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Novel formulations and delivery strategies for inactivated polio vaccines : new routes with benefits

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Novel formulations and delivery strategies for inactivated polio vaccines

New routes with benefits

Heleen Kraan

The research described in this thesis was performed at the Institute of Translational Vaccinology (Intravacc), Bilthoven, The Netherlands

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About the cover: Upper photo depicts conventional liquid vaccine that needs to be administered via needle injection. However, the droplet reflects possible future (dried) formulations and a delivery strategy. Below, these formulations were depicted more clearly: a vial with freeze-dried polio vaccine and a IPV-filled Bioneedle.

Novel formulations and delivery strategies for inactivated polio vaccines

New routes with benefits

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General introduction and thesis outline

POLIO

POLIOMYELITIS IS A HIGHLY INFECTIOUS DISEASE CAUSED BY THE POLIOVIRUS, A HUMAN ENTEROVIRUS BELONGING TO THE PICORNAVIRIDAE FAMILY. THE POLIOVIRUS IS A SMALL (30 NM) NON-ENVELOPED, POSITIVE STRAND RNA VIRION WITH A PROTEIN SHELL CALLED A CAPSID. THERE ARE THREE SEROTYPES OF WILD POLIOVIRUS (TYPE 1, TYPE 2 AND TYPE 3), EACH WITH A SLIGHTLY DIFFERENT CAPSID PROTEIN. THE MOST OFTEN SPREAD BY FECAL-ORAL ROU
MOUTH AND MULTIPLIES
ARE AS

INTRODUCTION

Polio

Poliomyelitis is a highly infectious disease caused by the poliovirus, a human enterovirus belonging to the Picornaviridae family. The poliovirus is a small (30 nm) non-enveloped, positive strand RNA virion with a protein shell called a capsid. There are three serotypes of wild poliovirus (type 1, type 2 and type 3), each with a slightly different capsid protein. The virus is most often spread by fecal-oral route; it enters through the mouth and multiplies in the intestine. Most polio infections are asymptomatic or lead to mild symptoms causing minor illness. In less than 1% of the cases, the poliovirus can invade the central nervous system, which may cause irreversible paralysis and even death in case breathing muscles become affected. Polio-infected individuals shed poliovirus into the environment for several weeks, where it can spread rapidly through the population, especially in lower developed areas with poor sanitation. Polio can strike at any age, but mainly affects young children. Since there is no cure for poliomyelitis, the only way to combat polio is by prevention through vaccination.

Polio vaccines

The first vaccine against polio was developed in the 1950s by Jonas Salk. This inactivated polio vaccine (IPV) consists of formalin-inactivated (killed) poliovirus strains of all three serotypes (type 1, Mahoney or Brunhilde; type 2, MEF-1; and type 3, Saukett strains). The vaccine is given via intramuscular or intradermal injection and confers protection against disease via the induction of serotype-specific antibodies in the blood. In the 1960s, Albert Sabin developed the oral poliovirus vaccine (OPV), a trivalent vaccine based on attenuated poliovirus (Sabin) strains. Major advantage of this orally administered vaccine is the induction of antibodies both in the blood and, like in natural infection, locally in the gut. Since the live-attenuated OPV is able to replicate in the intestine, it is able to elicit effective immunity at the primary site of poliovirus entry. Moreover, in areas of poor hygiene and sanitation, OPV vaccination can result in unintentional transfer of the vaccine to people who have not been vaccinated. Therefore, the use of OPV can rapidly stop person-to-person transmission and interrupt further spreading of the (wildtype) poliovirus through the whole community.

Both vaccines, OPV and IPV, are considered as safe, but in extremely rare cases the live-attenuated vaccine-virus in OPV might cause paralysis, the so-called vaccine-associated paralytic polio (VAPP). Moreover, also very rarely, when there is insufficient coverage in the community, the vaccine-virus may be able to circulate and might revert in a form with similar

transmissibility and neurovirulence as wild polioviruses. These circulating vaccine-derived polioviruses (cVDPVs) can cause new polio outbreaks. A major advantage of OPV is its affordability for low- and middle-income countries. Since IPV is not a live vaccine, it has no risk of VAPP, but IPV is more than five times more expensive than OPV. Thereby, conventional intramuscular vaccine administration using needle and syringes requires trained health workers, as well as sterile injection equipment and procedures to avoid re-use of needles or needle-stick injuries. Advantages and disadvantages of both OPV and IPV are listed in **table 1**.

