoilage during the crucial period when the vaccine moves from the city center out into the community for delivery.

2.4.2 Measles vaccine stability

Since the product consists of an intact, infectious virus, the stabilization of the measles vaccine has challenges not present with inactivated or subunit vaccines. Maintaining both the stability of the viral unit as well as the encapsulated RNA is crucial to retaining infectivity [90]. Significant work has been done in an attempt to solve this complex problem. Technology such as lyophilization and the addition of excipients to the final vaccine solution prior to packaging have been crucial in getting the stability of the

vaccine to its current state [91]. Further work focused independently on the two crucial times when vaccine wastage can occur.

First, measles infectivity during storage has been examined by a number of groups. The method by which the vaccine is dried before storage has been a crucial area of research. Spray drying has been shown to create a dry cake which retains significant infectivity after storage at elevated temperatures [92]. The researchers showed that while standard lyophilized measles vaccine lost 1 log($TCID_{50}$) after only a week at 37°C, their spray dried formulation did not lose this amount until 8 weeks of storage. It was hypothesized by the authors that spray-drying resulted in a lower final water content resulting in a more stable dry preparation. This work also identified a number of possible stabilizing excipients such as human serum albumin, various amino acids and cations. Other groups have examined drying using a novel process called CAN-BD which utilizes super-critical CO_2 to remove water content [93]. When this process was applied to measles vaccine it was shown to maintain significant stability after 1 week at 37^{o}C [94]. It was also found the type of sugar added to the vaccine solution prior to processing had the potential to alter both the final water content and eventual stability after storage.

Work has also been done to enhance the stability of the measles vaccine after reconstitution. The WHO currently recommends that measles vaccine must be delivered within 2 hours of reconstitution to ensure the proper dose is delivered [43]. This can result in vaccine wastage since the 10-dose vial is widely used during measles vaccination campaigns [95]. A major advance in the ability to rapidly screen measles formulation was the development of a measles vaccine virus which contained the genetic material for green fluorescent protein (eGFP-MeV) [96, 97]. Using this novel virus, investigators were able to rapidly screen a large number (>11,000) of compounds to assess their ability to minimize the loss in reconstituted measles vaccine [98]. They, like other researchers, found that a combination of sugars, amino acids and buffering components could greatly decrease infectivity loss at elevated temperatures. Their best

liquid formulation was able to withstand 8 hours at 40°C with less than 1 log($TCID_{50}$) loss in infectivity.

While promising, all of these efforts fall short of developing a measles vaccine preparation that completely mitigates infectivity loss at elevated temperatures. Further work is required to both increase the stability of the measles vaccine and to optimize transport and storage to the point where product wastage is no longer a pervasive problem.

2.5 Motivations for Research

Despite efforts to simplify delivery of the measles and polio vaccines, hurdles still exist. The injectable delivery systems currently used to administer both of these vaccines increase the cost of campaigns through a number of ways. The requirement for trained medical personnel limits the reach of large-scale campaigns while also lowering the speed at which doses can be delivered. The sharps waste created by the used needles is a significant burden that increases the cost and logistical complexity of vaccine campaigns. Finally, errors in reconstitution, delivery and vaccine storage result in the loss of a significant quantity of vaccine stock. Microneedles are a new delivery platform that has the potential to alleviate many of these concerns. Adapting the measles and inactivated polio vaccine for skin delivery using microneedles would represent a significant advance, removing many of the hurdles remaining in the eradication of both of these diseases.

CHAPTER 3 SPECIFIC AIMS AND HYPOTHESES

Aim 1: Determine the immune response to skin-vaccination of measles in a small animal model. *The live-attenuated measles vaccine can be adapted to existing microneedle patch systems and delivered to the skin of a cotton rat. We hypothesize that vaccination will result in a potent immune response comparable to a traditional injection.* The goal of this aim was to create a microneedle patch that had the ability to deliver a full human dose of the measles vaccine to the skin of a small animal. These patches were then used to vaccinate cotton rats.

Aim 2: Enhance stability of dried live-attenuated measles vaccine using an excipient screen. *We hypothesize that using a high-throughput infectivity screen, the stability of measles virus after drying can be significantly enhanced by the addition of excipients.* This aim focused on the creation of a simple, high-throughput assay to quickly asses the stabilizing potential of a number of pharmaceutical excipients. This assay resulted in the discovery that the amino acid threonine and the sugar sucrose, in combination, could mitigate loss after drying and storage for 6 months at room temperature.

Aim 3: Determine the immune response in the rhesus macaque to measles vaccination using a microneedle patch. *We hypothesize that delivery of measles vaccine to the skin using a dissolving microneedle patch will produce a potent immune response in the rhesus macaque.* The rhesus macaque is a commonly used model for testing of measles vaccines. It is often used as a key predictor of the immune response to vaccination in humans. Here, dissolving microneedle patches were created that contained a full dose of measles vaccine. These patches were used to deliver measles vaccine to the skin.

Aim 4: Determine the immune response in the rhesus macaque to inactivated polio vaccination using a microneedle patch. *We hypothesize that delivery of IPV to the skin using a dissolving microneedle patch will produce a potent immune response in the rhesus macaque*. The rhesus macaque is a commonly used model for testing polio vaccines. Here, dissolving microneedle patches were created that contained a full human dose of the inactivated polio vaccine. These patches were used to deliver IPV to the skin of rhesus macaques.

CHAPTER 4 Measles Vaccination in cotton rats using a Microneedle Patch

4.1 Introduction

Despite the widespread availability of an inexpensive and effective vaccine, measles virus is one of the leading causes of vaccine-preventable morbidity and mortality among children worldwide [27]. High levels of coverage are necessary for interruption of measles transmission. Measles vaccination programs have dramatically reduced the incidence of disease in both developed and developing countries [4, 99]. More than 4.5 million measles deaths have been prevented as of 2008 through implementation of the vaccination strategies developed by WHO and UNICEF. Global mortality has declined by 74% from an estimated 733,000 deaths in 2000 to 139,300 in 2010 [100]. Measles elimination, defined as the absence of endemic transmission of virus, has been achieved and sustained in the WHO Region of the Americas since 2002, and four of the five other WHO regions, European, Eastern Mediterranean and Western Pacific, have targeted measles for elimination by 2020 or earlier [101].

The measles vaccine is currently delivered by subcutaneous injection using a needle and syringe. This delivery method creates the requirement for specifically trained healthcare personnel to administer each vaccine dose, typically at centralized locations. In contrast, the global campaign to eradicate polio has been possible, in part, because of the simplicity of delivering the oral polio vaccine, which can be administered by

minimally trained personnel. Decreasing the logistical challenges associated with delivery of measles vaccine could increase vaccination coverage and reduce vaccination campaign costs.

Hypodermic injections create hazardous medical waste which must be safely destroyed. Preventing needle theft and reuse through responsible disposal methods adds significant costs to vaccination campaigns. For example, a relatively small measles vaccination campaign in the Philippines generated over 130,000 kg of sharps waste [102]. Another logistical challenge with the standard vaccination scheme is the requirement of a cold chain for vaccine storage and transport. After reconstitution, multi-dose vials must be used within 2 h or discarded [43]. This leads to vaccine wastage and increased program costs. A delivery system that eliminates the need for reconstitution and reduces or eliminates the need for cold storage and transport could enable more efficient use of measles vaccine and decrease the cost per delivered dose.

Measles vaccination using a microneedle patch may be able to address some of the limitations of conventional hypodermic injection and thereby facilitate measles mortality reduction and elimination programs. Microneedles are micron-sized needles made of metal or polymer that are designed to achieve the efficacy of hypodermic injection with the simplicity of a patch [12, 103]. Microneedles offer the possibility of eliminating or mitigating many of the logistical challenges associated with the current vaccination strategy, including reduced cost, simplified transport and storage, and increased safety. The microneedles used in this study remain on the patch after it is removed and could present a small risk for disease transmission as a sharps hazard. However, microneedles can also be fabricated from dissolving polymers in which case no

potentially infectious, sharps waste would be generated [2, 66]. Microneedles require a small amount of force to penetrate the skin, and once the barrier layer has been penetrated, the vaccine is rapidly released into the skin. The microscopic wound created by the patch is superficial and heals quickly [104]. With the correct excipient conditions other vaccines have been stabilized onto a microneedle patch [105, 106]. If this high level of temperature stability could be extended to the live-attenuated measles vaccine, the cost and logistical issues associated with vaccine transport and storage could be decreased significantly. Finally, the small size of the microneedle patch would limit sharps waste following large-scale vaccination campaigns. This would decrease transport costs while also minimizing the potential for reuse.

Measles vaccine has been previously delivered to the skin using a variety of methods including the Mantoux method [107] and jet injection [46, 49]. While some studies have shown improvements after intradermal delivery [108], others found lower neutralizing antibody titers when compared with traditional delivery routes [48, 109]. The inferior serologic response to intradermal vaccination seen in these studies could result from the low dose of measles vaccine delivered (as low as 5% of the standard dose). Neither study investigated the response to a standard subcutaneous dose (at least 10^3 $TCID_{50}$) delivered intradermally.

Stabilization of the measles vaccine in a dry state has also been previously examined. Viral infectivity loss after drying has been mitigated through both excipient selection and drying process optimization [92, 110]. Some of these dry powder vaccines were shown to be efficacious after delivery to the respiratory tract of non-human primates [5, 94, 110, 111]. However, these stabilization methods used drying processes such as

spray drying and lyophilization, which are not easily compatible with microneedle fabrication and coating.

Microneedles have been used successfully as an experimental delivery system for a number of different vaccines including live virus and bacteria, inactivated virus, virus-like particles, protein sub-unit, DNA and live viral vaccines against influenza and a number of other diseases [2, 13, 17, 20, 23, 80-83, 103, 112-116]. However, measles vaccine has never been studied before using microneedles. In this study, we first examined the ability of excipients to stabilize the live-attenuated measles vaccine during fabrication and storage. We then compared the immune response to vaccination using a microneedle patch to conventional subcutaneous injection in the cotton rat model.

4.2 Materials and methods

4.2.1 Preparation of live-attenuated measles vaccine

The measles vaccine strain, Edmonston-Zagreb, was obtained from the collection at the Centers for Disease Control and Prevention and this strain is used in many WHO pre-qualified measles vaccines. To achieve the high titers need for coating of the microneedles, the vaccine virus was propagated in Vero cells maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) and 2% fetal bovine serum (FBS,Gibco). Infected cells were harvested when the cytopathic effect was maximal; the cell suspension was freeze-thawed once before low-speed centrifugation to remove cellular debris [117]. The viral titer (50% tissue culture infective dose, $TCID_{50}$) was measured by end-point titration. The virus was then aliquoted and stored at -70^{o}C until use. For the end-point dilution assay, 10-fold dilutions of the viral stock were prepared in DMEM with 2% fetal bovine serum and used to infect multiple wells of Vero cell

monolayers in 24-well tissue culture plates. Plates were incubated for 7 days and scored visually for the presence or absence of viral cytopathic effect. The $TCID_{50}$ was then calculated using the Karber method [118].

4.2.2 Vaccine stability studies

Live measles vaccine virus with an initial viral titer of 10^6 $TCID_{50}$/mL was mixed with excipients at specific concentrations. Excipients used in this study included carboxymethylcellulose (CMC, CarboMer, San Diego, CA), trehalose (Sigma-Aldrich, St. Louis, MO), fish gelatin (Sigma-Aldrich), myo-inositol (Sigma-Aldrich), and Lutrol F68 (BASF, Mt. Olive, NJ). A 2 µl drop of each resulting solution was applied to a sterile chip of stainless steel measuring 3 mm by 4 mm to simulate the surface of a stainless steel microneedle. We used this simple method of coating to screen formulations since coating actual microneedles is more time consuming. Each 2 µl drop contained a mixture of 50% measles virus solution and 50% excipient solution. The chips were allowed to dry at room temperature (22 °C) in a Class II biosafety cabinet or an incubator (37 °C). In some cases, the air was de-humidified during storage by placing the stainless steel chip inside of a 50 mL plastic tube containing desiccant (Drierite, Sigma-Aldrich) and wrapped in Parafilm (Sigma-Aldrich). After specified storage times, the vaccine coated onto the chips were reconstituted in 1 mL DMEM and viral titers were measured in Vero cells as described above.

4.2.3 Microneedle fabrication and coating

Stainless steel microneedles were fabricated by first defining the microneedle shape lithographically and then etching the microneedles in a chemical bath. This produced patches each containing a single row of five microneedles that were 750 µm long and measured 200 µm by 50 µm at the base (Figure 4). We chose this microneedle patch design because the measles vaccine dose is sufficiently small that a full dose can be coated onto just five microneedles and because coated microneedles of similar design have been successfully used for vaccination and drug delivery in a number of published studies [12]. We chose a microneedle length of 750 µm because it matches the thickness of rat dorsal skin, which is generally reported in the range of 700 – 1000 µm [119, 120]. Thus, we believe vaccine coated on the microneedles was deposited along the needle track in the epidermis and dermis; it is possible that a small fraction of the vaccine was delivered to the subcutis in the case of thin skin.

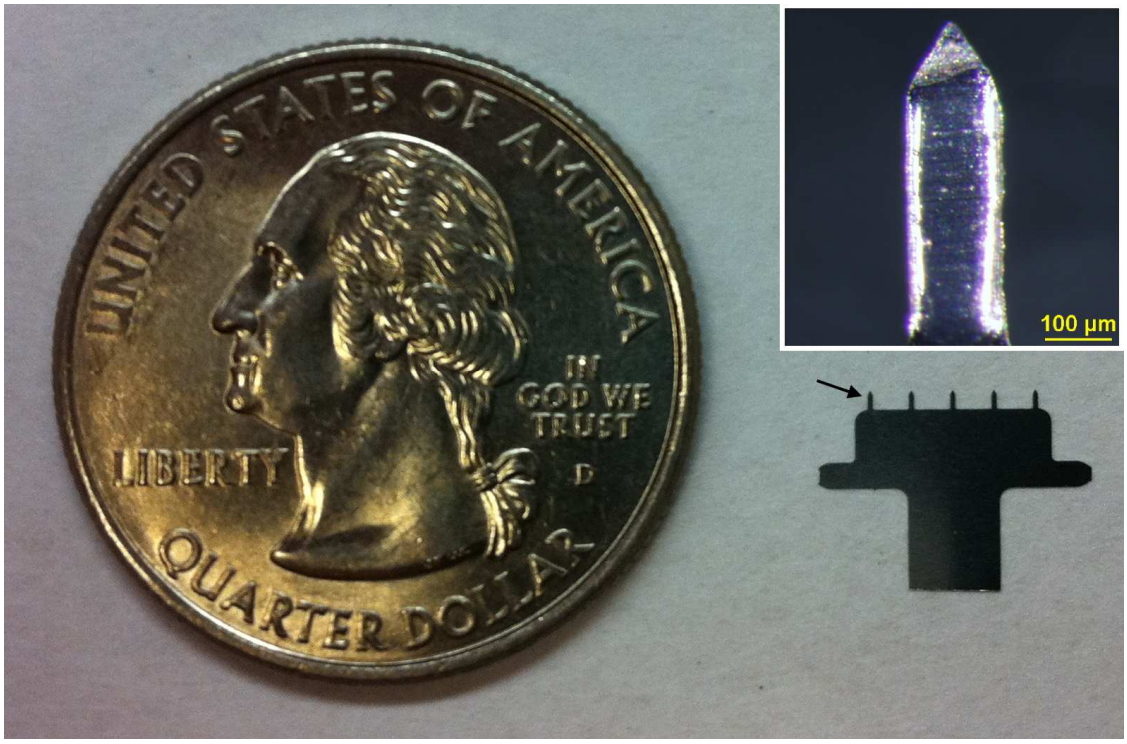

Figure 4: A five-needle microneedle array next to a U.S. quarter coin with a diameter of 24 mm. The arrow points at one of the microneedles mounted on the holder. Inset: A single microneedle coated with measles vaccine in a trehalose-based coating formulation.

Based on a method described previously [9], the microneedles were coated with live-attenuated measles vaccine by dipping the microneedles 6 times into a coating a solution containing 7.5% w/v trehalose, 1% w/v CMC, 0.5% w/v Lutrol F68 and $10^{5.6}$ $TCID_{50}$/mL measles vaccine in sterile DMEM for "full-dose" microneedles. "Low-dose" microneedles used a coating solution containing $10^{4.9}$ $TCID_{50}$/mL measles vaccine. In this way, a five-microneedle array was coated with 1000 $TCID_{50}$ after full-dose coating and with 200 $TCID_{50}$ after low-dose coating, as determined by dissolving the coatings from microneedles and measuring viral titers in Vero cells. The microneedles were stored in a sterile container sealed with Parafilm wrap at room temperature in a Class II biosafety cabinet for one day before use.

4.2.4 Immunization studies

The immunogenicity of measles vaccination using microneedles was tested in cotton rats (*Sigmodon hispidus*). Cotton rats were divided into seven groups of 5 animals each. Groups were assigned as follows: (1) full-dose and (2) low-dose vaccination using microneedles (MN); (3) full-dose and (4) low-dose vaccination by subcutaneous injection (SC); (5) full-dose and (6) low-dose vaccination by subcutaneous injection of vaccine eluted from microneedles (SC*); and (7) sham vaccination using sterile, uncoated microneedles.

Female, 6-week-old cotton rats were allowed at least 5 days to acclimate to the animal facility before vaccination. The day before vaccination, blood was collected from the rats via cheek bleeding. Animals were anesthetized using a ketamine/xylazine mixture during vaccination and blood collection [95]. In the microneedle vaccination groups (MN), the hair on the back of each rat was removed using electric shears followed by application of a depilatory cream (Nair, Princeton, NJ).

In the microneedle groups (MN), a microneedle array coated with the desired measles vaccine dose was pressed into the skin of the hairless region on the back of the animal. Each array was left in the skin for 10 min to ensure complete vaccine dissolution from the microneedles for delivery into the skin. Rats in the sham group were treated identically, except that no vaccine coating was applied to the microneedles. In the subcutaneous vaccination groups (SC), the stock measles vaccine was diluted using sterile phosphate-buffered saline (PBS) so that the desired dose was contained in 100 µl,

which was then injected subcutaneously using a 25-gauge hypodermic needle on the back of the animal. For the reconstituted subcutaneous groups (SC*), ten microneedle arrays coated with the desired measles vaccine dose were mixed with 1 ml sterile PBS in a 10 mL centrifuge tube and vortexed for 2 min to completely dissolve the vaccine. A 25-gauge hypodermic needle was used to withdraw 100 uL of this solution and inject it SC.

At the time of vaccination, no adverse effects were noted following any of the vaccination methods. A small grid of punctures at the site of microneedle application was faintly visible in the skin when the device was removed, but no bleeding was observed. Post-vaccination, the microneedle injection sites were examined daily by animal care staff and no adverse effects were seen. The small puncture grids were no longer visible 2-3 days post vaccination and no swelling, discharge or other abnormalities were observed at any time point. The hair that had been removed began growing back within one week and had returned to normal in all rats by the end of the investigation.

At multiple time points after vaccination, approximately 500 µL of blood was collected from each rat by performing a cheek bleed [95]. After 200 days, the animals were anesthetized using a ketamine/xylezine mixture and euthanized by injecting 1 mL of Beuthanasia-D (Intervet, Summit, NJ) into the heart. The protocol for the cotton rat experiments was approved by the Animal Care and Use Committees of the CDC and the Georgia Institute of Technology.

4.2.5 Neutralizing antibody measurements

Measles neutralizing antibody titers in serum samples obtained from the cotton rats were determined by the standard plaque reduction neutralization assay [121]. For these studies, two-fold dilutions of serum were tested beginning at a dilution of 1:4.

4.2.6 Statistics

All statistics were calculated using Prism software version 5.04 (Graphpad, La Jolla, CA). Comparisons between individual samples were done using an unpaired t-test with a significance cutoff of $p < 0.05$. For comparisons between 3 or more samples, a two-way ANOVA with a Bonferroni post-test was used.

4.3 Results

4.3.1 Vaccine stabilization

One of the advantages of vaccination using a microneedle patch is that the vaccine is stored in a dry state and is administered to the patient without reconstitution. When coating microneedles, the thin coating film dries within seconds, leaving no time for transfer to a lyophilization chamber. Therefore, it was necessary to optimize formulation during this rapid drying step to maintain vaccine viability during patch fabrication.

In a first assessment of virus stability during drying, vaccine stock solution with an initial titer of $10^{5.6}$ $TCID_{50}$/ml infectivity was dried onto microneedles without additives; this resulted in a greater than 10-fold reduction in virus titer ($p<0.02$; Figure 5A). Then, excipients were added to the solution to make thick, uniform coatings on the

microneedles. Carboxymethylcullose (CMC) was used to increase the solution's viscosity and surfactant (Lutrol F68) to lower surface tension. These additives (CMC and Lutrol F68) in the coating solution destabilized the measles virus even further and reduced the $TCID_{50}$ of eluted virus by more than 100 fold compared to the stock solution ($p<0.001$; Figure 5A).

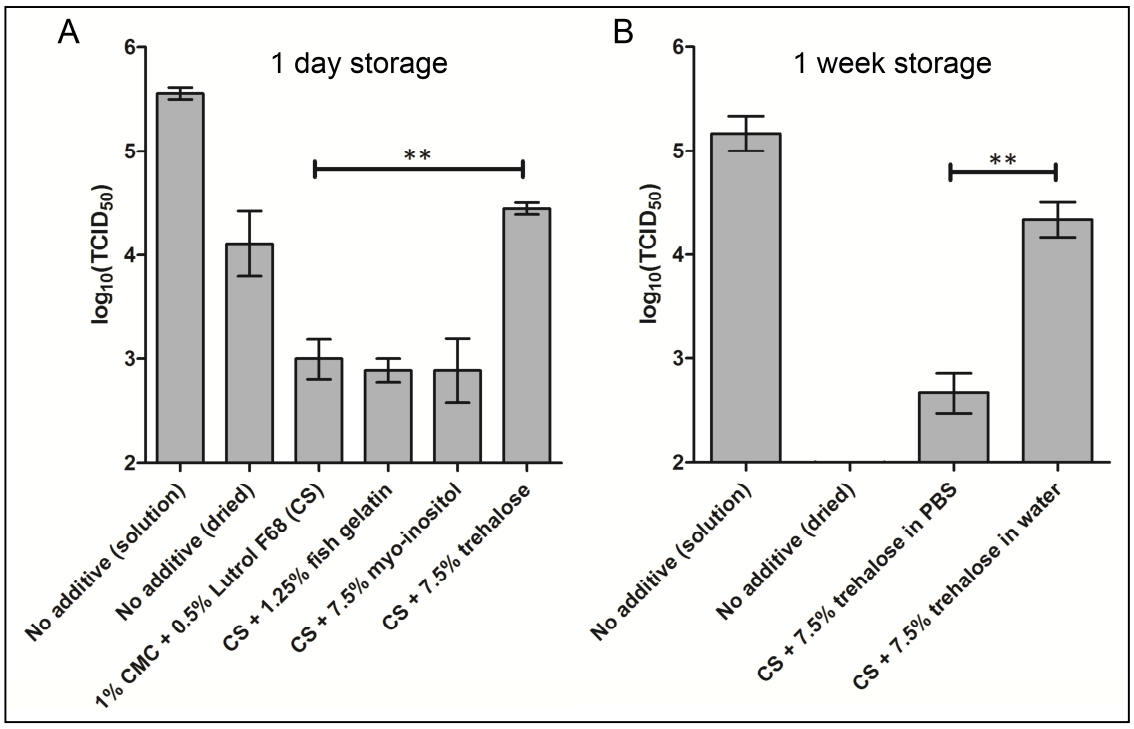

Figure 5: Effect of coating formulation on measles virus infectivity after drying onto microneedle surfaces. Coatings were dried and then stored at room temperature (~22ºC) and relative humidity (~50%) for (A) 24 h or (B) 1 week. The coating solution (CS) contained 2% CMC and 1% Lutrol F68. In (A), all coating solutions were prepared using phosphate-buffered saline (PBS). In (B), coating solutions were prepared with and without PBS, as indicated on the graph. Asterisk (**) indicates a significant difference ($p<0.005$). Data points represent the average ± standard error of the mean (SEM) from n = 3 independently tested samples.

To minimize loss of viral infectivity, a collection of excipients previously shown to stabilize lyophilized measles vaccines and approved for use in humans were evaluated

[94]. Fish gelatin and the sugar, myo-inositol, provided no significant improvement of infectivity compared to the use of coating solution without these additives ($p > 0.5$; Figure 5A). Addition of the sugar trehalose at a concentration of 7.5%, however, significantly reduced loss of infectivity compared to the coating solution without additives ($p<0.005$) and the titer of the eluted vaccine was within approximately 1 $\log_{10}(TCID_{50})$ of the vaccine stock solution (Figure 5A). Unfortunately, after one week of storage at room temperature using this formulation (coating solution and 7.5% trehalose in PBS), virus infectivity decreased by more than 2 $\log_{10}(TCID_{50})$ ($p<0.03$; Figure 5B).

The coating solutions used so far were all prepared in PBS. To address possible osmotic effects, a coating solution was prepared with trehalose but without PBS. Use of this coating solution significantly increased stability relative to the saline-containing solution ($p<0.005$) and resulted in a loss of just 0.8 $\log_{10}(TCID_{50})$ after drying and storage at room temperature and ambient humidity for 1 week (Figure 5B). This formulation containing trehalose and lacking PBS was used for all remaining experiments in this study.

The current WHO standard for stability of lyophilized measles vaccine is less than 1 $\log_{10}(TCID_{50})$ unit of infectivity loss after 30 days at 25°C or 1 week at 37°C [122]. The next set of experiments was designed to determine if measles virus coated onto microneedles could meet the WHO standard. These tests were performed with the addition of desiccant to control for humidity. First, a control sample of virus diluted in standard DMEM was seen to rapidly lose activity at 25°C and no infectivity was reported by the 1 week time point. Following the addition of the stabilizing solution, samples dried at room temperature exhibited a viral titer loss of 0.57 $\log_{10}(TCID_{50})$ units of infectivity

after 30 days of drying at 25ºC. At 37ºC, the loss in viability after one week was 0.85 $\log_{10}(TCID_{50})$, although longer exposure resulted in additional loss of viability up to 2.91 $\log_{10}(TCID_{50})$ (Figure 6). Therefore, the optimized coating formulation developed in this study was sufficient to meet one of the WHO standards for measles vaccine stability, although further improvements in stability would be desirable and are currently being evaluated.

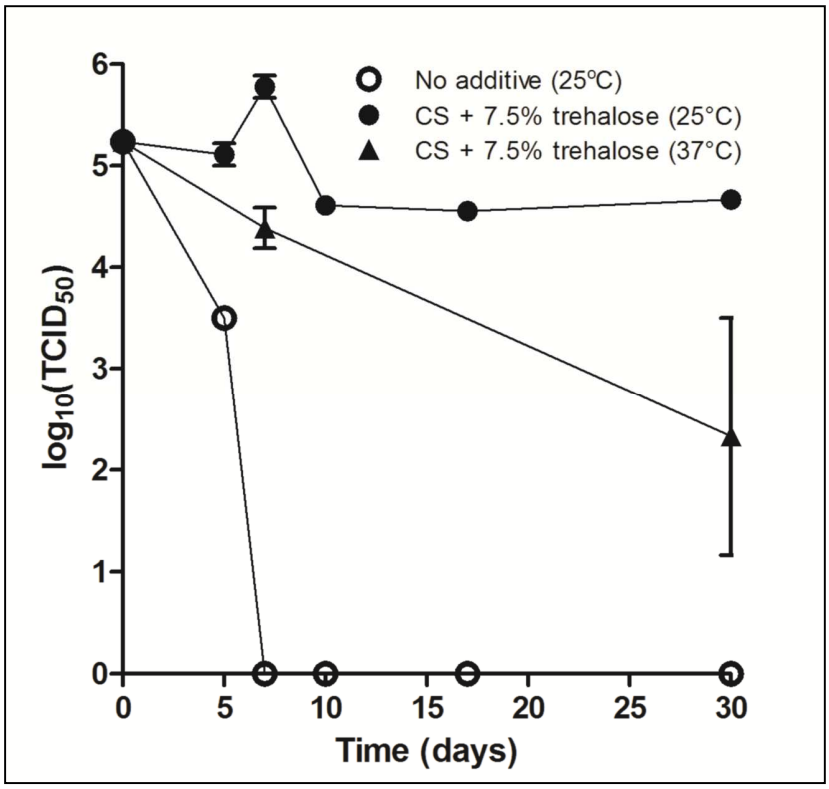

Figure 6: Loss of measles virus infectivity over time as a function of formulation and storage temperature. Coatings were dried at room temperature and humidity and then stored in sealed pouches with desiccant for 30 days at 25ºC or 37ºC. Formulations included no additives (i.e., no coating solution or trehalose) and coating solution (CS) with 7.5% trehalose. Data points represent the average ± SEM (N = 3). The data points for the 10, 17 and 30 day time points for the 25ºC samples had a SEM of 0 because all replicates had the same values.

4.3.2 Immunization studies

The immunogenicity of measles vaccination using a microneedle-based vaccine was evaluated in the cotton rat. This animal model was chosen because it is a well-studied, small-animal model for measles viral infection and is commonly used in measles vaccination experiments [123]. The goal of the study was to compare the immunogenicity of measles vaccination using a microneedle patch with the immunogenicity of the same vaccine dose delivered by subcutaneous injection. One group (n=5) received subcutaneous injection (SC) as a positive control to represent the current approach used in human vaccination. A second subcutaneous group received an injection containing measles vaccine that had been dried on a microneedle patch and reconstituted in PBS prior to subcutaneous injection (SC*). This group was included to account for the possible loss of immunogenicity of the measles vaccine during fabrication of the microneedle patches independent of the route of administration. The microneedle group (MN) received a single microneedle patch applied to the skin of the back. Groups were immunized either with a standard human dose of measles vaccine (1000 $TCID_{50}$) or a reduced dose (20% of standard dose, 200 $TCID_{50}$) to investigate possible dose-sparing associated with vaccination in the skin.

All vaccinated animals demonstrated a detectable antibody response after day 20. Additionally, in all vaccinated groups (MN, SC, SC*) at both doses, the time course of the neutralizing antibody response was similar (Figure 7). At the day 10 time point, both MN groups had statistically higher titers than the subcutaneous controls at the same dose ($p<0.005$). This suggests that microneedle delivery of the measles vaccine may generate a more rapid antibody response than subcutaneous injection, but this observation needs to

be tested further. Peak neutralizing antibody titers occurred in all groups at approximately 30 days post vaccination with no statistically significant differences in titer observed (Table 1, $p>0.05$). The peak titers achieved in all vaccinated groups were statistically indistinguishable among the standard dose groups (Table 1, Figure 7A, $p>0.05$) and among the reduced dose groups (Table 1, Figure 7B, $p>0.05$). All vaccinated groups achieved peak titers significantly greater than the sham control group ($p<0.005$), which had no detectable neutralization activity.

Blood was also collected 200 days after vaccination to examine long-term antibody responses. Both the MN (full dose) and SC (full dose) groups showed a statistically significant decrease in titer from the peak over time ($p<0.05$). It is notable; however, that neutralizing antibodies were detected in all vaccinated groups more than 6 months after immunization. Peak titers among the MN and SC groups vaccinated with the standard dose were approximately three-fold greater than those among MN, SC and SC* groups vaccinated with the reduced dose (Table 1, $p<0.005$).

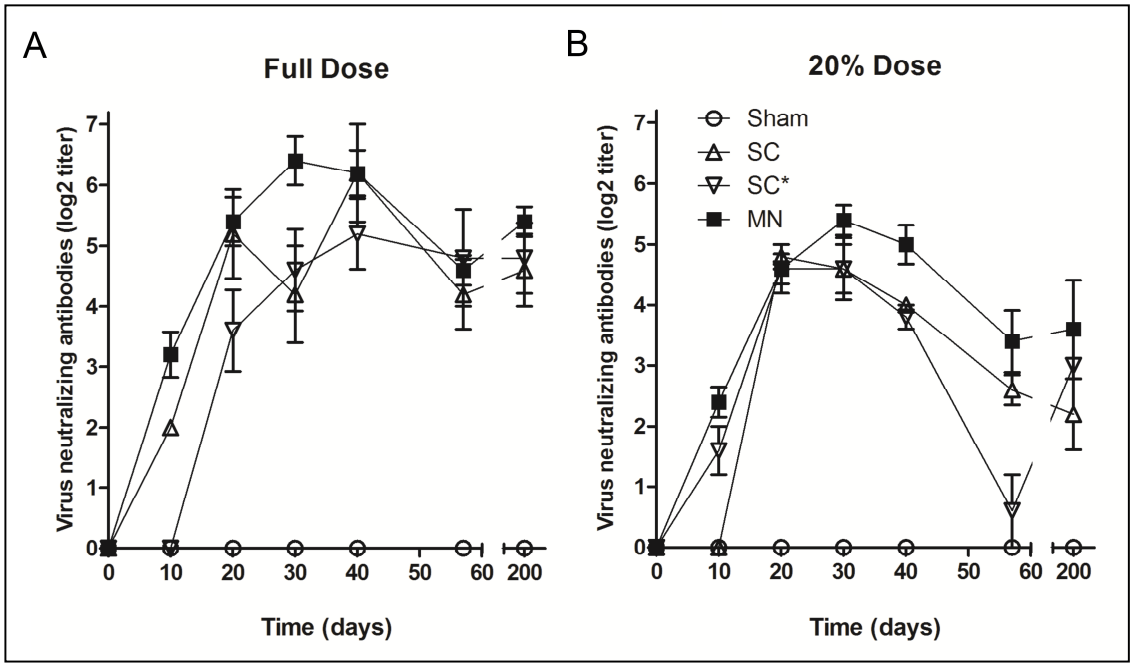

Figure 7: Neutralizing antibody responses after vaccination using microneedles compared to subcutaneous injection. Cotton rats were vaccinated with (A) a full human dose (1000 $TCID_{50}$) or (B) 20% of a full human dose (200 $TCID_{50}$). Vaccination was performed with microneedles (MN), subcutaneous injection of unprocessed vaccine (SC), subcutaneous injection of vaccine coated onto microneedles and reconstituted before injection (SC*) or as a sham vaccination using microneedles with a vaccine-free coating. Antibody titers were determined by plaque neutralization. Blood was collected from each animal at the given time points and tested independently. Data points represent the average ± SEM (N = 5).

Table 1: Immune response characteristics of measles vaccination using microneedles.

Group[2]	T_{MAX} ± SEM (days)[3]	Peak titer ± SEM (log_2 antibody titer)	Day 200 Titer ± SEM (log_2 antibody titer)
SC (Full)	32 ± 4.9	6.8 ± 0.2	4.6 ± 0.6
SC* (Full)	32 ± 3.7	6.0 ± 0.3	4.8 ± 0.6
MN (Full)	28 ± 2.0	6.8 ± 0.2	5.4 ± 0.2
SC (20%)	24 ± 2.4	5.2 ± 0.2	2.2 ± 0.6
SC* (20%)	20 ± 0.0	4.6 ± 0.4	3.0 ± 0.0
MN (20%)	26 ± 2.4	5.6 ± 0.2	3.6 ± 0.8

[1] Reported values were determined from antibody timecourse data for each animal and then averaged. The corresponding average antibody timecourse data are shown in Figure 7.
[2] Vaccination was performed by subcutaneous injection of unprocessed vaccine (SC), subcutaneous injection of vaccine coated onto microneedles and reconstituted before injection (SC*) or microneedles (MN) using the full human dose (Full) or 20% of the human dose (20%)
[3] T_{MAX} is the average time at which antibody titers peaked.

4.4 Discussion

The goal of this study was to evaluate a microneedle patch for delivering measles vaccine. The microneedles were designed to be applied as a skin patch without the need for reconstitution. In contrast to conventional subcutaneous injection, administration of the microneedle patch should require minimal training, and therefore, reduce the need for injections by highly trained healthcare professionals. This simple delivery method could

reduce the cost of vaccination, and facilitate mass vaccination campaigns aimed at achieving regional measles elimination and future eradication

The microneedle patch was also designed for cost-effective manufacturing. The vaccine-free microneedle patches can be mass produced at a cost that should be similar to or even less than the cost of a needle and syringe. Coating vaccine onto microneedle patches was carried out as a simple, automated, dip-coating process that can readily be scaled up for low-cost mass production as well. Indeed, microneedle patches coated with parathyroid hormone have already been manufactured commercially and used in Phase I and Phase II human clinical trials [124]. For these reasons, we anticipate that mass-produced microneedle vaccine patches may be manufactured at cost similar to conventional lyophilized measles vaccine. However, as a single-dose presentation, microneedle patches should reduce the extensive wastage currently associated with measles vaccine in multi-dose vials [65].

In this study, immunogenicity after measles vaccination using microneedles was statistically indistinguishable from vaccination by the traditional subcutaneous route in the cotton rat model, including the time course of the immune response, peak titers and titers measured >6 months after vaccination. This shows that vaccination using a microneedle patch can induce an antibody response to measles virus that is equivalent to the response following standard subcutaneous injection. Though the optimal animal model for evaluating measles vaccines is the rhesus macaque [5], cotton rats were chosen for this study because they provided a low cost, small animal model for evaluating the ability of the microneedle vaccines to reconstitute *in vivo* and generate a neutralizing antibody response. Cottons rats are an accepted small animal model for measles, and this

model has been used in many research projects. Both vaccine and wild-type strains of measles have been shown to replicate in cotton rats [123].

In this study, a vaccine coating formulation was developed that enabled vaccine-coated microneedles to meet the accelerated stability criterion of the WHO, i.e., storage for 1 week at 37^{o}C while retaining at least 10% virus viability [122]. Removal of the salts in PBS, which probably reduced osmotic stresses during drying, and addition of the sugar trehalose, which is believed to stabilize vaccine antigen structure, maintained vaccine virus viability for 1 month at 25^{o}C in the presence of desiccant.

The use of trehalose in the coating formulation was important to maintaining stability of the measles virus. This disaccharide has been widely used as a stabilizer in many different biological systems [125-127]. It is thought that trehalose replaces the water around hydrophilic protein regions during drying, thereby preventing protein denaturation [128].Other stabilizers tested in this study were not effective. Fish gelatin and myo-inositol were chosen because they are included in the formulations of other currently available vaccines and they have been shown to have stabilizing effects on measles virus in the literature.[94]

A dose-sparing effect has sometimes been seen when using microneedles with other vaccines [21, 129], but dose sparing was not seen in this study with the measles vaccine at the doses used. As the mechanism of dose sparing in the skin is still under investigation [50], the reasons why dose sparing was not seen in this study are not clear.

4.5 Conclusion

This study compared administration of live-attenuated measles vaccine using a microneedle patch to conventional subcutaneous injection for the first time. We showed that the measles virus can be coated and dried onto metal microneedles with acceptable stability during storage. Vaccination of cotton rats showed that microneedle vaccination produced antibody titers similar to vaccine delivered using a conventional subcutaneous injection. However, unlike subcutaneous injection, measles vaccination with a microneedle patch is rapid and simple to administer, which could dramatically decrease the training required for measles elimination campaigns [130]. The patches themselves are small and lightweight, easy to dispose of, and expected to require low-cost manufacturing in mass production. We conclude that delivery of measles vaccine with a microneedle patch can be efficacious and could provide a means to significantly increase vaccine coverage as many regions advance towards measles elimination.

4.6 Acknowledgments

This work was carried out with the help of Paul Rota, Marcus Collins and the Measles Lab on the campus of the Centers for Disease Control and Prevention. I would also like to thank Vladimir Zarnitsyn for designing and building the system used to coat the needles and giving support during its use; Samir Patel and Mark Papania for helpful discussions, James Norman for help with statistical analysis and Donna Bondy for administrative support. This work was supported in part by a grant from the Georgia Research Alliance. Mark Prausnitz is an inventor on patents and has a significant

financial interest in a company that is developing microneedle-based products. This potential conflict of interest has been disclosed and is being managed by Georgia Tech and Emory University.

CHAPTER 5 Discovering stabilizing excipients for the measles vaccine using a high-throughput eGFP assay

5.1 Introduction

Measles is a highly infectious respiratory disease that still kills more than 150,000 people worldwide [117]. The primary way to prevent infection is successful vaccination. Since its introduction in the 1960's, the live-attenuated measles vaccine has led to significant advancements in the fight against measles [131]. Endemic measles was eliminated from the Americas in 2002 and currently 4 of the 5 WHO regions have set elimination goals [117]. It has been estimated that interruption of measles transmission requires vaccine coverage rates in excess of 90% [132]. This high bar requires a coordinated effort between vaccine manufacturers, public health experts and delivery personnel in the field. Effective vaccination also requires an effective vaccine. While the live-attenuated measles vaccine is extremely effective when given correctly, storage requirements can impede widespread dissemination. Additionally, the vaccine is known to rapidly lose activity when exposed to elevated temperatures, even in its lyophilized state. The WHO estimates that due to spoilage, mishandling, or improper reconstitution more than 60% of the measles vaccine delivered into the field is not utilized [133]. Even for a product as inexpensive as measles this can lead to significant increases in the cost of mass vaccination campaigns. Increasing the stability of the live-attenuated measles vaccine would decrease this wastage and ultimately result in more product available for eventual administration.

The traditional method for determining the titer of live-virus vaccines is some form of plaque assay. This typically requires infection of a monolayer of cells followed by incubation, staining and analysis. The standard unit of infectivity for the measles vaccine is the 50% tissue culture infectivity dose [121]. This assay requires a 5-7 day incubation time and then manual identification of the resulting plaque formation. It has been shown that the same measles sample read by different labs and personnel can result in nearly a 1 log unit difference in reported titer [134]. This type of assay does not easily lend itself to a high-throughput screen of excipients for the purposes of increasing measles viral stability.

The introduction of a genetically modified measles virus has opened up new avenues for infectivity detection. An altered measles virus strain (eGFP-MeV) was created which encodes the genetic material for green fluorescent protein [96]. Following infection, viral replication causes the production of eGFP. This makes it possible to detect measles activity rapidly using the resulting fluorescence. This virus has been previously used to examine viral stability in a large-scale screening study [98]. The previous technique was complicated by a number of additional steps including the addition of an agent which prevents cell fusion. It also required computer software to count and analyze the halted viral propagation. An assay which could simplify the process of measuring fluorescent activity after infection would be a positive development.

The goal of this study was to discover excipients which could improve the stability of the live-attenuated measles vaccine. In order to accomplish this a new assay was created which utilized eGFP-MeV to measure measles infectivity quickly, efficiently and reproducibly. Using this assay, a screen was performed to determine the stabilizing activity of a number of commercially available excipients.

5.2 Materials and methods

5.2.1 Propagation of eGFP-measles vaccine virus

Measles vaccine virus that had been genetically engineered to produce eGFP during replication was acquired from the lab of Dr. Paul DuPrex at Boston University [97]. This stock was then propagated in Vero cells as previously described to increase the viral titer [122]. The final titer was measured using a $TCID_{50}$ assay to be 3.0 x 10^5 viral units/mL.

5.2.2 Selection of excipients

The excipients used for the screening portion of this study were selected based on a number of characteristics. A through literature search resulted in an initial list that had been specifically shown to stabilize the live-attenuated measles vaccine virus. This list was further expanded by adding additional compounds which had been shown to stabilize other vaccines, including excipients found in commercially approved vaccine preparations. The final list also included compounds that had not been previously studied in vaccine stabilization experiments. These were selected by expanding the number of certain types of compounds that already appeared on the list in high numbers. This resulted in the addition of a significant number of carbohydrate substances and amino acids. All excipients chosen for this study either appear on the Generally Regarded as Safe (GRAS) list or are already approved for injection into humans. The final excipient list contained 46 different compounds or concentrations of compounds (Table 2). All listed percentages represent a weight/volume percent.

5.2.3 Green fluorescent protein infectivity assay

All excipient formulations were mixed in a 1:1 ratio with a stock of eGFP-measles vaccine virus (eGFP-MeV) with a titer of 3.0 x 10^5 $TCID_{50}$/mL. A 3 µL sample of this formulation was then coated onto stainless steel chips in a manner previously described [122]. These chips were placed into 1.5 mL centrifuge tubes (Epindorf, Hauppauge, NY). The samples were then placed into an opaque bag along with color-changing desiccant (Drierite, Sigma-Aldrich, St. Louis, MO). The bag was then sealed using a heat sealer to prevent against moisture contamination. All samples were dried for 24 hours at 22ºC in a fume hood before storage. After removal from storage the desiccant of each sample was checked for indication of moisture. If any contamination was detected the sample was discarded.