Table 1 Pros and cons of both polio vaccines: the live-attenuated oral polio vaccine (OPV) versus inactivated polio vaccine based on Salk strains (Salk IPV).

	OPV	Salk IPV
Pros	Highly affordable Ease of administration Mucosal immunity (intestinal sIgA)	Safe (no risk of VAPP[1]) Relatively stable High efficacy (1-2 dose)
Cons	Risk of VAPP[1] Risk of circulating VDPV[2] Very poor thermostability	High costs No mucosal immunity Trained health personnel needed to administer

[1] *VAPP – vaccine-associated paralytic polio*
[2] *VDPV – vaccine-derived polioviruses*

Polio eradication

Since the introduction of the Global Polio Eradication Initiative (GPEI) in 1988, the incidence of polio has decreased by more than 99.9%. In the late 1980s, polio paralyzed more than 1000 children every day, whereas only 22 cases of wildtype and 91 cases of vaccine-derived polioviruses were reported last year (2017) (**Figure 1**). However, polio remains endemic in three countries that have never stopped polio transmission: Afghanistan, Nigeria and Pakistan.

Of the three wildtype polioviruses, two of them are eradicated. The last case of type 2 was reported in 1999 and the most recent case of wildtype poliovirus type 3 dates to November 2012. However, stopping the last polio cases to reach global polio eradication proved to be challenging due to conflicts, political instability, hard-to-reach populations, and poor infrastructure. Therefore, in 2013 the GPEI launched a comprehensive strategic polio endgame plan that describes several steps to reach and maintain a polio-free world. The first objective of the plan is to discontinue the transmission of all wildtype poliovirus, but also to rapidly stop new outbreaks due to circulating VDPVs. This should be reached

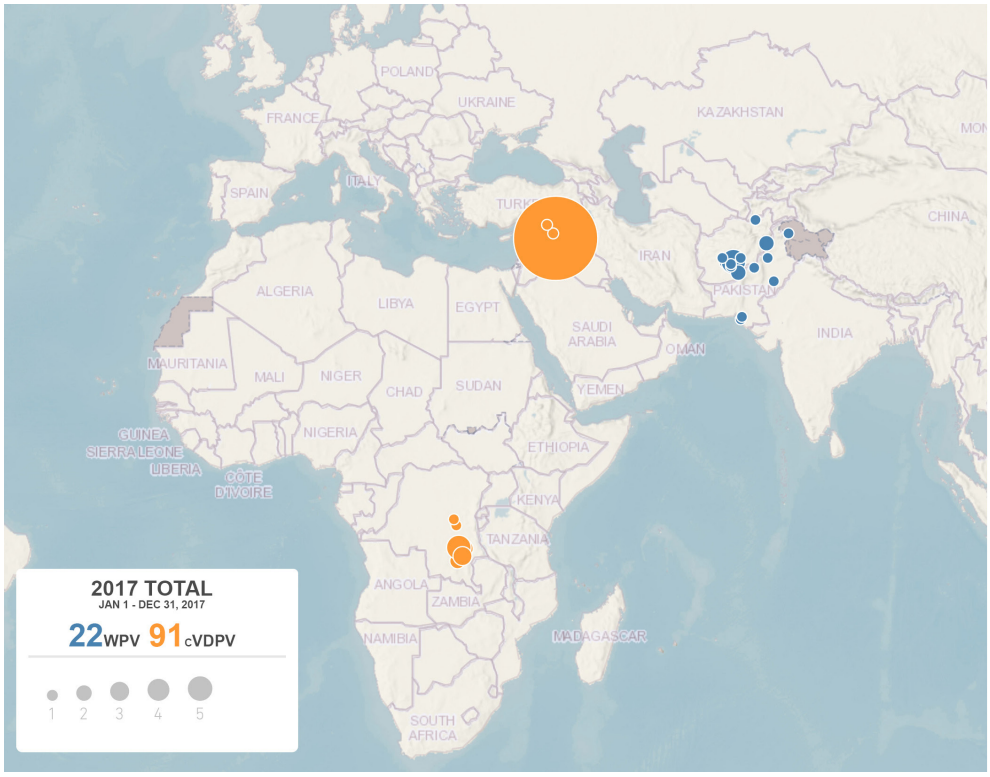

Figure 1 Geographical distribution of polio cases reported in 2017. Both wildtype polioviruses (WPV, blue dots) and circulating vaccine-derived polioviruses (cVDPV, orange dots) are shown.
source: www.polioeradication.org