Each centrifuge tube, which contained a single dried measles virus preparation, was filled with 1 mL of a solution of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) containing 2% fetal bovine serum (FBS, Gibco). This was then vigorously mixed to re-suspend the dried viral sample. The samples were tested for fluorescent activity using 96-well plates (Immunolon 2HB, Thermo Scientific, Waltham, MA) containing a monolayer of Vero cells. To each well in the plate, 100uL of the reconstituted viral solution was added. The plates were incubated for 72 hours at 37ºC to encourage viral propagation. After incubation each well was washed with 300 uL of sterile phosphate-buffered saline (PBS, Sigma-Aldrich) to remove media and uninfected viral particles. Any remaining solution was aspirated from the wells before testing. Detection of florescence was accomplished by measuring each well using a 96-well plate reader with an excitation wavelength of 485 and an emission wavelength of 520. The

detected signal of each sample was compared to a positive control containing the same concentration of liquid eGFP-MeV.

5.2.4 Stabilizing excipient screen

A first screen was performed to investigate the ability of all of the excipients selected to prevent infectivity loss in a dried preparation of measles vaccine virus. Each sample was mixed to the concentration indicated and dried with a solution of measles vaccine virus as described above. For the initial screen, each prepared sample was stored in an incubator at 37^{o}C for 7 days. After testing for eGFP expression each sample was then ranked based on percent of activity remaining as compared to a liquid control sample of eGFP-MeV. A cutoff value of 10% remaining activity was chosen to eliminate samples with minimal stabilizing activity.

A subset of the initial excipients was then subjected to a more stringent secondary screen. Theses samples were prepared and stored for 1 month at 37^{o}C. After storage the samples were tested and ranked according to remaining activity. The results from this storage condition led to the addition of excipient combinations to the testing regimen. The combinations of excipients were tested at the same storage time and temperature conditions as the secondary screen. After eGFP florescence detection a cutoff value of 40% remaining activity was chosen for advancement to the final storage condition.

The final screen was performed by storing samples for 1 month at 45^{o}C. The results from this screen resulted in the final excipient combination which was then subjected to a variety of temperature and storage conditions.

5.2.5 Extended storage study

A stabilizing solution consisting of 300 mM of threonine (Sigma-Aldrich) and 15% w/v sucrose (Sigma-Aldrich) in DI-H2O was created for the final storage experiment. Samples were created using the method described above and stored at 4ºC, 22ºC and 45ºC for between 1 and 24 weeks. A control consisting of a dried sample of eGFP-MeV containing no stabilizers stored at 22ºC for 1 to 4 weeks was also included.

5.2.6 Statistics

All statistics for this study were calculated using Prism software version 6.02 (Graphpad, La Jolla, CA). Comparisons between individual samples were done using an unpaired t-test was a significance cutoff of $p<0.05$. Comparisons between multiple samples was done using a two-way ANOVA with a Tukey post-test and a significance cutoff of $p<0.05$. The exponential best fit line was determined using Excel 2013 (Microsoft, Redmond, WA). Averages of all results represent the arithmetic mean of the tested samples.

5.3 Results

5.3.1 Assay development

Before proceeding to the stability screen, initial experiments were performed to better understand the parameters of the eGFP assay. Multiple 96-well plates containing confluent layers of Vero cells were infected with decreasing concentrations of eGFP-MeV and then allowed to incubate for 2, 3 or 4 days to examine how florescence activity changed over time. The initial eGFP-MeV titer used was measured to be 500 $TCID_{50}$/mL. The goal was to determine the shortest incubation time which still delivered a robust

florescence curve. The florescent activity was measured using the method described in Section 5.2.3. The results show that after 3 days of incubation there is a linear correlation between viral concentration and florescent intensity starting at a concentration of 250 $TCID_{50}$/mL (Figure 8). As a result, we used an incubation time of 3 days and a maximum viral titer of 250 $TCID_{50}$ per sample for all stability experiments.

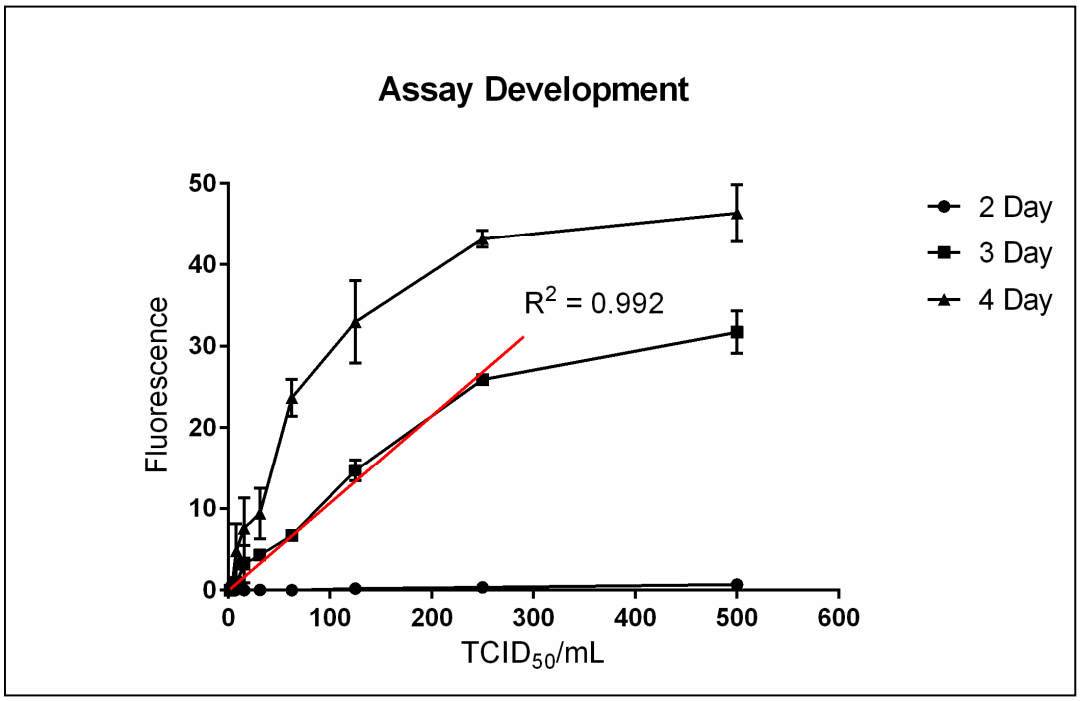

Figure 8: Development of the eGFP-MeV assay.
A dilution series of eGFP-MeV was used to infect a confluent layer of Vero cells. The infected cells were allowed to incubate for 2, 3 or 4 days. At that time the cells were washed and the florescence was measured. The results show that after 3 days of incubation florescence has a linear relationship to viral titer beginning at a concentration of 250 $TCID_{50}$/mL. Each point represents the average (N = 2) ± SD. The red line indicates a linear best fit curve excluding data collected from the 500 $TCID_{50}$/mL sample. The R^2 value was calculated to be 0.992.

5.3.2 Stability screen

In order to rapidly assess the stabilizing potential of the initial list of excipients, a high temperature, short time storage study was performed. The goal of this study was to eliminate any excipients which had minimal or no detectable effect on vaccine activity.

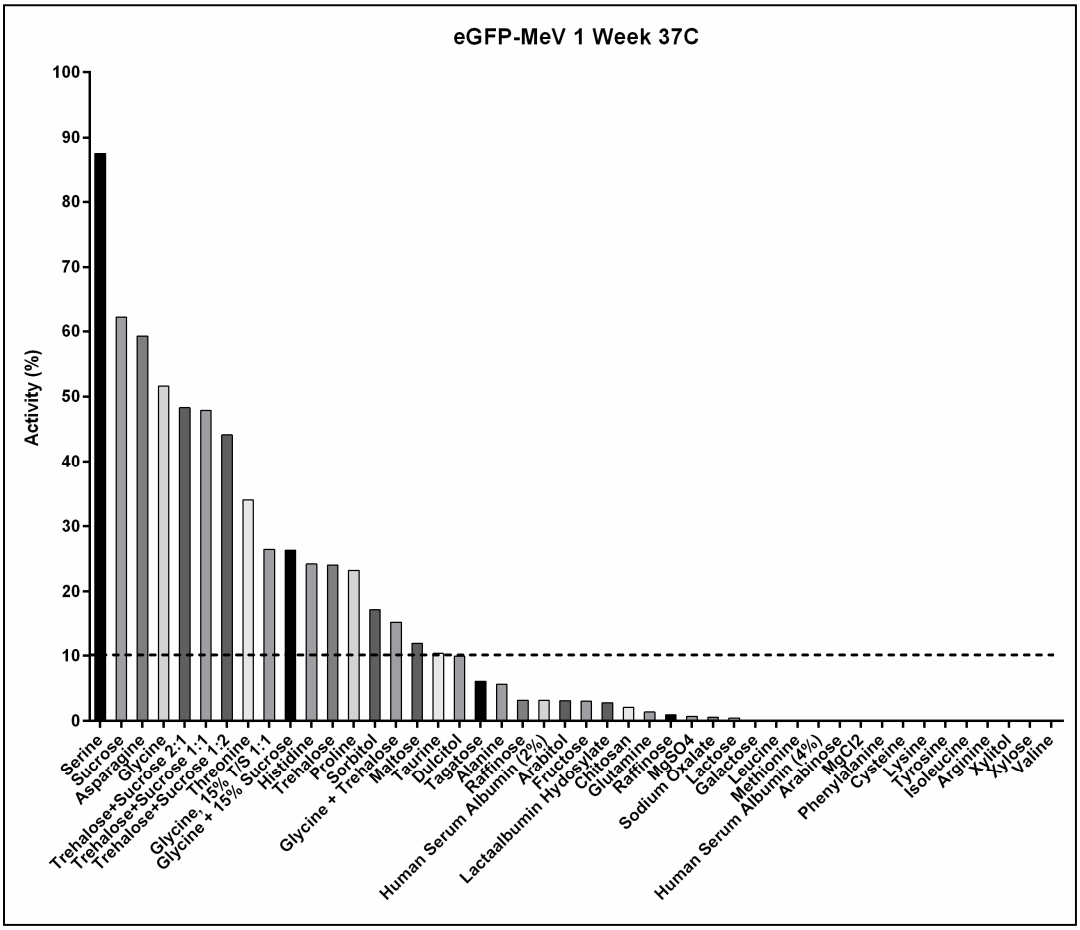

Figure 9: Initial screen of excipient using eGFP-MeV assay.
The initial list of excipients was screened for stabilizing activity using the eGFP-MeV assay. Samples were dried overnight and stored for 1 week at 37ºC with humidity control. After storage each sample was incubated in cell culture for 3 days and then assayed for fluorescence activity. Each bar represents a comparison between the detected fluorescence of a control MV sample and a dried sample. The dotted line represents the cutoff point of 10% remaining activity after storage. Each sample was tested once to maximize throughput.

As expected, the initial screening study resulted in the elimination of the vast majority of the chosen excipients. After storage, approximately 41% of the tested samples retained less than 1% of their initial activity. The cutoff value of 10% remaining activity

was chosen because it corresponds to the WHO requirement for live-attenuated measles vaccine stored for 1 week at 37ºC [118]. This resulted in the elimination of about 61% of the initial list (Figure 9). After the initial screen, 14 samples were chosen for further investigation in the second screening experiment.

In order to further assess the stabilizing potential of the remaining excipients, a longer storage condition was chosen for the second screen. Since the goal of this study was to produce a single excipient mixture for long-term evaluation, the secondary screen attempted to eliminate all of the excipient which could not significantly stabilize eGFP-MeV under these revised conditions. After 1 month at 37ºC, only 4 samples demonstrated remaining activity of more than 1% (Figure 10). During this secondary screen we noticed that when the excipients glycine and sucrose were tested individually they exhibited extremely low stabilizing activity (0.53% and 0.61% respectively). When these excipient were added together their ability to stabilize eGFP-MeV increased significantly to 30.45% ($p<0.0005$) (Figure 11). This led us to investigate the effect of using a carbohydrate sugar and an amino acid in combination to stabilize the measles vaccine. Combinations were made of all excipients in these two categories that exhibited any stabilizing activity during the initial screen. This resulted in 21 combinations. All of the amino acids were also tested individually to serve as a control. After storage for 1 month at 37ºC the results confirmed our earlier observation. Every amino acid tested exhibited a higher stabilizing ability when paired with a carbohydrate sugar (Figure 12). Sucrose was the most potent secondary stabilizer. Combinations including sucrose had the highest remaining activity for 6 of the 7 tested amino acids. A cutoff limit of 40% was used for this screen to exclude stabilizing combinations that had lower activity.

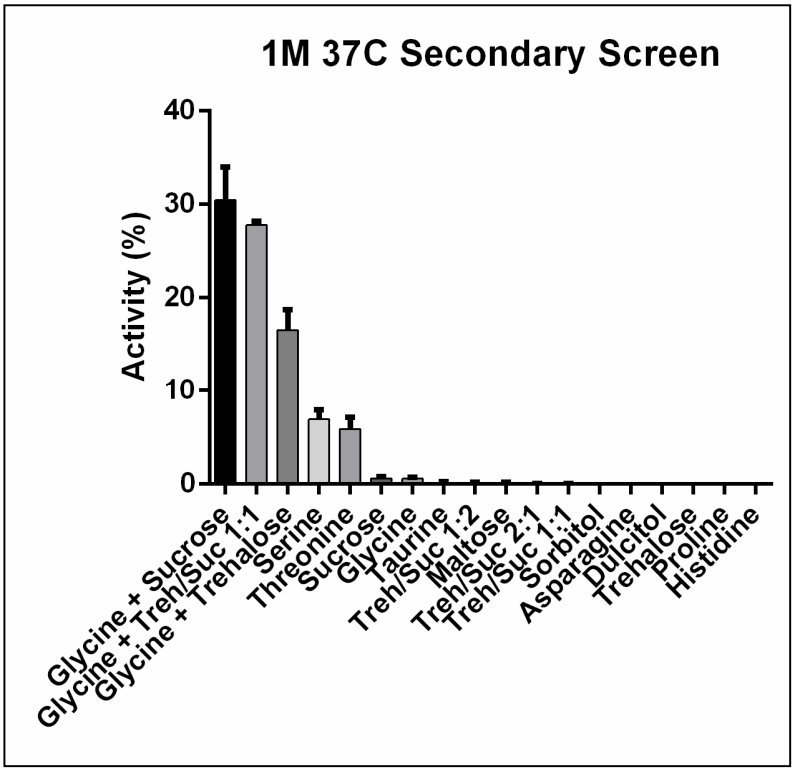

Figure 10: Secondary screen of excipients using eGFP-MeV assay.
After removal of low-performing excipients a second screen was performed. These samples were prepared in the same manner as before and stored for 1 month at 37ºC. After storage the samples were incubated in cell culture for 3 days and then assayed for fluorescent activity. The percentage represents a comparison between the fluorescent activity of a control samples of eGFP-MeV and the dried samples. Each bar represents the average (N = 3) ± SD.

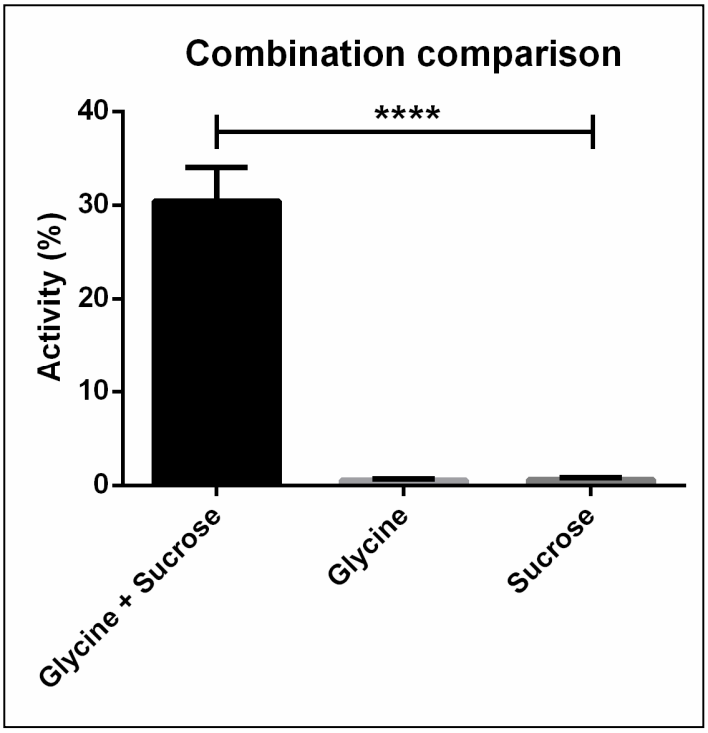

Figure 11: Effect of combining excipients on their overall stabilizing ability.
It was determined that a combination of glycine and sucrose had significantly higher stabilizing ability than either excipient possessed individually. The bar represents the average (N = 3) ± SD. Asterisk (****) indicates a significant difference ($p<0.0005$) as determined by ANOVA.

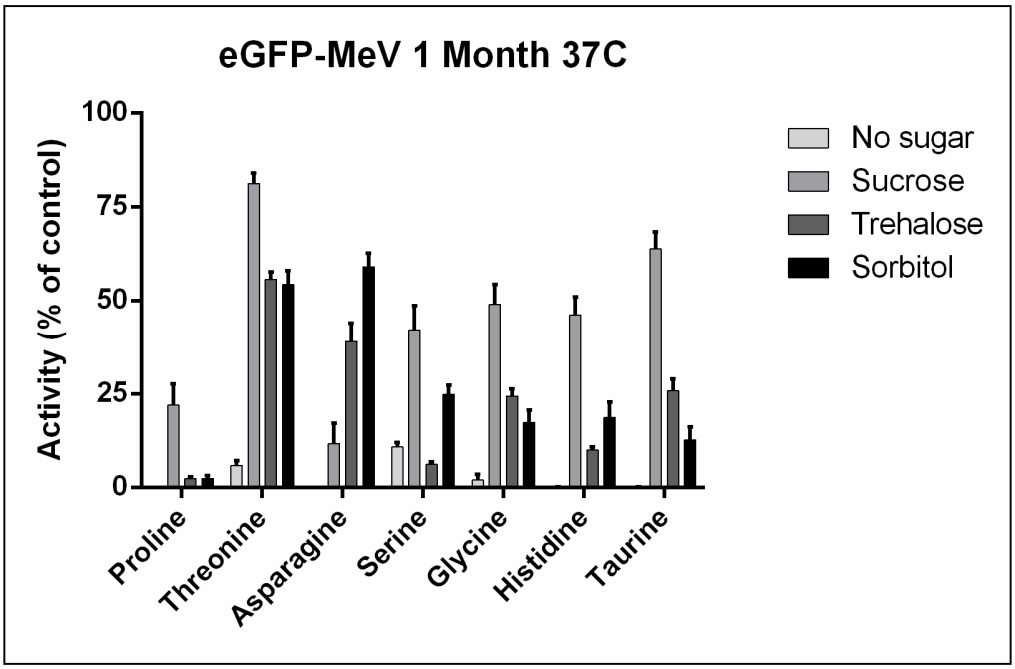

Figure 12: Stabilizing ability of excipient combinations tested with an eGFP-MeV assay.
All possible combinations of amino acids and sugars that had previously shown stabilizing activity were made. These samples were tested and compared to individual amino acid solutions. The bars represent the average (N = 3) ± SD.

A final screen was carried out to determine the best combination of excipients to use for more extensive stability experiments. After storage for 1 month at 45ºC the remaining activity of each sample was tested. This screen showed that a combination of the amino acid threonine and the sugar sucrose had the highest stabilizing potential (Figure 13). This combination was able to retain nearly 14% of its original activity after storage at this harsh temperature condition.

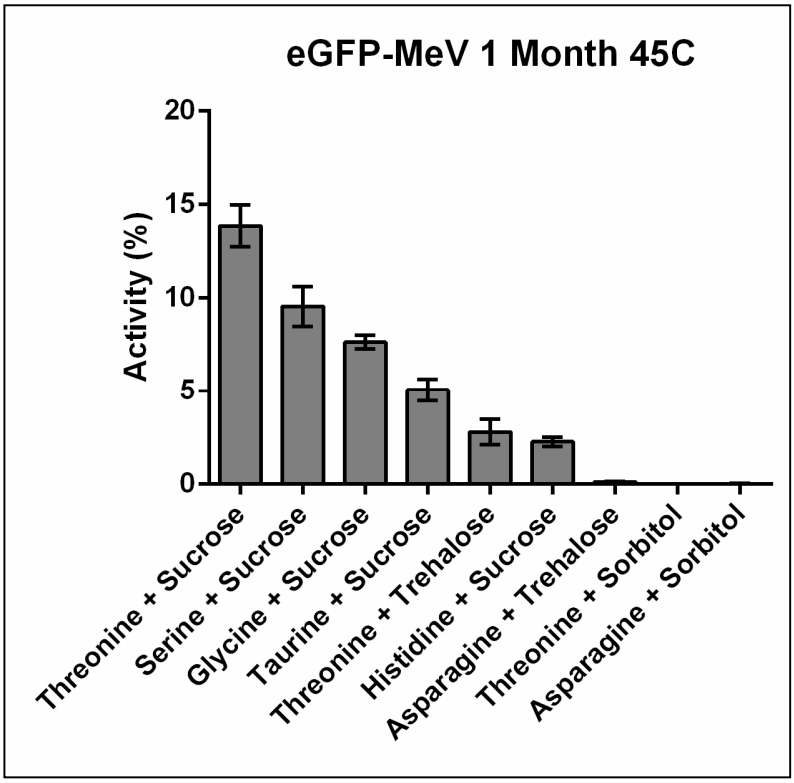

Figure 13: Final excipient screening study using eGFP-MeV assay.
Excipient combinations that showed potent stabilizing activity in the previous screen were subjected to a higher storage temperature. These samples were dried and stored for 1 month at 45ºC. After storage the samples were incubated in cell culture for 3 days and then assayed for fluorescence. The activity represents a comparison between the detected fluorescence of a control stock of eGFP-MeV and the dried samples. The bars represent the average (N = 3) ± SD.

5.3.3 Extended storage study

After screening, the highest performing excipient mixture was subjected to a longer term study to further examine its ability to maintain the infectivity of eGFP-MeV. Samples were stored at a range of temperatures for up to 2 month. After storage for 1 week at room temperature (25ºC), the control sample which included no stabilizing excipient had lost 100% of it infectivity as measure using the eGFP assay. Samples which included the stabilizing solution (threonine + sucrose) performed much better (Figure 14). After some initial detected loss at the 1 week time point, the samples stored at 4ºC and

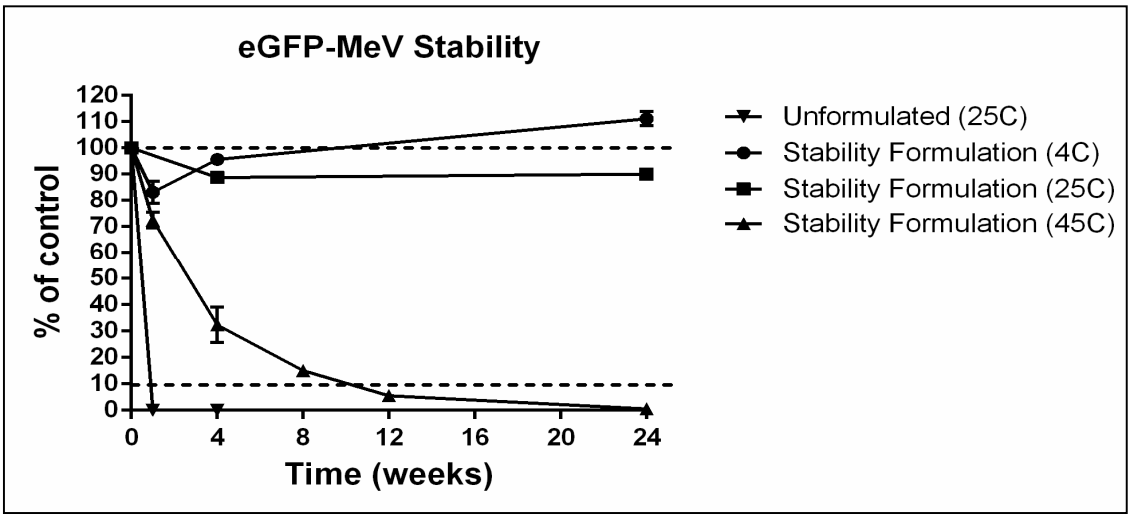

Figure 14: Extended stability of threonine-sucrose formulation.
The best performing formulation from the final screening study (threonine + sucrose) was added to eGFP-MeV and stored for 0 – 24 weeks at three different temperatures. A samples of eGFP-MeV without excipients was also dried and stored at 25ºC. All samples were compared to a liquid control of eGFP-MeV to obtain the stability percentage. The samples stored at 4ºC and 25ºC retained more than 90% of their activity. The samples stored at 40ºC had greater activity loss over time. The data points represent the average (N = 3) ± SD.

25ºC retained on average 95% and 89% of their activity respectively after 1 month of storage. Samples stored at the higher temperature (45ºC) had significantly higher loss at this time point. They retained only 32% of their infectivity as measured using eGFP florescence detection. At the final 6 month time point, the samples stored at the 2 lower temperatures proved to be very stable. The 4ºC and 25ºC samples retained 100% and 90% of their original infectivity respectively. The samples stored at 45ºC maintained less than 1% of their infectivity at this time point. The rate of decay for the 45ºC sample as calculated by an exponential best-fit line with an $R^2 = 0.9961$ was found to be k = -0.216. The rate of decay for the 4ºC and 25ºC samples was not calculated because they had not lost a significant amount of activity by the 6 month time point.

5.4 Discussion

Measles vaccine spoilage represents a significant problem for large-scale vaccination campaigns. The WHO estimates that more than 50% of all possible vaccine doses are never delivered due to handling error or spoilage [135]. Wastage of measles vaccine in particular is even more acute. A study in Bangladesh found that on average 70% of possible measles doses were wasted due to spoilage or reconstitution error [133]. Increasing the ability of the measles vaccine to withstand adverse temperature conditions could greatly reduce these levels of waste.

Stabilization of the live-attenuated measles vaccine has been previously studied [92, 94, 98]. Ohtake et al examined a variety of excipient combinations for their effect on measles vaccine stability following a traditional spray drying procedure. They found that many compounds expected to decrease vaccine loss at elevated temperatures such as surfactants, ions and plasticizers did not have a beneficial effect. It was discovered that the addition of arginine, an amino acid, to the stabilizing formulation resulted in a significant increase in stability after 4 weeks of drying. Their best formulation also included sucrose, potassium phosphate and human serum albumin. Their final drying condition of 12 weeks at 37ºC resulted in an infectivity loss of approximately 1.2 $\log_{10}(TCID_{50})$. The stability of liquid measles has also been studied by Schlehuber et al [98]. Their best excipient formulation also included amino acids (glycine and serine) and sugars (trehalose and sucrose) in addition to a buffering agent (tricine) and a few other compounds. With this formulation they were able to limit activity loss to less than 0.4 $\log_{10}$ after 8 hours of incubation at 37ºC as measured by an eGFP-MeV assay. This result corresponded well to the same formulation measured using a traditional $TCID_{50}$. After the same 8 hours of incubation the detected loss using this assay was 0.5 $\log_{10}$.

Remaining water content after drying has also been shown to be important to measles vaccine stability. Burger et al measured the remaining water content of the

measles vaccine following the use of a novel bubble-drying procedure [94]. They found that depending on the excipient formulation and process conditions the final water content could range from 0.5% to greater than 1%. They hypothesized that minimizing the amount of water left in the sample would have a beneficial effect on long-term vaccine stability.

Drying under ambient temperature and pressure is an alternative that has not been well studied. Many vaccine delivery systems such as microneedle patches are not well-suited to compounds dried using spray drying, lyophilization or similar methods. This study examined the ability of commercially available, human-approved excipient compounds to stabilize the measles vaccine after drying and subsequent storage. Another goal of this study was to develop a high throughput assay to aid in the rapid screening of excipients.

The current method of determining the titer of the measles vaccine is by a 50% tissue culture infectivity dose ($TCID_{50}$) assay. This assay is both time-consuming and uses a large quantity of materials. Each sample requires the use of a single 24-well plate, with requirements increasing when samples are run in duplicate or triplicate. This assay has also been shown to have a high level of variability from test to test. A collaborative study found that laboratory variability when testing the same sample of measles vaccine was as high as 1 log($TCID_{50}$) unit [134]. This range could cause major problems for a study attempting to examine vaccine potency measurements at a variety of storage conditions. This study utilized a measles vaccine virus variant which has been engineered to express green fluorescent protein during replication [96]. This vaccine virus has been previously using in a high throughput assay for similar purposes [98]. That assay utilized a number of additional steps including chemical inhibition of cell fusion post-infection and computer-aided detection of florescent plaque formation. That assay measured total infected cells rather than overall florescence. This study shows that simple florescence detection after infection can reliably demonstrate the infectivity potential of dried

samples after reconstitution. Using this assay, a variety of different excipients were rapidly screened for the presence or absence of stabilizing activity.

Over the course of the screening experiment, an interesting phenomena was observed. Excipient formulations which contained both a sugar and an amino acid demonstrated far superior stabilizing potential than either of the excipients showed when tested individually. Many commercial vaccine solutions include a compound from each of these categories but to date there have been no study which directly showed this synergistic response. The ability of amino acids to stabilize proteins has been well-studied [136]. Glycine, for example, has been shown to increase the melting temperature of model proteins such as bovine serum albumin (BSA) by up to 13^{o}C [137]. It has been hypothesized that the amino acids react indirectly, creating a preferential hydration state that allows the protein to maintain a minimum level of water contact even after drying [138]. It is also well known that sugars can have a stabilizing effect on proteins. Microbes which can maintain activity after extreme water loss have been known to increase their intracellular trehalose concentrations as a coping mechanism [139]. The many hydroxide groups present on this and other sugars have been shown to interact directly with proteins and lipids, taking the place of water around the hydrophilic domains [128]. This allows for the maintenance of the native “wet” state even in the absence of water.

It is possible that the stabilizing effect seen in this study is a result of both of these phenomena acting in concert. The amino acid in the solution may be increasing the protein’s ability to accept hydration. This allows for the sugar molecules to more easily replace water around the hydrophilic domains as the sample dries. More work needs to be done to elucidate the exact mechanism of action for this interesting synergistic response.

5.5 Conclusion

A method which could stabilize the measles vaccine to prevent spoilage would be a major benefit for the global measles elimination campaign. Vaccine wastage represents a serious problem, impeding efforts to increase the reach of these campaigns away from population centers. This work shows that by using a high-throughput assay, excipients which stabilize the live-attenuated measles vaccine after drying can be found. The best formulation, consisting of a mixture of threonine and sucrose, was able to completely mitigate infectivity loss at 4ºC and 25ºC. It was also able to maintain more than 10% activity at 45ºC for more than 8 weeks. These advances could allow for future microneedle patch systems which incorporate measles vaccine to be more stable at elevated temperatures, mitigating current cold chain requirements. Future work will attempt to increase stability even further towards a goal of removing measles vaccine from the cold chain completely.

5.6 Acknowledgements

I would like to that Betul Benzer for her help preparing the samples used in the excipient screen. I would also like to thank Marcus Collins and Paul Rota at the Centers for Disease Control and Prevention for their help designing and implementing the eGFP-MeV assay. I would also like to thank Donna Bondy for administrative assistance. This work was supported in part by the CDC's Global Immunization Division and the Georgia Research Alliance.

CHAPTER 6 Measles vaccination of non-human primates using a microneedle patch

6.1 Introduction

In the past decade, substantial progress has been made in the fight against measles. Since the year 2000, deaths from measles have decreased by more than 82%. They have fallen from more than 850,000 to an estimated 158,000. This sustained decrease in mortality is due in large part to the availability of a safe, cheap and effective vaccine. The live-attenuated measles vaccine costs just $0.10 to produce and has efficacy rates approaching 90% when two doses are given [131]. A plan to implement high levels of two-dose coverage in the WHO Region of the Americas led to the elimination of measles in 2002 [117]. By 2012, 4 of the 5 remaining WHO regions had set a goal for measles elimination by, or before 2020. Unfortunately, the drive towards measles elimination has stalled in recent years. This is primarily due to the highly infectious nature of measles. It is estimated that coverage rates in excess of 95% are required to interrupt viral transmission [4]. Increasing global measles vaccine coverage is the only way to continue current reductions in measles morbidity and mortality.

In 2012, the research priorities for global measles control and eradication were published and these included improved vaccine delivery methods [140]. In resource limited settings, the traditional vaccine delivery methods have can pose significant logistical drawbacks. Currently, the measles containing vaccine must be administered by hypodermic injection. This necessitates the need for trained personnel to deliver each dose of vaccine. Compared to using minimally trained volunteers, this requirement significantly increases the cost of a mass vaccination campaign while simultaneously

decreasing its scope. Door to door vaccinations, which are used for the oral polio vaccine, are simply not conducive to injectable vaccines.

Needle and syringe delivery also creates safety hazards for both the vaccinator and patient. Sharps and other medical waste must be properly disposed of to avoid disease transmission or needle reuse. Even small vaccination campaigns can generate significant amounts of medical waste [102]. Transport and storage of this byproduct constitutes a significant portion of the overall cost of large public health projects such as measles elimination.

Finally, the lyophilized measles vaccine is typically packaged in multi-dose vials which must be kept in the cold chain, reconstituted prior to use, and discarded within 6 hours after reconstitution. Errors in reconstitution of the vaccine, as well as the need for refrigerated storage can lead to significant vaccine wastage [95]. A technology which could mitigate the need for the cold chain has the potential to drastically lower the cost and complexity of mass delivery of the measles vaccine.

An innovative delivery method which could alleviate many of the problems associated with syringe injection is the microneedle patch. Microneedle patch systems have the potential to substantially improve vaccine delivery. Microneedles are micron-scale (<1000 μm), solid or dissolving needles containing a dry formulation of vaccine that rapidly dissolves in the skin upon patch application [13]. Numerous vaccines and other biologics have previously been delivered using microneedle systems [15-17, 19, 22, 82, 87, 101, 115, 141]. The simplified application provided by a microneedle-based measles vaccine could move trained medical personnel, a limited resource in developing countries, into a supervisory role leaving vaccinations to be performed by minimally-trained personnel. Microneedle patches cause little or no pain and can adhere to the skin in a manner as easy as applying a bandage. Microneedle patches would also be formulated to deliver a single dose without the need for reconstitution. This limits vaccine waste associated with errors in reconstitution such as using expired or incorrect

reconstitution fluid. Additionally, microneedles may allow for less stringent cold-chain requirements, minimizing vaccine spoilage. The microneedle vaccine patch is also expected to require lower packaging requirements compared to the current combination of lyophilized vaccine, hypodermic needles and reconstitution vials. This advancement would require less storage, transportation, and disposal capacity. These benefits could greatly enhance current efforts to attain the high vaccination coverage needed to achieve measles elimination goals.

In this study, the live-attenuated measles vaccine was delivered for the first time using a dissolving microneedle patch in a rhesus macaque. This vaccination route was then compared to the same dose delivered using a traditional needle and syringe.

6.2 Materials and methods

6.2.1 Preparation of live-attenuated measles vaccine

The Edmonston-Zagreb measles vaccine strain was acquired from the collection at the Centers for Disease Control and Prevention. The viral stock was then propagated in Vero cells to increase the titer as previously described [122].

6.2.2 Microneedle fabrication

6.2.2.1 Creation of microneedle mold

Molds consisting of a 10 x 10 array of 300 x 300 x 600 µM pyramidal microneedles with a tip-to-tip spacing of 640 µM were fabricated as previously described [142, 143].

6.2.2.2 Measles vaccine filling

Measles vaccine solution at a titer of 1.0 x 10^5 was added in a 1:1 ratio to a solution consisting of 15% w/v sucrose (Sigma-Aldrich, St. Louis, MO), 300mM threonine (Sigma-Aldrich) and 2% w/v carboxymethyl cellulose (Sigma-Aldrich). This solution was mixed and applied to the microneedle mold. The solution was spread using a pipet tip to ensure the entire mold surface was covered. This mold was then placed on custom-made vacuum system and suction was pulled from the bottom of the mold at a pressure of approximately -3.59 kPa for 20 minutes. The mold was removed from the vacuum system and allowed to dry for 40 minutes. At this time any vaccine solution remaining on the surface of the mold was removed by tape stripping.

6.2.2.3 Polymer matrix filling

To create the matrix for the dissolving patches, 8 g of sucrose (Sigma-Aldrich) and 8 g of poly-vinyl alcohol (Sigma-Aldrich) was mixed into 15 mL of DI-H_2O. This solution was then heated at 60°C for 3 hours. The solution was allowed to cool overnight at 25°C. The PVA/sucrose matrix material was spread in a thin layer over the microneedle mold using a spatula. Care was taken to ensure that the entire mold surface was covered. The molds were placed back into the vacuum system and suction was applied at a pressure of -3.59 kPa for 60 minutes. The molds were then allowed to dry for 48 hours at 25°C in a fume hood. To remove the needle patches, a circle of dextrin (Sigma-Aldrich) was coated with double-sided tape (MacTac, Stow, OH) and applied to the back of the mold. This disc was gently peeled away from the PDMS mold, taking care to prevent damage to the needles. The completed microneedle patches were then lyophilized for 24 hours to strengthen the needles and remove any remaining water.

Patches were stored in a sealed pouch with color-indicating desiccant (Drierite, Xenia, OH) and protected from light until insertion.

6.2.3 Immunization studies

The immune response to microneedle delivery of measles vaccine was testing in rhesus macaques (*Macaca mulatta*). The animals were divided into groups of 4 monkeys each as follows: (1) full-dose delivered by subcutaneous injection and (2) full-dose delivered by dissolving microneedle patch.

Female, 2 year old rhesus macaques were kept in quarantine for 4 weeks and tested for antibodies to measles, influenza, polio and canine distemper virus. One week before the first vacation, blood was collected from a leg vein using Vacutainer tubes (BD, Franklin Lake, NJ). Animals were anesthetized by animal facility staff using ketamine during vaccination and blood collection. For the microneedle vaccination group, a section of hair on the upper back of the animal was removed using electric shears followed by an application of depilatory cream (Nair, Princeton, NJ).

For the microneedle group, a single dissolving patch containing roughly 3500 $TCID_{50}$ infectivity units of measles vaccine was pressed into the skin at the site of hair removal. The patch was left on the skin for 10 minutes to ensure complete dissolution of the polymer needles. In the groups receiving a subcutaneous injection, the stock vaccine solution was diluted using sterile phosphate-buffered saline so that the target dose was containing in 500 µL. This solution was then injected subcutaneously using a 25-gauge needle into the back of the animal. The upper back was chosen for all vaccinations to prevent the animals from scratching the site and causing irritation.

Following vaccination, no adverse effects were noted after microneedle insertion or subcutaneous injection. After microneedle patch removal, a small grid of puncture

sites was faintly visible and some redness existed where the edges of the patch pressed into the skin. No bleeding was observed for any of the vaccinations. The microneedle insertion site was examined daily by animal care staff and no adverse effects were seen. The grid of puncture sites was no longer visible 2-3 days after insertion and no swelling, discharge or other abnormalities were observed at any point during the study.

Eleven weeks after the initial measles vaccinations, the animals in this study were subsequently given the inactivated polio and inactivated influenza vaccines. Due to their inactivated and non-adjuvanted nature we do not believe the subsequent vaccinations had any impact on measured measles antibody titers.

Once a week approximately 10 mL of blood was removed from a leg vein on each animal. Following the completion of the study, all animals were transferred to other protocols within the CDC. The protocol for the rhesus macaque study was approved by the Animal Care and Use Committees of the CDC and the Georgia Institute of Technology.

6.2.4 Neutralizing antibody measurements

Measles neutralizing antibody titers were measured from collected sera using methods previously described [121]. In this study, two-fold dilutions of serum were tested starting at a dilution of 1:4.

6.2.5 IgG and IgM ELISA measurements

A commercially available, indirect enzyme-linked immunosorbent IgG assay (Measles IgG ELISA II, Wampole Laboratories, Cranbury, NJ) and an IgM assay developed at the Centers for Disease Control and Prevention (CDC, Atlanta, GA) were

used for the detection and qualitative determination of IgG and IgM antibodies to measles virus in serum specimens. Positive, equivocal, and negative status of sera was determined using the cut-offs specified by the manufacturer based on index standard ratio (ISR) values. Seronegativity was defined as a serum ISR value of ≤0.90; sera with ISR values of 0.91–1.09 were considered equivocal and sera with ISR values of ≥1.10 were defined seropositive.

6.2.6 Stability studies

In order to test the stability of the microneedle patches used in this study samples were stored at multiple temperatures for up to two months. Dissolving microneedle patches containing 4.5 log($TCID_{50}$) infectivity units of measles vaccine were created using the method described above. The titer in each patch was increased over the patches used in the immunization experiments to create a higher ceiling for the eventual detection of titer loss. After lyophilization, all patches were stored with color-indicating desiccant in an opaque pouch. The pouches were sealed twice using an industrial grade heat sealer (AIE-300, American International Electric, Inc., City of Industry, CA) to ensure that no moisture could penetrate the sample. Each sample was stored in duplicate at 4^{o}C, 22^{o}C and 40^{o}C for 1 week, 1 month and 2 months. After the completion of storage, the sample pouches were opened and the titer was measured using a $TCID_{50}$ assay. The color-indicating desiccant was inspected for each sample. If evidence of moisture contamination was present the sample was discarded. All infectivity results were compared to fresh microneedle patches tested immediately after processing had been completed.

6.2.7 Statistics

All statistics for this study were calculated using Prism software version 6.02 (Graphpad, La Jolla, CA). Comparisons between individual samples were done using an unpaired t-test was a significance cutoff of $p<0.05$. Comparisons between multiple samples was done using a two-way ANOVA with a Tukey post-test. Averages of log($TCID_{50}$) results represent the geometric mean of the samples. All other averages represent the arithmetic mean of the samples.

6.3 Results

6.3.1 Formulation of microneedles

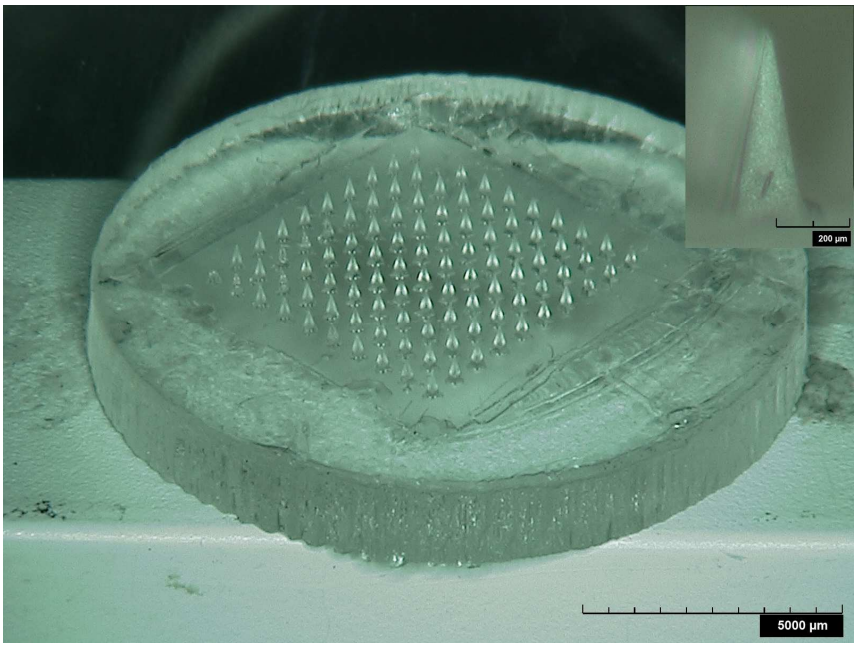

Figure 15: Dissolving microneedle patch containing measles vaccine.
The microneedle patch was made predominately of a PVA and sucrose mixture. Each patch contained 100 pyramidal needles. The insert shows a single dissolving needle at high magnification.

The needles used in this study were designed to enable simple skin insertion, rapid, complete dissolution in the skin and scalability to meet the needs of a global vaccination campaign.

Skin insertion of microneedles is known to depend on sharpness of the needle tip [100]. After removal from the polymer mold, microneedle patches were examined under high magnification (Figure 15). Tip sharpness was determined to be less than 5 µm.

Needle dissolution was tested by inserting fully made needles into pig skin and leaving them for different amounts of time. Typical needles after 1, 5 and 10 minutes of insertion are shown in Figure 16. The tips of the needle dissolved almost instantly upon insertion in the skin with the top third of the needle gone after only 1 minute. Longer insertion resulted in dissolution of a majority of the needle shaft after 5 minutes while the remainder of the needle base was gone after 10 minutes.

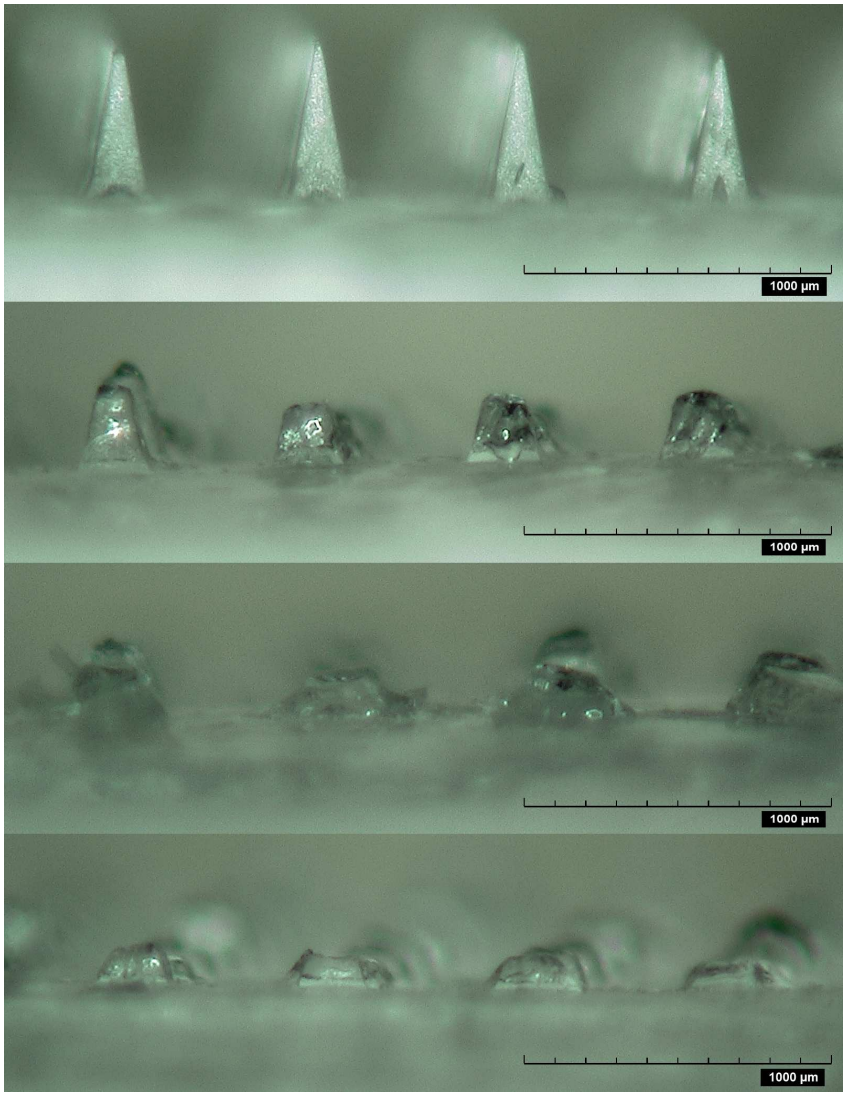

Figure 16: Microneedle dissolution after insertion into pig skin.
Dissolving microneedle patches were inserted into pig skin for 0, 1, 5 and 10 minutes. The patches were then removed and examined at high magnification. After 10 minutes of insertion all needles had completely dissolved.

The needle casting process was designed to be simple and efficient. The fabrication process itself utilized a custom vaccine system which was designed to scale easily depending on the number of patches required. The system is simple and requires only a vacuum source to make sharp, strong patches. Since vaccine wastage is a major concern the addition of the vaccine solution and polymer matrix were split into separate steps ensure that nearly all of the measles vaccine would be concentrated in the needle

rather than the backing of the patch. This was confirmed by measuring the vaccine content before and after insertion into the monkeys. Prior to insertion, each patch contained approximately 3.5 log($TCID_{50}$) of live-attenuated measles vaccine. After removal from the skin the remaining polymer was tested for measles infectivity. We found that more than 90% of the dose in the patch was delivered into the animal after a 10 minute application (Figure 17).

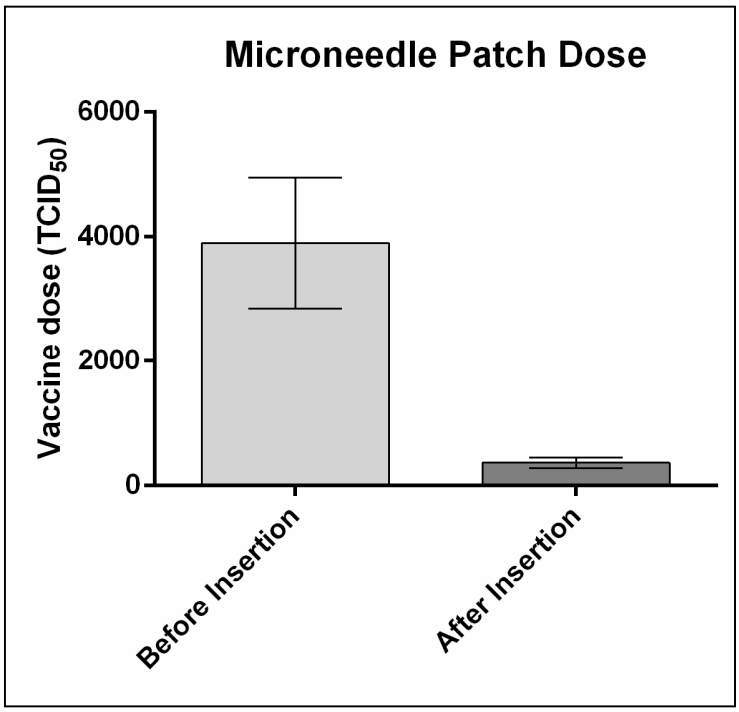

Figure 17: Measles vaccine delivery efficiency using a microneedle patch. Dissolving microneedle patches containing measles vaccine were tested for viral infectivity using a $TCID_{50}$ assay before and after insertion into the skin of a rhesus macaque. The patches were inserted for 10 minutes. The data presents the average (N = 3) ± SD.

6.3.2 Immunogenicity of the microneedle patch

The immune response following dissolving microneedle patch delivery of the measles vaccine was evaluated in a non-human primate model. The rhesus macaque is an extremely well-studied model for measles vaccination studies [130]. Rhesus macaques have previously been used to evaluate vaccine immunogenicity and have a strong

correlation to humans with regard to immune response [50]. In this study, one group of monkeys (n = 4) received a full human dose (~3500 $TCID_{50}$) of the live-attenuated measles vaccine via a dissolving microneedle patch and the other group (n = 4) served as a positive control and received the same dose of vaccine via subcutaneous injection using a needle and syringe. A third group of rhesus macaques received the same dose of measles vaccine delivered using a metal microneedle to confirm the results obtained in the previously conducted cotton rat experiment [122]. Serologic testing during pre-vaccination quarantine confirmed that the animals did not have existing neutralizing antibodies to measles or canine distemper virus.

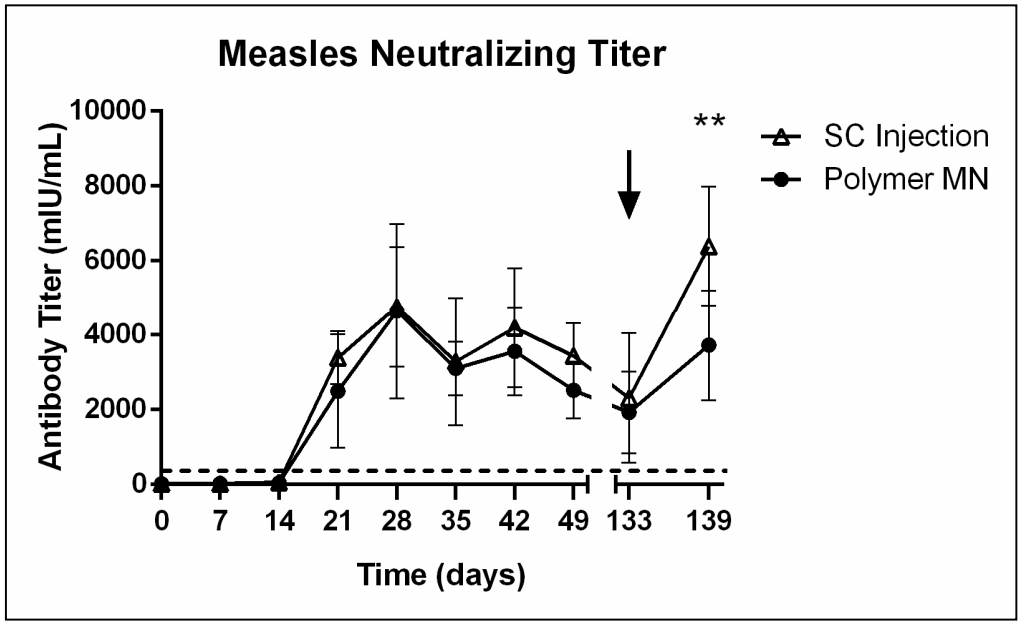

Figure 18: Measles neutralizing antibody titers following vaccination.
Neutralizing titers were obtained from rhesus macaque sera using the method described earlier. Samples were tested weekly. The arrow indicates the date that a 100% dose of monovalent measles vaccine was delivered to all animals subcutaneously. The neutralizing titers between the two groups were not statistically different except at day 139. The asterisk (**) represents a significant difference ($p<0.005$) as measured by ANOVA. The data points represent the average titer (N = 4) ± SD. The dashed line represents 200 mIU/ml which is generally recognized as the minimum titer required for protection.

Serum samples were obtained weekly and tested for antibodies to measles virus. Neutralizing antibodies were detected at low titers beginning 21 days post vaccination and increased to a peak on day 28 (Figure 18). The time to peak titer was not significantly different between the two groups. Peak titers were not significantly different between the two groups indicating a potent immune response both from the subcutaneous injection and the microneedle patch. After 133 days all animals were given a measles boost consisting of 1000 $TCID_{50}$ of a licensed, monovalent formulation of measles vaccination via subcutaneous injection. One week after the boost, all animals showed an increase in titer. The average titer following the boost was statistically higher for the subcutaneous group compared to the microneedle patch group ($p<0.005$). Later time points did not show this statistical significance between groups but the data was confounded by the application of an MMR shot 1 week after the measles-only boost. All animals which received a measles vaccination using a coated, metal microneedle displayed neutralizing titers which followed a similar curve as compared to the other tested delivery routes. However, these animals displayed statistically lower titers when compared to the subcutaneous control on days 28, 42 and 133 following vaccination (Figure 30). All animals in this group also demonstrated a marked increase in titer after the boost.

We tested for the presence of systemic IgG using a type-specific ELISA. As expected, serum IgG antibodies were detected in all animals (Figure 19) indicating that all animals had a robust immune response following vaccination. A general rise in IgG titer was detected in both groups which peaked at day 21. We did not detect any correlation between IgG OD values and plaque neutralizing antibody titers. The difference in IgG ELISA OD values in the two vaccinated groups were not statistically significant at any time point.

Serum IgM levels were also detected using a measles-specific ELISA (Figure 20). During the immune response to vaccination, IgM levels typically precede the appearance of IgG [144]. The presence or absence of these antibodies is often used as a first detection

method to ensure a vaccination has activated the immune system. Measles specific IgM was detected for every animal in both groups indicating generation of a primary immune response to measles vaccine. In both groups the IgM ELISA OD values rose to a peak by day 14 then quickly receded. All animals had positive IgM levels by 21 days following vaccination.

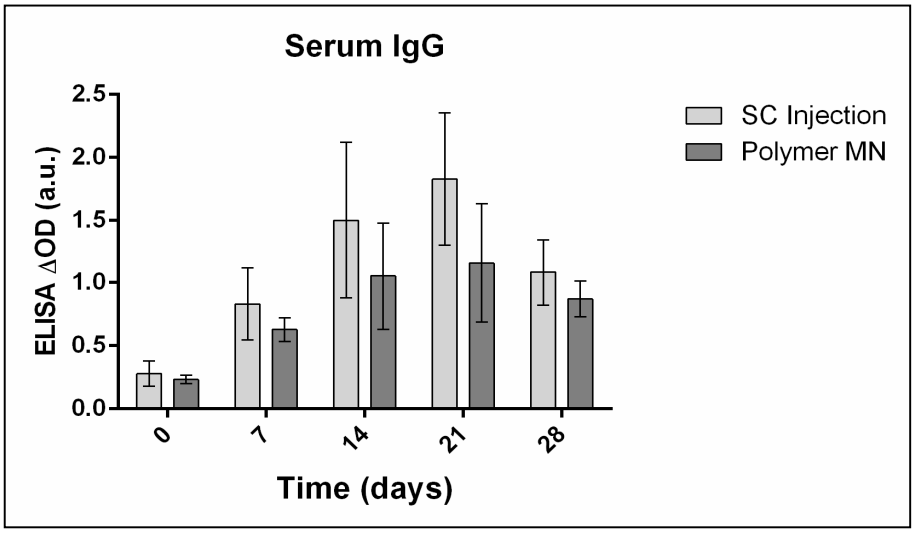

Figure 19: Serum IgG levels as measured by ELISA.
Sera was tested weekly for the first 28 days following vaccination for the presence of measles-specific IgG. The bars represent the average (N = 4) ± SD.

Vaccination using dissolving microneedles resulted in a lower day 14 OD value when compared to the subcutaneous vaccination group. This difference was statistically significant ($p<0.0005$). The difference remained significant on day 21 ($p<.005$) but was no longer significant on day 28. Measles IgM ELISA OD values have not been shown to correlate with eventual neutralizing titer values. While the lower values produced by the dissolving microneedle group was unexpected it did not result in lower neutralizing titers at any point after the vaccination.

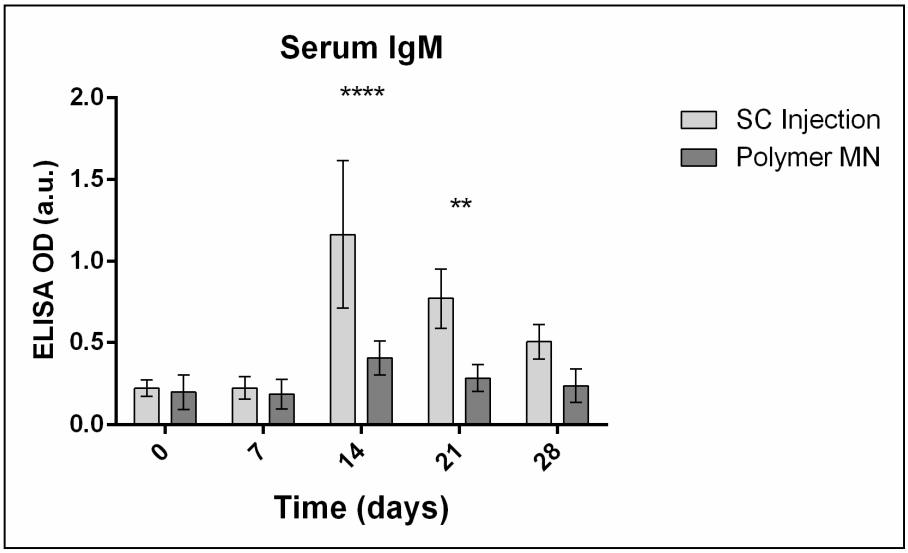

Figure 20: Serum IgM levels as measured by ELISA.
Sera was tested weekly for the first 28 days following vaccination for the presence of measles-specific IgM. The asterisk (** or **) represents a significant difference ($p<0.00005$ or $p<0.005$ respectively) as measured by ANOVA. The bars represent the average (N = 4) ± SD.**

6.3.3 Safety

The microneedle patches were well tolerated in all animals with no adverse effects seen by the investigators or animal care staff. Following removal of the patch, some mild redness was present around the site of insertion. One week after patch removal the redness had dissipated and the microneedle insertion site was indistinguishable from other shaved portions of the animal (Figure 21). The veterinary staff reported no study-related health problems in the animals and no irritation or evidence of discomfort was seen in any animal at the application site.

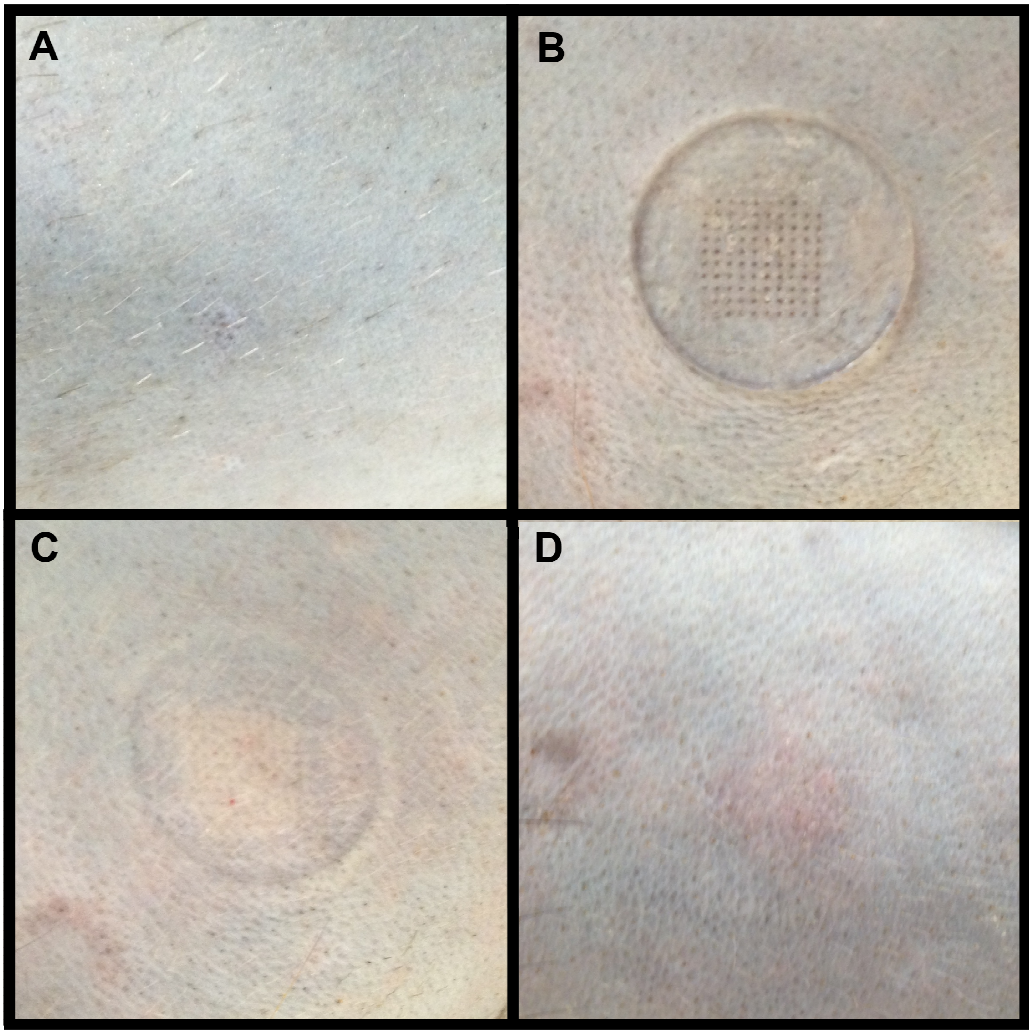

Figure 21: Rhesus macaque skin before and after microneedle patch insertion. The pictures represent the same area of skin at different time points before and after microneedle patch insertion. A: After hair removal and immediately before insertion. B: After insertion of the patch. C: Immediately after patch removal. D: 1 week after patch removal.

6.3.4 Microneedle patch stability

Complete microneedle patches containing a high dose of measles vaccine were stored at multiple temperatures to examine their stability over time. Previous studies have shown that the addition of excipients have the potential to significantly increase the

ability of the dried measles vaccines to retain activity over time [92, 122]. This was the first study to examine the stability of measles vaccine which had been encapsulated in dissolving microneedle patches.

Though there was some variability in the early data, microneedle patches containing both measles vaccine and stabilizing excipients retained complete infectivity as measured by $TCID_{50}$ after 2 months of storage at both 4°C and 22°C (Figure 22). There was no detected loss in either of the samples at the end of the study. The lower infectivity titers at early time points could be the result of variability between microneedles or could

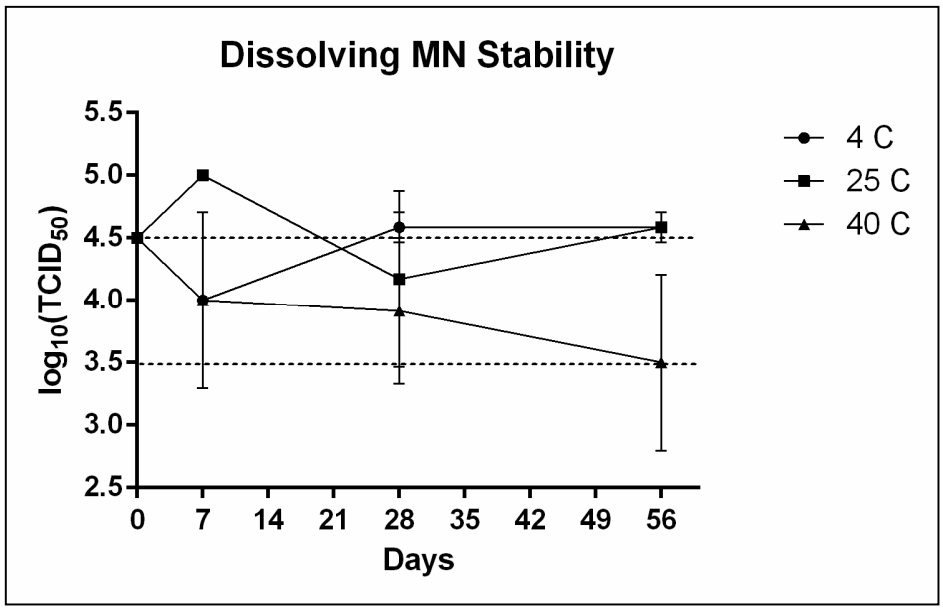

Figure 22: Stability of measles virus after microneedle creation and storage. Microneedle patches were created which encapsulated measles vaccine. The vaccine was mixed with the stabilizers sucrose and threonine during needle processing as indicated in Section 6.2.2.2. The patches were stored for the number of days indicated at 3 different temperatures and then tested for viral infectivity using a $TCID_{50}$ assay. Humidity was controlled using a color-indicating desiccant. The data points represent the average (N = 2) ± SD. The dashed line represents the range between 100% activity and 1 log unit loss of infectivity compared to the starting titer.

have been caused by the inherent error present in the $TCID_{50}$ assay.

The infectivity loss at the higher temperature was more pronounced. The microneedle patch lost 32% of its infectivity after the first week of storage. At the end of 2 months the encapsulated vaccine retained, on average, approximately 10% of its original potency. The data from this study was compared to the stability results obtained from the eGFP-MeV assay used in Chapter 5 (Section 5.2.3). We found that the average activity at the elevated temperature condition was similar at the 1 month and 2 month time points for both the eGFP and traditional $TCID_{50}$ assays (Figure 23). As expected, the standard deviation for the $TCID_{50}$ assay was much larger.

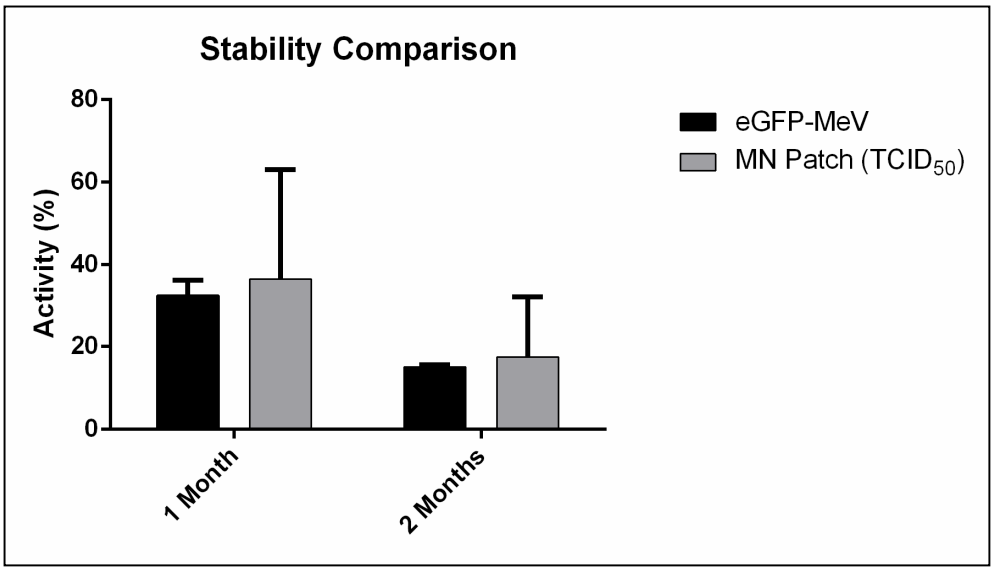

Figure 23: Stability after drying as measured by two different assays.
The activity of the measles virus after drying and storage was measured independently using multiple assays. For the eGFP-MeV test, a small amount of eGFP measles vaccine was placed on a metal chip and dried as indicated in Section 5.2.3. This sample was stored at 45ºC for the time indicated and the fluorescence was compared to a liquid sample of eGFP-MeV. For the MN patch test, the samples were created and stored at 40ºC as indicated in Section 6.2.6. These patches were tested for activity using a $TCID_{50}$ assay and the infectivity was compared to a control MN patch. The bars represent the average (N = 3 or 2 for the eGFP-MeV and MN Patch groups respectively) ± SD.

6.4 Discussion

The current method of delivering the measles vaccine, subcutaneous injection, has a number of logistical drawbacks. These include the requirement for trained personnel at the time of delivery, dangers associated with sharps disposal and the inefficiency caused by vaccine wastage. Any new vaccine delivery method must also generate a potent immune response following delivery. Dissolvable microneedle patches have the potential to mitigate many of these problems and help advance the current measles elimination goals.

The creation of a dissolving microneedle patch that met these requirements was a significant advancement. A patch that was difficult to insert into the skin or did not deliver a full dose of measles vaccine would be insufficient to address the hurdles which are impeding the measles elimination program. Previous work in our lab has shown that microneedle patches consisting of 100 needles was able to insert into the skin with as little as 3 N of force [100]. This is comparable to pushing a button on an elevator. This would represent a significant simplification of the current injection-based delivery method for measles vaccine. Our needles were proven to be both sharp enough to enable easy insertion while simultaneously having the strength necessary to withstand this force without fracturing. Our dissolving microneedle patch requires no additional applicator and we envision a system as simple as applying an adhesive bandage as the final product. By removing the requirement for trained medical personnel, microneedles could allow for door to door measles immunizations. This has the potential to significantly increase the scope of mass vaccination campaigns, helping to accomplish the goal of greater than 95% vaccine coverage in the third world.

Dissolvable needles, such as those used in this study, also eliminate the risk posed by medical sharps waste. Following insertion, the needles on our patch were shown to completely dissolve in the skin. No needle tips remained on the patch after removal. Used

microneedle patches have no potential for reuse, further increasing the safety of mass vaccination campaigns. Additionally, the disposal of non-sharps medical waste requires fewer precautions and has a lower cost than the disposal required for used needles. We imagine the disposal of microneedle patches to entail a similar burden as disposing used bandages.

Vaccine spoilage due to improper handling and temperature conditions remains a significant contributor to campaign costs [95]. Our microneedle patch represents a new mechanism for delivering single vaccine doses which requires no prior modification or reconstitution. The elimination of the reconstitution buffer removes a possible source of handling error which can result in wasted vaccine. The simplification provided by a single contained dosing system would also lower transport and storage costs, which also has the potential to expand the scope of mass vaccination efforts. Our work has shown that encapsulation of the measles vaccine in a microneedle patch can result in lowered loss at elevated temperatures. Current measles vaccine standards require that after reconstitution all doses must be delivered in 6 hours to minimize spoilage. Our patches maintained complete infectivity in both refrigerated and ambient conditions for 2 months. Additionally, at elevated temperatures we found that our patch could last for 2 months before losing 1 log($TCID_{50}$) unit of infectivity. Other studies involving more complicated drying methods showed similar results after storage at elevated temperatures [92]. While the loss in potency at elevated temperatures was significant it still represented a major improvement from our previous work. That study showed that dried vaccine stored at 37^{o}C lost more than 2.5 log units after only a month [122]. This advancement could allow for the possibility of short-term removal of measles vaccine patches from the cold chain during delivery. This would be hugely beneficial as vaccine campaigns moved from major population centers into less developed regions. This would represent a major step towards a safer, cheaper, more effective vaccine delivery system.

We also showed that our microneedle patch was effective and generated a potent immune response after insertion. This response was assessed in rhesus macaques, a well-established, non-human primate model for measles [50]. Previous studies in our lab found that measles delivered using a microneedle was immunogenic and non-inferior to syringe delivery in the cotton rat model [122]. However, microneedle vaccination has never been assessed in a non-human primate model. Rhesus macaques are a well-studied model for measles and produce a robust, easily quantified immune response to both wild-type and live-attenuated virus [130]. For this study, slightly more than a full human dose (~3000 $TCID_{50}$) of measles virus was delivered either subcutaneously or into the skin using a microneedle patch. Both groups had neutralizing antibody levels that were significantly higher than the 200 mUI/ml level that is generally regarded as the minimum titer need for protection. The antibody titers were not statistically different between the two vaccination groups. Titers decreased slightly over time, but remained well above the threshold of protection for over 4 months following a single vaccination. We also tested for systemic serum IgG and IgM levels using an ELISA. Though IgM OD levels were found to be statistically lower in the microneedle group these titers have not been shown to correlate with eventual neutralizing antibody levels. We did show that all animals had a positive IgM response by day 21 which is an important initial marker of vaccine efficacy. IgG OD levels were not found to be significantly different between the groups and all animals had a potent response following vaccination. Therefore, our microneedles have the ability to insert into the skin and dissolve, allowing the released vaccine to reconstitute *in vivo* and subsequently generate a robust immune response.

6.5 Conclusion

This study examined for the first time the immune response in rhesus macaques to measles vaccine delivered using a novel microneedle patch created using a safe, bio-absorbable polymeric compound. These needles were shown to be sharp, strong and able to successfully penetrate the skin to deliver a standard dose of measles vaccine. In addition, the process used to fabricate these patches is simple, and scalable to the extent required by global measles elimination efforts. This work shows that microneedles are a suitable carrier for the measles vaccine and represent a solution to many of the problems that currently face measles mass vaccination programs. We believe measles vaccine delivery using microneedle patches has the potential to greatly aid the measles elimination campaign in its goal to significantly expand global vaccination coverage rates.

6.6 Acknowledgments

I would like to thank the animal facility at the Centers for Disease Control and Prevention for their help with animal handling and care. Specifically, Dr. Robyn Engel and Ryan Johnson's help with anesthesia and bleeding was crucial to the success of the study. I would also like to thank Donna Bondy for administrative assistance. This work was supported in part by a grant from the CDC's Global Immunization Division.

CHAPTER 7 Inactivated polio vaccination using a dissolving microneedle patch in the rhesus macaque

7.1 Introduction

Due in large part to the efforts of the WHO and the Global Polio Eradication campaign, worldwide confirmed polio cases have reached their lowest level in history [5]. The current target for the total eradication of the disease is fast approaching [145]. In the quest to reach this outcome, significant financial resources in conjunction with an extremely effective vaccine are crucial. Poliomyelitis is caused from an infection by one of 3 serotypes of poliovirus. Each serotype contains different capsid proteins which dictate cellular receptor specificity and viral antigenicity [146]. Since protection from one strain does not protect against the others, most polio vaccines contain antigens for all three serotypes to confer complete protection from the disease. Of the two vaccine types, the oral polio vaccine (OPV) is the easiest to deliver, extremely inexpensive to produce and has a high immunological efficacy [107]. Unfortunately, due to its nature as a live-attenuated vaccine, OPV carries with it an ability to revert back to its virulent, wild-type form which can result in the transmission of vaccine-derived polio viruses [108]. The WHO has recommended that as polio eradication progresses, countries should begin to transition from OPV to the inactivated polio vaccine (IPV) [110]. The inactivated polio vaccine does not have any risk of reversion, making it more suitable to a world without poliomyelitis. IPV is currently delivered using a standard needle and syringe injection which presents many problems in a mass vaccination setting. Intramuscular injections require trained personnel to ensure proper vaccine delivery, vaccine spoilage can lead to

significant product wastage and the disposal of medical sharps constitutes a significant burden for large-scale campaigns.

The requirement for trained personnel to deliver IPV is a significant barrier to its wide use in third-world campaign settings. The door to door delivery of OPV is a major factor in the resulting high vaccination rates [147]. Alternative delivery methods which could simplify vaccine administration have the potential to significantly decrease the cost of adapting IPV to the existing OPV framework. Additionally, IPV is traditionally delivered using multidose vials. The number of undelivered doses which are wasted varies significantly from campaign to campaign and can result in the wastage of a nontrivial portion of the potential vaccine stock [148]. Mitigating this wastage would allow for a higher percentage of the purchased vaccine to make it into patients. Finally, transport, storage and eventually disposal of the resulting medical waste presents a problem for large-scale vaccination campaigns [63]. This burden is in addition to the increased risk of needle reuse and misuse created by delivery using syringe injection. Removing the risk of sharps and lessening the burden of medical waste disposal from delivery of IPV would be a major benefit to the global polio eradication campaign. A promising technology with the ability to achieve targeted skin delivery is the microneedle patch.

Microneedles are small, micron-scale needles which penetrate the skin and have the potential to deliver biologics to the upper layers of the skin in a targeted manner [12]. Microneedles have previously been used to delivery other vaccines such as measles, influenza and BCG [111, 115, 122]. Delivery to skin of these vaccines has resulted in either equivalent or enhanced immune response when compared to delivery using a traditional needle and syringe. Microneedle patches are also simple to insert and require minimal training to achieve a successful delivery. This would remove the burden of trained delivery and allow for a better allocation of scarce personnel resources. Microneedle patches can also be fabricated out of bio-adsorbable polymers [2]. After

insertion into the skin, the needles are removed along with the vaccine leaving a patch without the risk posed by contaminated sharps. This reduces the risk to both the vaccinator and patient. Additionally, the patches can then be simply disposed in a manner similar to used bandage. Finally, microneedle patches would require a lower cost to ship and store as a result of their small size and integrated design. Microneedle patches could be designed to contain a single dose of IPV in a compact package that required no secondary support materials. Rather than transporting needles, syringes and vials independently, a single microneedle patch could incorporate everything needed to deliver the vaccine.

This study, for the first time, examines the immune response to delivery of IPV in the skin using a microneedle patch. Dissolving microneedle patches containing a full human dose (40, 8, 32 D-antigen units of IPV type 1, 2 and 3 respectively) were inserted into the skin rhesus macaques. This delivery system was compared to the same dose of IPV delivered using an intramuscular injection utilizing a standard needle and syringe.

7.2 Materials and methods

7.2.1 Concentration of inactivated polio vaccine

Unformulated, monovalent, bulk inactivated polio vaccine was kindly provided by GlaxoSmithKline Biologicals (Rixensart, Belgium). The starting antigen concentration was measured to be 2023, 831, 1081 D-antigen units of IPV type 1, 2 and 3 respectively. Concentration of the bulk IPV was done using Amicon Ultra centrifuge spin filters with a 100kDa molecular weight cutoff (Merck, Whitehouse Station, NJ). The stock IPV was concentrated approximately 38x by volume and the final antigen concentration was measured to be 56,300, 39,500, and 52,300 D-antigen/mL for type 1, 2 and 3 respectively. All D-antigen values were determined by ELISA.

7.2.2 Microneedle fabrication

7.2.2.1 Inactivated polio vaccine filling

Molds consisting of a 10 x 10 array of 300 x 300 x 600 µM pyramidal microneedles with a tip-to-tip spacing of 640 µM were fabricated as previously described [142, 143]. Polio vaccine solution was concentrated as described above. This concentrated stock was added in a 1:1 ratio to a solution consisting of 15% w/v sucrose (Sigma-Aldrich, St. Louis, MO) and 300 mM threonine (Sigma-Aldrich). This solution was mixed and applied to the microneedle mold. The solution was evenly spread over the entire mold surface using a pipet tip. This mold was then placed in a custom-made vacuum system and suction was pulled through the bottom of the mold at a pressure of approximately -3.59 kPa for 20 minutes. The mold was removed from the vacuum system and allowed to dry in a fume hood for 60 minutes. Adhesive tape (Sigma-Aldrich) was applied to the dried mold. This tape was then quickly peeled away to remove any remaining vaccine solution present on the mold.

7.2.2.2 Polymer matrix filling

The backing matrix for the dissolving microneedles was composed of a 40 wt% solution of fish gelatin (Sigma-Aldrich) and 15 wt% sucrose (Sigma-Aldrich) in DI-H_2O. The fish gelatin solution was mixed for 1 hour at 25°C before use. The backing matrix material was spread using a spatula over the tape-stripped microneedle mold. The molds were placed back into the vacuum system and suction was applied at a pressure of approximately -3.59 kPa for 90 minutes. The molds were then allowed to dry for 48

hours at 25°C in a fume hood. To remove the needle patches a circle of dextrin (Sigma-Aldrich) was coated with double-sided tape (MacTac, Stow, OH) and applied to the back of the mold. This disc was gently peeled away from the mold taking care to prevent damage to the needles. Patches were stored in a light-protected, sealed pouch with color-indicating desiccant (Drierite, Xenia, OH) until insertion.

7.2.3 Immunization studies

The immune response to microneedle delivery of inactivated polio vaccine was tested in the rhesus macaque (*Macaca mulatta*). The animals were divided into groups of 4 monkeys each as follows: (1) full-dose delivered by intramuscular injection and (2) full-dose delivered by microneedle patch.

Female, 2 year old rhesus macaques were kept in quarantine for 4 weeks and tested for antibodies to measles, influenza, and canine distemper virus. In order to determine that the animal had no previous exposure to polio, blood was collected from a leg vein using Vacutainer tubes and analyzed by plaque micro-neutralization (BD, Franklin Lake, NJ). Animals were anesthetized by animal facility staff using ketamine during vaccination and blood collection. For the microneedle vaccination group, a section of hair on the upper back of the animal was removed using electric shears followed by an application of depilatory cream (Nair, Princeton, NJ).

After characterizing the dose in each patch, 4 monovalent dissolving patches (2 IPV type 1 patches and 1 patch of type 2 and type 3) containing a total of 47.4, 8.2, 38.2 D-antigen units of IPV type 1, 2 and 3 respectively were pressed into the back, between the shoulder blades, of the each animal. This site was chosen to prevent the animals from scratching the site and causing irritation. The patches were left on the skin for 15 minutes to ensure complete dissolution of the polymer needles. In the groups receiving an

intramuscular injection, the un-concentrated GSK stock solution was diluted using sterile phosphate-buffered saline so that the target dose (40, 8 and 32 D-antigen units of type 1, 2 and 3 respectively) was contained in 500 µL. The intramuscular injection was delivered in a single trivalent preparation using a 25-gauge needle into the thigh muscle of the animal.

Eight weeks after the initial vaccination all animals were given a 2nd dose of IPV. The booster dose was delivered using the same route as the initial vaccination and consisted of the same dose of IPV.

Once a week approximately 10 mL of blood was removed from a leg vein on each animal. Following the completion of the study all animals were transferred to other protocols within the CDC. The protocol for the rhesus macaque study was approved by the Animal Care and Use Committees of the Centers for Disease Control and Prevention and the Georgia Institute of Technology.

7.2.4 Neutralizing antibody measurements

Neutralizing antibody titers to poliomyelitis were measured from collected sera using methods previously described [149]. In this study, two-fold dilutions of serum were tested starting at a dilution of 1:4.

7.2.5 Enzyme-link immunosorbant assay

Antibody-capture ELISA was used for the detection of D-antigen poliovirus. Poliovirus-specific monoclonal antibodies (mAb) were used as both capture and detection antibodies. Type 1 (NBP1-05101, Novus Biologicals, St. Louis, MO) type 2 (HYB294-06, Thermo Fisher Scientific, Waltham, MA), or type 3 (HYB300-05, Thermo Fisher

Scientific) specific antibodies were diluted in 0.05M carbonate-bicarbonate buffer, pH 9.6. Type 1, 2 and non-D-antigen type 3 capture mAb were diluted 1:1000. For the D-antigen specific type 3 ELISA the capture antibody (HYB300-06, Thermo Fisher Scientific) was diluted 1:500. Fifty microliters of diluted antibody was dispensed into Immulon 2HB high-binding 96-well plates (NUNC, Inc., Penfield, NY) which were incubated for a minimum of 16 hours or up to 7 days at 4°C in a moist chamber. The antibody-coated plates were washed four times in wash buffer (0.01 M PBS, pH 7.2 with 0.05% Tween 20), followed by blocking with 100 µl of dilution buffer (0.01 M PBS, pH 7.2 with 0.5% gelatin and 0.25% Tween 20) for 60 min at 37°C in a humid chamber. The plates were then washed four times with wash buffer and 50 µl of was added to each well of the antibody-coated plates and incubated for 60 min at 37°C in a moist chamber. For detection, horseradish peroxidase labelled (HRP) poliovirus type-specific monoclonal antibodies were prepared with a Lightning Link Conjugation kit (HRP, 1 x 100 µg reaction) (Novus Biologicals). All HRP-conjugated antibodies were diluted at 1:1000 in dilution buffer. The plates were washed four times in wash buffer and 50 µl of diluted serotype-specific HRP-labelled mAb was added to each well. The plates were incubated for 60 minutes at 37°C in a moist chamber, washed four times with wash buffer, and 50 µl of SureBlue Reserve TMB Microwell Peroxidase Substrate (1-Component) (KPL, Gaithersburg, MD) was added to each well. The plates were incubated at room temperature for 15 minutes and the reaction was stopped by addition of 50 µl of TMB BlueSTOP Solution (KPL). Plates were then evaluated on a plate spectrophotometer at a wavelength of 620 nm.

7.2.6 Statistics

All statistics for this study were calculated using Prism software version 6.02 (Graphpad, La Jolla, CA). Comparisons between individual samples were done using an unpaired t-test was a significance cutoff of $p<0.05$. Comparisons between multiple samples was done using a two-way ANOVA with a Tukey post-test.

7.3 Results

7.3.1 Microneedle patch testing

The patches created during the course of this study were designed to be simple to manufacturer, easy to insert and able to contain a full dose of the inactivated polio vaccine. Using a vacuum filling system, dissolving microneedle patches were molded out of a fish gelatin and sucrose solution. The resulting patches contained a 10 x 10 array of pyramidal needles which were approximately 650 µm in height (Figure 24). These patches were first tested to ensure they possessed both the strength to successfully insert into the skin and the correct dose of inactivated polio vaccine. Needle strength was assessed by inserting fully-made patches into pig skin and examining the ability of the needles to pierce the skin. This method has been previously used to test needle strength [24]. Upon insertion into the skin, small holes are created in the stratum corneum. These holes can be detected with the application of a dye. Microneedle patches were inserted into pig skin for 15 minutes and then examined under magnification. After staining, an insertion success rate of 100% was calculated by counting the detected holes and comparing it to the 100 needles on the microneedle patch (Figure 25). Subsequent testing was done to ensure that the needle fully dissolved after insertion. After 15 minutes of insertion into pig skin the vast majority of both the needle shaft and needle base were dissolved (Figure 27). This test confirmed that the microneedle patches created for this

study were both strong enough to pierce the skin intact and also rapidly dissolved after insertion.

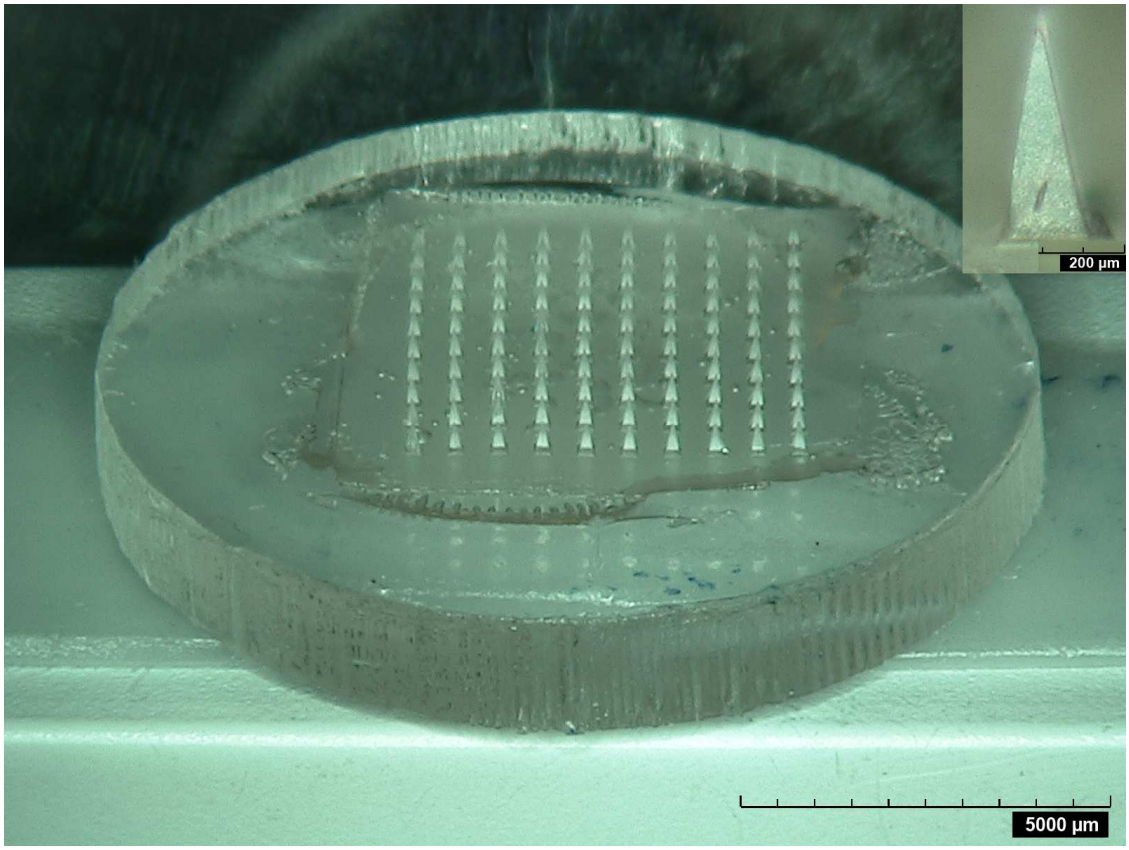

Figure 24: Microneedle patch containing IPV at high magnification.
Dissolving microneedle patches were created out of fish gelatin using a vacuum filling process. The needles were approximately 650 µm in height. A 10 x 10 array is shown. The insert shows a single needle from this array at high magnification.

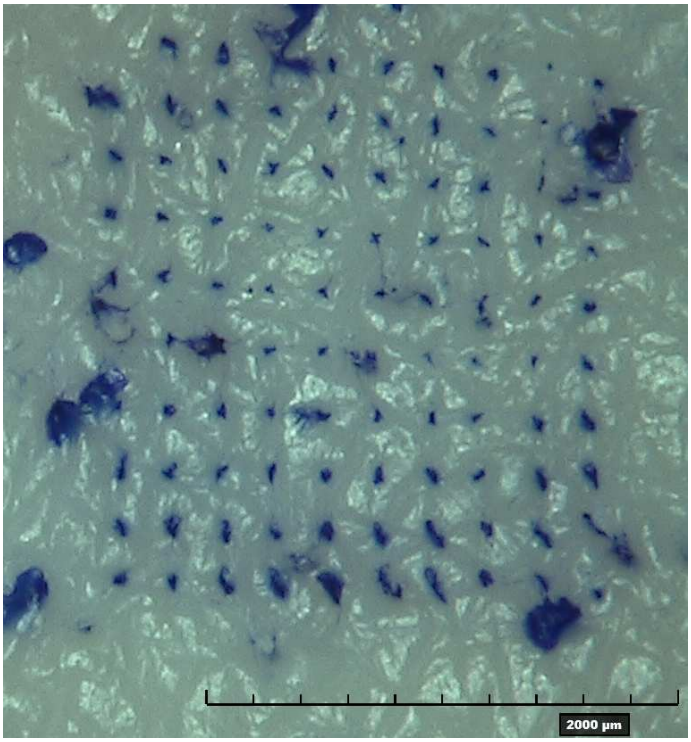

Figure 25: Pig skin after microneedle patch insertion and staining.
A single microneedle patch was inserted into excised and shaved pig skin. The patch was removed after 15 minutes of insertion. The skin was then stained to show the puncture sites.

The needles were then assayed for their IPV content using a serotype-specific ELISA. Initial testing found that each monovalent patch contained an average of 23.7, 8.7 and 38.2 D-antigen units of IPV type 1, 2 and 3 respectively. After the completion of the study it was found that the antibody in the ELISA used to measure the dose of IPV type 3 was not specific for the D-antigen conformation of the antigen. Testing was done to compare type 3 patches on both the old and a new ELISA which utilized a D-antigen specific antibody. It was found that the delivered D-antigen dose for type 3 was approximately 66% lower than expected ($p<0.05$) (Figure 26).

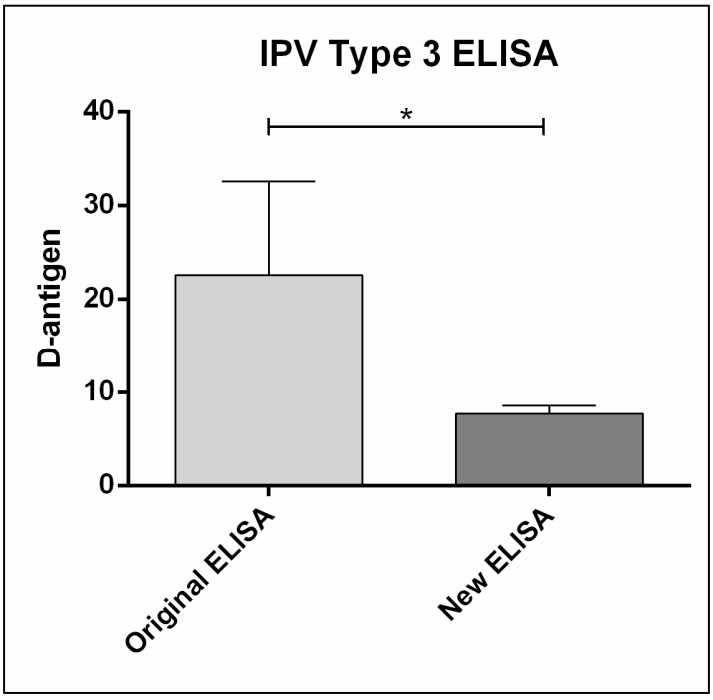

Figure 26: IPV 3 dose tested with multiple ELISAs.
The D-antigen amount loaded into multiple microneedle patches was assayed using two different ELISA tests. The original ELISA utilized an antibody that was not specific for the D-antigen region of IPV type 3 while the new ELISA used a different ELISA that had this specificity. The results show that after needle creation, much less of the IPV type 3 retains the D-antigen conformation than originally estimated. Each bar represents the average (N = 3) of individually tested patches. The asterisk (*) denotes a significant difference ($p<0.05$) as measured by an un-paired t-test.

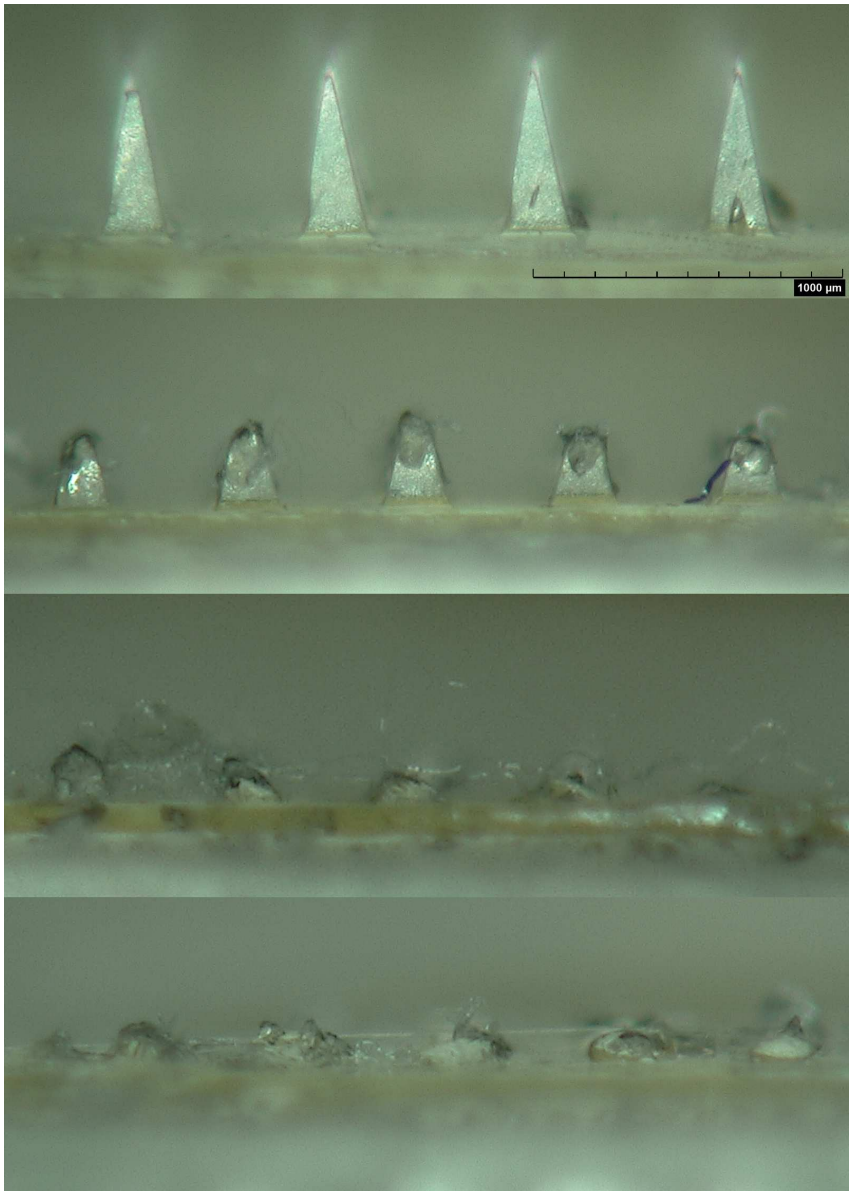

Figure 27: Microneedle patch before and after insertion into pig skin.
Microneedle patches were created and then inserted into pig skin to determine the dissolution time. From top to bottom, this figure shows microneedles at high magnification 0, 1, 5 and 15 minutes after insertion and removal.

7.3.2 Immunization Study

The rhesus macaque was chosen for this study because it has historically been used to test inactivated polio vaccine efficacy and viral potency [150]. Prior to vaccination, each animal was tested for pre-existing, neutralizing antibodies to each polio serotype. For this study two groups were used. The first group (n = 4) received at least a full human dose of IPV type 1 and 2 (47.4, 8.7 D-antigen units) delivered using a dissolving microneedle patch. These animals only received approximately 13.06 D-antigen units of type 3 as a result of incorrect initial ELISA information. The second group (n = 4) received a full human dose delivered intramuscularly using a needle and syringe. The animals were bled weekly and the sera were tested for neutralizing antibodies using the gold-standard micro-neutralization assay [151]. For polio serotype 1, 75% of the animals which received a dissolving patch had a positive titer ($\geq$ 3.0 $\log_2$ units) by 8 weeks after vaccination while the IM injection group displayed 50% seropositivity. The average titer for the dissolving microneedle and IM injection groups at this time point was 3.9 and 4.3 respectively. All animals exhibited higher antibody titers to a single dose of IPV type 2 when compared to the other IPV serotypes. The enhanced immunogenicity of IPV type 2 after a single polio immunization has also been seen in vaccination studies done in children [152]. For type 2, the seropositivity at 8 weeks was 100% for both groups while the average titer was 5.5 and 6.3 for the microneedle and IM groups respectively. The immunologic response to IPV type 3 was the weakest amongst the three serotypes. No animals in either group were seropositive at the 8 week time point.

The low titers observed at 8 weeks post-vaccination for IPV type 1 and type 3 necessitated the need for a second vaccine dose, delivered 8 weeks after the initial vaccination. The booster dose also produced no adverse events. Following the second dose, almost all of the animals exhibited a priming response. The average $\log_2$ titer for IPV 1 peaked at 10.1 and 11.4 for the dissolving needle and intramuscular injection groups respectively. The average titer for IPV 2 peaked at 11.9 and 12.8 for the two groups. The neutralizing antibody responses were not significantly different between the two groups for IPV type 1 or 2 ($p > 0.05$). In addition, the average titer on each day was statistically similar for each group ($p > 0.05$). This indicates that the immune response to IPV type 1 and type 2 was indistinguishable when comparing microneedle delivery to an intramuscular injection. This was not the case for IPV type 3. Following the boost, the average titer for the intramuscular injection peaked at 9.3 while the peak titer for the dissolving needle group was 3.7. The average titers between the two groups were statistically different over time ($p<0.005$). This inferior response could be due to the low dose delivered for type 3 which was detected after the completion of the study using a D-antigen specific type 3 ELISA. Animals were also vaccinated using coated, metal microneedle patches. The response to this delivery route proceeded comparably to the other two groups. The neutralization titers were statistically lower when compared to the subcutaneous control at a number of time points after the boost for all IPV serotypes (Figure 31). The final IPV type 3 seropositivity results were also lower in the metal microneedle group.

At the conclusion of the study, the seropositivity rates were 100%, 100%, 25% for the dissolving microneedle patch group and 100%, 100%, 75% for the group receiving an IM injection for types 1, 2, and 3 poliovirus respectively (Figure 28).

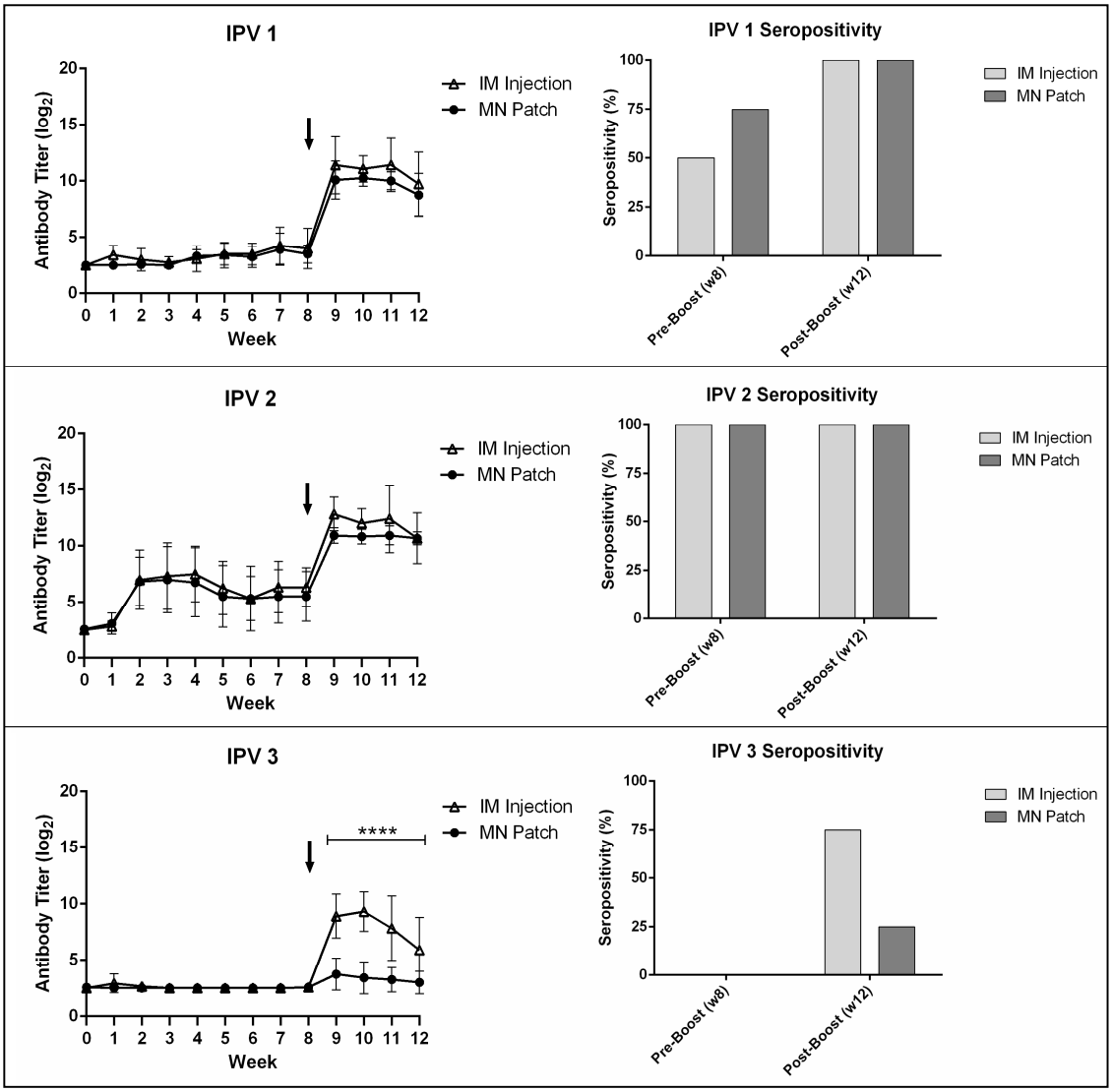

Figure 28: Neutralizing titers to IPV following vaccination.
Sera was taken from rhesus macaques weekly following vaccination. Neutralizing titers were determined using a serotype-specific plaque micro-neutralization assay. We detected no significant difference in titer between the groups for IPV type 1 or 2. There was a difference between the groups for IPV type 3. Each data point represents the average (N = 4) ± SD. The asterisk (****) represents a significant difference ($p<0.00005$) as measured by ANOVA. Seropositivity was defined as a titer greater than or equal to 3.0 log_2.

7.3.3 Safety

Following either vaccination, no adverse effects were noted after microneedle insertion or intramuscular injection. After removal of the microneedle patches a small grid of puncture sites was faintly visible and minor redness existed where the edges of the patch pressed into the skin (Figure 29). The grid of puncture sites was no longer visible 2-3 days after insertion. No bleeding was observed for any of the vaccinations. The vaccination sites were examined daily by animal care staff and no study-related adverse effects were seen. No swelling, discharge, irritation or other abnormalities were observed at any point during the study.

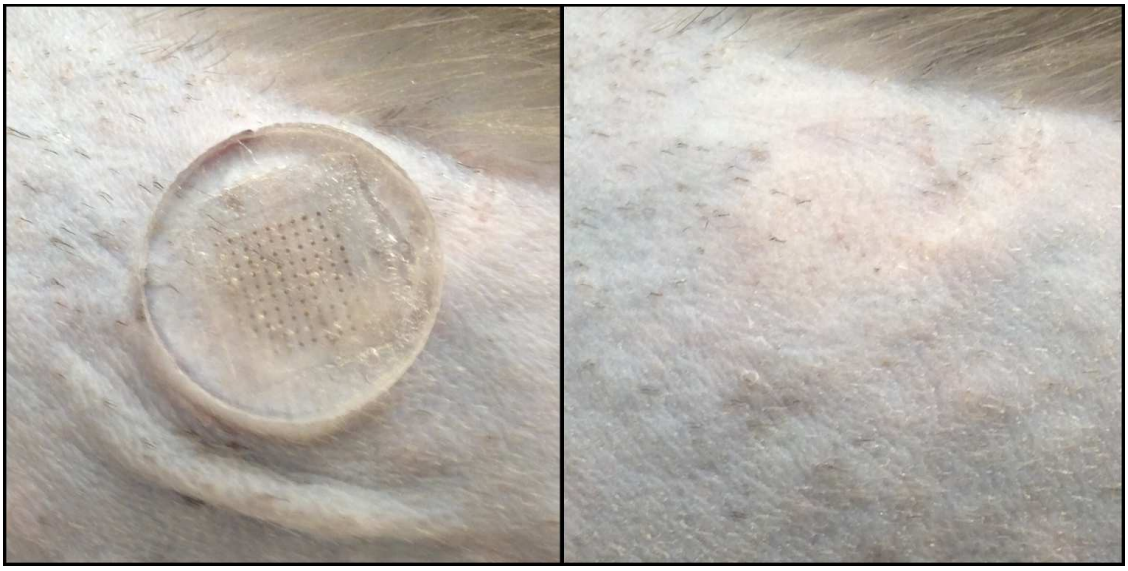

Figure 29: Rhesus macaque skin during and after microneedle patch insertion.
A 10 x 10 array of dissolving microneedles were inserted in the skin of the rhesus macaque between the shoulder blades after hair removal. These patches were removed after 15 minutes of insertion. The left image shows the patch in the skin. A grid can be seen where the needles have punctured the skin. The right image is the same area of skin immediately after patch removal. A faint grid can be seen which dissipated after 2-3 days.

7.4 Discussion

The WHO has recommended that member countries begin transitioning to the inactivated version of them polio vaccine as eradication progresses due to in large part because this vaccine has no danger of reverting to a virulent form. IPV is currently delivered using a needle and syringe which introduces a number of drawbacks when compared to the oral delivery route utilized by OPV. Syringe injection requires the presence of trained personnel and increases the risk of disease transmission due to needle reuse. The transition to IPV would be greatly aided by an improved method of delivering the inactivated polio vaccine [153].

We have created a microneedle patch using a process that is simple, repeatable and importantly, scalable to the demands required by the polio eradication campaign. These patches can be inserted into the skin without the use of a secondary applicator, simplifying administration. Microneedle patches would also likely not require medically trained personnel to ensure proper insertion. This change would shift the vaccination scheme towards the more efficient method currently used with the oral polio vaccine. Dissolving microneedles in particular have the potential to eliminate the risk of sharps contamination since the needles no longer remain on the patch after insertion into the skin. Finally, a single-dose packaging system could decrease both shipping costs and medical waste, which are significant factors in large-scale vaccination campaigns. Our patches were tested in pig skin and found to easily insert with near-complete needle dissolution within 15 minutes. These are important qualifications for a delivery device intended to overcome many of the hurdles posed by the upcoming transition from OPV to IPV.

The microneedles patches were then inserted into the skin of a rhesus macaque and delivered at least a full human dose of inactivated polio vaccine types 1 and 2. The vaccine remained immunologically active and induced a potent neutralizing antibody

response after two doses. The serologic response to IPV type 1 and type 2 delivered using a microneedle patch was statistically indistinguishable from a similar dose delivered using a traditional needle and syringe. The positive response to IPV type 2 is especially important. The final stage of polio eradication calls for the administration of a bivalent oral vaccine protecting against polio type 1 and 3 with a supplementary dose of IPV type 2 [110]. It is expected that this delivery schedule will continue throughout the final years of the eradication [154].

The immune response to IPV type 3 proved to be inferior when microneedle delivery was compared to an intramuscular injection. The D-antigen component of polio is thought to be the primary immunogenic region of the virus [155]. We hypothesize that this region of IPV type 3 was damaged during the creation of the microneedle patches. When comparing the results of our original type 3 ELISA to an improved type 3 ELISA based on a D-antigen specific antibody, testing showed that the delivered dose of IPV was much lower than predicted. We believe this was the primary factor which contributed to the lowered immune response. Further testing will be done to improve the needle creation process with the goal of minimizing antigenic loss for IPV type 3.

One alternative method that has been previously used to deliver IPV into the skin is the liquid jet injector [156]. Jet injectors are devices which propel a liquid formulation of the vaccine at speeds high enough to penetrate stratum cornea, depositing their payload in the dermal or subcutaneous layers of the skin [119]. While suited to deliver many doses in rapid succession, care must be taken to prevent disease transmission due to nozzle contamination [120]. This problem has been largely solved through the use of disposal parts, though this would require a fresh applicator for each patient adding weight to both the initial shipment and eventual medical waste elimination. These devices are also not ideal for delivering vaccines in a door to door setting, limiting the potential reach of large-scale vaccination campaigns. We believe that microneedles have the potential to safely and effectively deliver IPV into the skin without these associated drawbacks.

7.5 Conclusion

Dissolvable microneedle patches represent a significant advance over traditional delivery of the inactivated polio vaccine. They are simple to administer, contain no risk of sharps and their small size has the potential to reduce the cost of vaccine storage and transport. This study showed that this alternative delivery method can induce a potent immunogenic response to skin delivery of IPV. More work needs to be done to optimize this new delivery platform. Future studies integrating all three serotypes into a single patch and work towards a Phase I clinical trial are ongoing. As the endgame nears for the global campaign to eliminate poliomyelitis, microneedles represent a possible solution to many of the final hurdles.

7.6 Acknowledgements

I would like to thank Naomi Dybdahl-Sissoko and Dr. Will Weldon for their help planning and carrying out this study. I would also like to thank Ryan Johnson and Dr. Robyn Engel from the Veterinary staff at the Centers for Disease Control and Prevention for their assistance bleeding and anesthetizing the animals. I would also like to thank Dr. James Norman for his help calculating the statistics used in this study. Finally I would like to thank Donna Bondy for her administrative assistance. This work was funded in part by the Bill and Melinda Gates Foundation, the World Health Organization and the Centers for Disease Control and Prevention's Global Immunization Division.

CHAPTER 8 CONCLUSIONS

Microneedles have been shown to be an effective delivery vehicle for administration of the live-attenuated and inactivated polio vaccines. This project encompassed the development of microneedle patches for measles and polio vaccines from concept to a preclinical, non-human primate study. A summary of the relevant advances from each study are detailed below:

8.1 Measles Vaccination in Cotton Rats using a Microneedle Patch

The goal of this study was to adapt the live-attenuated measles vaccine into currently existing metal microneedle patch technology. We have shown that the measles vaccine can be dried and stored for short time periods with acceptable levels of activity loss. This loss can be minimized with the addition of stabilizing excipients. We also showed that after delivery to the skin, microneedles produce a neutralizing antibody titer statistically equivalent to delivery using a standard subcutaneous injection. Specific advances are as follows:

- The live-attenuated measles vaccine rapidly loses activity after drying. More than 1.0 log($TCID_{50}$) units of infectivity loss was seen after 24 hours.
- Infectivity loss can be mitigated by the addition of excipient compounds. While fish gelatin and malto-dextrin had minimal effect on stability, the sugar trehalose proved to be highly efficacious.
- A combination of trehalose and protection from humidity resulted in a formulation that was stable for 30 days at 25^{o}C.

- This formulation was less stable at elevated temperatures. A loss of more than 2.0 log($TCID_{50}$) units was seen after 1 month of storage at 37°C.
- Microneedle delivery of measles vaccine was shown to be equivalent to the same dose delivered using a subcutaneous injection. Peak neutralizing titers for the full dose of vaccine were 6.4 and 6.2 log_2 units for the microneedle and injection groups respectively.
- No dose sparing was seen after delivery of the measles vaccine to the skin. The titers for a 20% dose delivered using microneedles or a subcutaneous injection were equivalent. Peak titers for the 20% dose were 5.4 and 4.6 log_2 units for the microneedle and injection groups respectively.

8.2 Discovering Stabilizing Excipients for the Measles Vaccine using a High-throughput eGFP assay

The goal of this study was to develop an assay which could rapidly screen excipients for their ability to stabilize the live-attenuated measles vaccine after drying and storage. We found the eGFP-MeV could be used in a 96-well plate assay to quickly and simply measure the activity of measles vaccine after storage at a variety of temperatures. This assay allowed us to screen a large number of excipients for their ability to stabilize the measles vaccine virus after drying. The results of this study are summarized below:

- Measles vaccine virus encoding green fluorescent protein was used to develop an assay to rapidly assess viral infectivity. This significantly increased the throughput of infectivity testing over the standard $TCID_{50}$ assay.
- This assay was able to measure the infectivity of 96 separate samples of measles virus in less than 5 minutes and after only 3 days of incubation.

- This assay was used to test the stabilizing activity of more than 40 common commercial excipients.
- It was determined that a combination of an amino acid and a sugar had significantly higher stabilizing activity than either compound individually.
- The best formulation was determined to be a combination of the amino acid threonine and the sugar sucrose.
- This formulation maintained nearly complete measles vaccine activity after 6 months of storage at 4^{o}C or 25^{o}C.
- Activity loss was greater after storage at 45^{o}C. The virus maintained at least 10% of its initial activity until the 12 week time point. After 6 months the activity level was detected to be <1% of a control sample.

8.3 Measles Vaccination of Non-human Primates using a Microneedle Patch

This study examined the delivery of measles vaccine using a dissolving microneedle patch using a non-human primate model. Dissolving microneedles were created that encapsulated active measles vaccine. These needles were strong enough to penetrate the skin and rapidly dissolved after insertion. Furthermore these needles retained infectivity following creation and storage at elevated temperatures. Following administration into the skin of a rhesus macaque, dissolving microneedles produced equivalent antibody titers to the same dose of vaccine delivered using a subcutaneous injection. The important findings of this study are summarized below:

- Dissolving microneedles made from poly-vinyl alcohol were created which encapsulated the live-attenuated measles vaccine.
- These patches were strong enough to penetrate pig skin during initial testing. Force testing determined the fracture force for each needle to be approximately

0.5 Newtons. Following insertion into pig skin, the bulk of the needle completely dissolved within 10 minutes.

- Needles were inserted into the skin of a rhesus macaque and tested for their ability to deliver the measles vaccine. Following insertion it was found that >90% of the encapsulated vaccine was delivered into the skin.
- Both the dissolving microneedle patch and subcutaneous injection groups produced a potent neutralizing antibody response. The levels between the two groups were found to be statistically equivalent.
- All animals demonstrated a positive serum measles-specific IgM response as measured by ELISA 14 days after vaccination. This is considered an important marker of a successful measles vaccination.
- All animals demonstrated increased measles-specific serum IgG titers as measured by ELISA following vaccination. The levels between the two groups were not statistically different.
- Following a boost of monovalent measles vaccine delivered subcutaneously, all animals demonstrated a measurable increase in antibody titer.
- Microneedle patches containing measles vaccine proved to be stable after extended storage. The patches demonstrated no activity loss after 2 months of storage at 4^{o}C or 25^{o}C.
- Lower activity was seen after storage at 40^{o}C. These patches showed a 1.0 log($TCID_{50}$) lower titer after 2 months of storage.

8.4 Inactivated Polio Vaccination using a Dissolving Microneedle Patch in the Rhesus Macaque

This study focused on development of a dissolving microneedle patch containing a full human dose of the trivalent inactivated polio vaccine. Microneedle patches, created out of fish gelatin, were used to deliver IPV into rhesus macaques and the response to skin delivery was compared to the same dose delivered using a standard intramuscular injection. The major findings of this study are summarized below:

- Initial testing showed that these patches successfully maintained antigenicity after creation as measured by a serotype-specific ELISA.
- Following administration into the skin of a rhesus macaque, a robust neutralizing antibody response was seen to IPV serotype 2 but not to serotypes 1 or 3. This phenomena was also observed with the same dose of vaccine delivered using an intramuscular injection.
- Following a boost delivered using the same routes, a marked increase in type 1 and type 2 antibody titers was seen in both groups. An increase in type 3 titers was seen after intramuscular injection but not after microneedle delivery.
- It was determined that the ELISA used to detect IPV type 3 was not D-antigen specific. A new ELISA was developed which was specific for the immunogenic D-antigen region of IPV type 3.
- This new ELISA demonstrated that the microneedle patches only contained approximately 30% of the targeted dose of IPV type 3 before insertion.
- Neutralizing antibody titers for type 1 and type 2 IPV were equivalent between immunized groups. Titers to type 3 were significantly lower in the microneedle group. This is thought to be a result of the substantially lower dose delivered.

CHAPTER 9 FUTURE DIRECTIONS

9.1 Improving the dissolving measles microneedle patch

The studies performed showed that the measles vaccine could be encapsulated into a dissolving microneedle patch which maintained activity at 4°C and 25°C for 2 months. The success of these results were tempered by the fact that the patches stored at 40°C lost nearly 90% of their activity by the same time point. More work can be done to further increase measles vaccine stability. Removing measles vaccine from the cold chain would require the creation of a much more stable patch. A more complete screen of excipients using the developed eGFP-MeV assay could be performed which may result in formulations with an even higher level of stabilizing activity. Current WHO standards state that measles vaccine must not lose more than 1.0 log($TCID_{50}$) after 2 years of storage at 4°C. This is also a good target for high temperature stability. If a microneedle patch could be created which met this condition at 40°C, it would provide a compelling scientific argument to allow for a measles patch to be transported and stored outside of the cold chain. Since the temperature fluctuates significantly between night and day, patches may not need to maintain activity at this elevated temperatures for an extended period of time. Transient temperature stability may be sufficient.

Further testing must also be performed on measles microneedle patches before a product could be approved. While patches were tested for measles activity after storage, they were not tested for insertion ability or immunogenicity. Since the patches rapidly dissolved in the presence of moisture in the skin, testing must be performed to ensure that the needles don't lose mechanical strength following storage. Even packaging designed to minimize humidity may result in a small amount of water retention in the patch. It is

unknown whether this could result in microneedles that are no longer strong enough to pierce the skin. Mechanical testing must be performed after extended storage to ensure that the microneedle patches remain sharp and retain the ability to easily insert into the skin. Immunogenic testing also needs to be performed after storage. While the $TCID_{50}$ assay directly measures viral infectivity, this is not sufficient to confirm that patches retain the ability to elicit a potent immune response after delivery. Patches should be stored for an extended period of time at a range of temperatures and then inserted into cotton rats to determine their immunogenicity. Cotton rats would be recommended since rhesus macaques would be prohibitively expensive for this type of study.

9.2 Measles + Rubella patch development

Currently, the measles vaccine is often co-administered with the rubella vaccine during mass vaccination campaigns. This is designed to rapidly expand rubella vaccine coverage in addition to the standing goal of measles elimination. A microneedle patch which could simultaneously deliver both of these antigens would be extremely beneficial to these joint goals. Developmental steps for this patch could follow a similar track as the experiments in aim 3. Short term stability of a combination patch should be tested in the presence of stabilizing excipients. Once confirmed, patches could be tested in the rhesus macaque. Preliminary studies carried out during aim 3 showed that rubella vaccine is infectious in the rhesus macaque and stimulates a potent antibody response. Further optimization could then focus on increasing the stability of each vaccine component. Unfortunately no rapid screening assay yet exists for rubella, so testing would need to be done using a traditional infection and staining assay.

9.3 Dissolving inactivated polio microneedle patch enhancement

While the inactivated polio vaccine dissolving microneedle patch was successful in inducing a potent immune response to IPV types 1 and 2, the response to type 3 was subpar. This was determined to be the result of inaccurate dosing information provided by our type 3 ELISA. Since this assay was not specific for the D-antigen region of IPV type 3, it did not detect a substantial drop in activity after microneedle casting and creation. After development of a more specific assay, it was found that the dose delivered using microneedles was only about 30% of the target full human dose. More work must be done to determine the cause of this activity loss and eliminate it. Once a patch is created which can maintain the short-term antigenicity of all three polio serotypes, another rhesus vaccination could be performed. This would hopefully result in a potent antibody response to the entire trivalent vaccine. This is a crucial hurdle that must be overcome before this product could move into clinical testing.

9.4 Transgenic mouse polio challenge study

While neutralizing antibody titers can be predictive of protection from wild-virus a true challenge study would be very beneficial to any eventual FDA clinical trial application. Unfortunately, very few animal models currently exist for this kind of study. One that is well-studied and WHO-approved, is the Tg-PVR21 mouse model. This consists of a genetically modified mouse that has been altered to express the human poliovirus receptor. This animal displays clinical signs of infection after intracranial or intramuscular application of a sufficient quantity of wild-type poliovirus. It is currently used by the WHO during studies to verify the efficacy of polio vaccine candidates. This model could be used to examine the protective ability of IPV delivered using a

microneedle patch. Mice would be vaccinated using both dissolving needle patches and a traditional intramuscular injection. After approximately 1 month, the animals would be challenged using a 10x PD_{50} dose of wild-type poliovirus. Since each serotype of IPV only protects against the companion wild-type strain, a single serotype would need to be chosen for testing. IPV type 2 is the mostly widely used in the literature and requires the lowest dose to achieve seroconversion. The animals typically display signs of paralysis within 10 days of infection, so daily monitoring of activity and weight would be required. This could prove as an important preclinical study proving the efficacy of microneedle patch delivery of IPV.

APPENDIX

A.1. Tables

A.1.1. Excipients used in stability screening study.

Table 2: Excipients and concentrations chosen for stability study.
All excipients were acquired from Sigma-Aldrich, St. Louis, MO. All amino acids were in the L-configuration and all sugars were in the D-configuration unless indicated otherwise.

Proteins 300 mM Alanine BioXtra, USP-grade, >98.5% pure 300 mM Arginine Reagent-grade, >98% pure 300 mM Asparagine Bioreagent, suitable for cell culture, >98% pure 300mM Cysteine NI-grade, >97% pure 300 mM Glutamine ReagentPlus-grade, >99% pure 300 mM Glycine BioUltra-grade, >99% pure 300 mM Histidine USP-grade, cell culture tested 300 mM Isoleucine Reagent-grade, >98% pure 300 mM Leucine USP-grade, >98.5% pure 300 mM Lysine >98% pure	300 mM Methionine Reagent-grade, >98% pure 100 mM Phenylalanine Reagent-grade, >98% pure 300 mM Proline USP-grade, cell culture tested 300 mM Serine USP-grade, >98.5% pure 300 mM Taurine >99% pure 300 mM Threonine Reagent-grade, >98% pure 300 mM Tyrosine Reagent-grade, >98% pure 300 mM Valine USP-grade, >98.5% pure

Sugars 15% Arabinose L-form, microbiology-grade, >99% pure 300 mM Arabitol Premium quality level, >98% pure 1% Dulcitol >99% pure 15% Fructose Premium quality level, >99% pure 15% Galactose >99% pure 5% Lactose Monohydrate, USP-grade 15% Maltose Monohydrate, BioUltra-grade, >99% pure 15% Raffinose Pentahydrate, BioXtra-grade, >99% pure 15% Sorbitol >99% pure 15% Sucrose BioUltra-grade, >99.5% pure	15% Tagatose >98.5% pure 15% Trehalose Dihydrate, BioReagent-grade, >99% pure 15% Xylitol >99% pure 15% Xylose BioUltra-grade, >99% pure **Other** 5% Chitosan Low molecular weight 2%, 4% Human Serum Albumin Lyophilized powder, >97% pure 2% Lactaalbumin Hydosylate Microbiology-grade, >11% nitrogen impurity 1 M MgCl2 BioUltra-grade, solution in water 1 M MgSO4 Molecular biology-grade, solution in water 1% Sodium oxalate BioXtra-grade, >99% pure

A.2. Figures

A.2.1. Neutralizing antibody titers after measles vaccination using metal microneedles in the rhesus macaque.

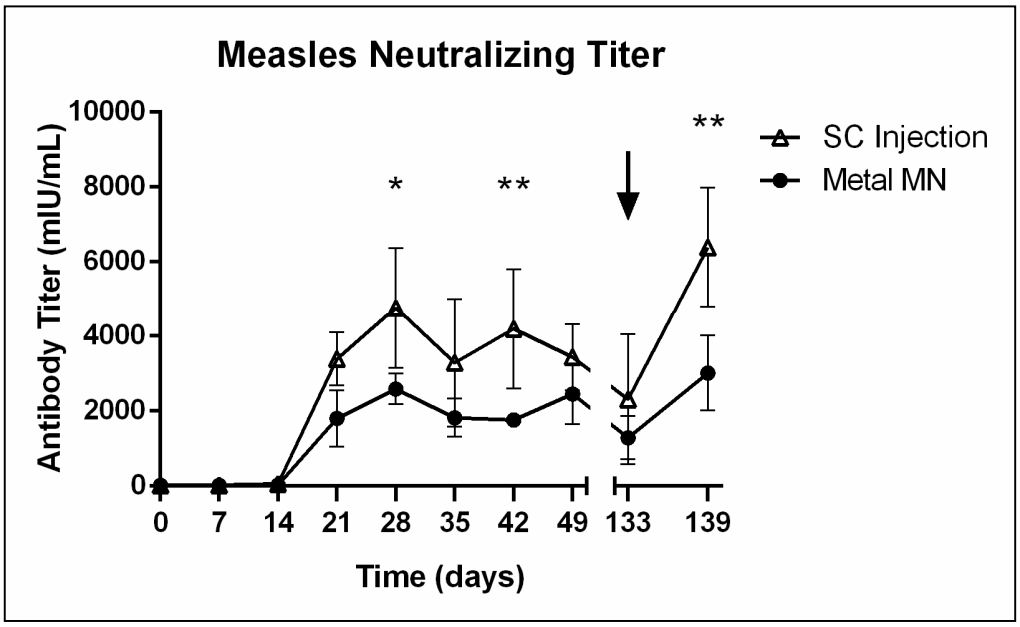

Figure 30: Measles plaque neutralization data (metal microneedle). Neutralizing titers were obtained from rhesus macaque sera using the method described earlier. Samples were tested weekly. This data represents the groups which received either a subcutaneous injection or a coated, metal microneedle insertion. The arrow indicates the date that a 100% dose of monovalent measles vaccine was delivered to all animals subcutaneously. The neutralizing titers between the two groups were not statistically different except at days indicated. The asterisk (*, **) represents a significant difference ($p<0.05$ or 0.005) as measured by ANOVA. The data points represent the average titer (N = 4) ± SD.

A.2.2. Neutralizing antibody titers after IPV delivery using metal microneedles in the rhesus macaque.

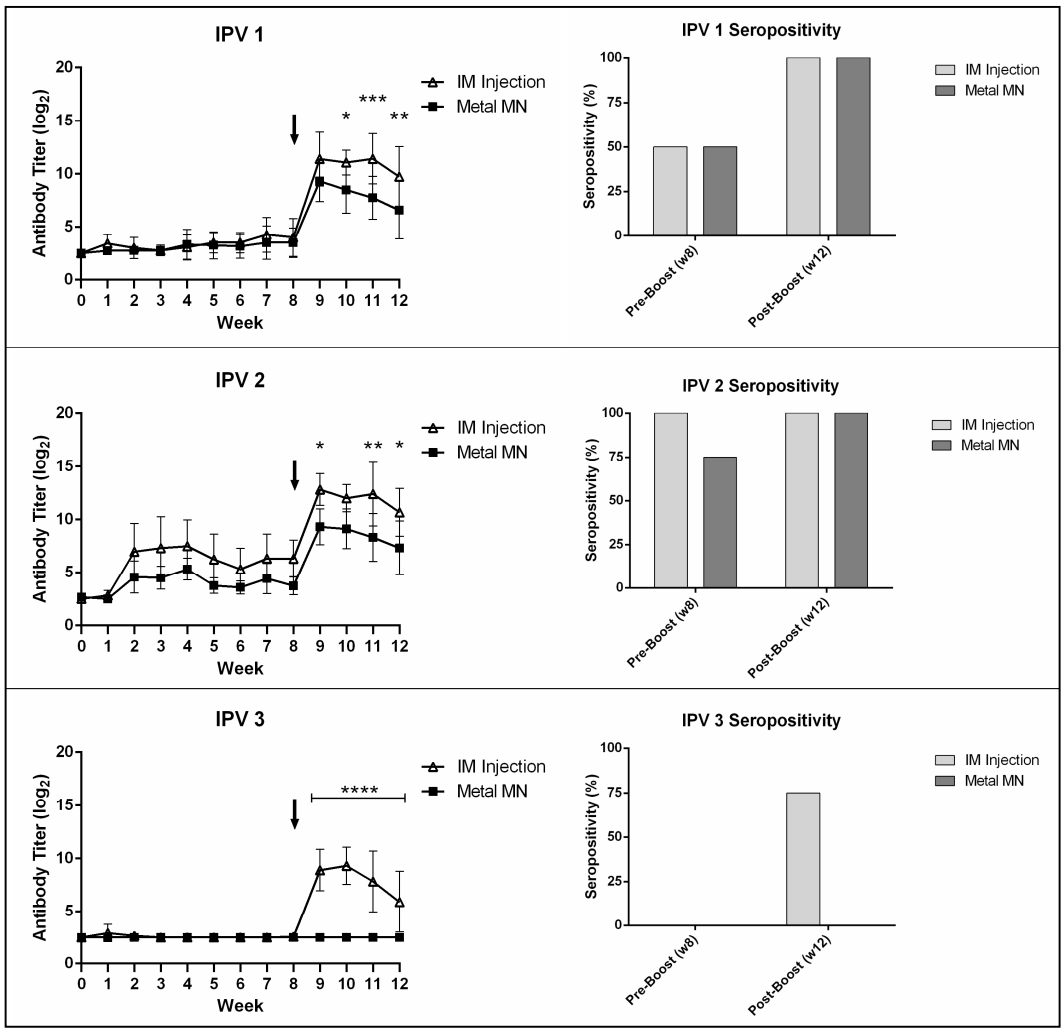

Figure 31: Neutralizing titers to IPV following vaccination (metal microneedle). Sera was taken from rhesus macaques weekly following vaccination. Neutralizing titers were determined using a serotype-specific plaque micro-neutralization assay. The titers for each day were not statistically significant unless otherwise indicated. There was no statistical difference in titer for any serotype until after the boost was delivered. Each data point represents the average (N = 4) ± SD. The asterisk (*, **, *, or ****) represents a significant difference ($p<0.05$, 0.005, 0.005 and 0.00005 respectively) as measured by ANOVA. Seropositivity was defined as a titer greater than or equal to 3.0 log_2.**

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