by enhancing global polio surveillance, improving the quality of OPV campaigns in the remaining endemic countries and ensuring rapid outbreak response. The second objective includes strengthening of immunization programs and the phased withdrawal of OPV in order to hasten the interruption of all poliovirus transmission. As wildtype poliovirus type 2 was eradicated since 1999 and main cause of VDPV outbreaks is currently the type 2 component of OPV (91 VDPV type 2 cases reported), every country using trivalent OPV switched to bivalent OPV to reduce the risks of new circulating VDPVs. Thereby, the introduction of at least one dose of trivalent IPV into all routine immunization programs is part of the plan to strengthen immunization systems, especially in areas of highest risk. All these efforts resulted in the current situation with fewer cases reported from fewer areas in fewer countries than ever before (Figure 1).

Another aim of the Polio Eradication & Endgame Strategic Plan encompasses the certification of all regions to be polio-free and the guarantee that all poliovirus stocks are safely contained. Strict requirements for safe handling and biocontainment of polioviruses, retained

at only a small number of facilities, are essential to minimize the risk of reintroduction of the poliovirus in the post-eradication era. Currently, the GPEI is developing a post-certification strategy to ensure maintenance of a polio-free world after complete eradication. Polio legacy planning, as fourth objective of the endgame plan, and its subsequent implementation into public health programs should ensure that the world learns from all investments made in polio eradication for future health care.

While the program had many successes, eradication goals were not reached yet due to both external (e.g., growing conflict areas) and internal factors (e.g., suboptimal management, inappropriate surveillance, tight IPV supply). As the virus' prevalence is declined to very limited parts of the world, the eradication of polio depends on the success of health workers to identify and vaccinate every high-risk child, even living in dense urban environment, in extremely remote and hard-to-reach area, or being on the move.

Towards and beyond polio eradication

The inclusion of IPV into all global routine immunization programs and the possible eradication of polio spur the need for improved and affordable IPV formulations. Important variables for the development of new IPV formulations are the route of administration, the selection of adjuvants, the vaccine formulation, and the use of (non-invasive) delivery methods. Ideally, a new generation of IPV have the benefits of OPV and should therefore be easy to administer, provide mucosal immunity, and be affordable for low-income countries. IPV formulations with improved thermostability that can be kept outside the cold chain may simplify logistics and increase vaccine availability in remote areas. Furthermore, the ideal polio vaccine should be safe to manufacture and have a long shelf-life with both characteristics being even more critical in the period after polio eradication, respectively to reach biosafety goals and for stockpiling purposes.

Towards polio eradication and also in the period thereafter, there will be a market for better IPV formulations. Besides, it is important to build up stocks of a polio vaccine that can be used as outbreak intervention in case of reemergence of poliovirus in the post-eradication era and even in the period post-vaccination.

THESIS SCOPE AND OUTLINE

The aim of this thesis was to develop improved formulations and novel delivery strategies for polio vaccination using IPV as staring point. In chapter 2, the current status of alternative polio vaccine delivery strategies is provided. The feasibility of these strategies is discussed by highlighting challenges, hurdles to overcome, and formulation issues relevant for optimal vaccine delivery.

Chapter 3 describes the development of a dried IPV having minimal loss during the lyophilization process and improved stability when compared with the conventional liquid IPV. A certain thermostable vaccine formulation should allow distribution and storage at unrefrigerated conditions, at least long enough for their transport to remote areas. In chapter 4 the potency of lyophilized IPV was evaluated. Moreover, an approach to obtain a hexavalent vaccine by reconstituting lyophilized IPV with liquid pentavalent vaccine, which contains diphtheria toxoid, tetanus toxoid, whole cell pertussis, Haemophilius influenza type B and hepatitis B (DTwP-Hib-HepB), is described.

The potential of the Bioneedle-technology as syringe-free alternative delivery system for polio vaccination is described in chapter 5. Bioneedles are small biodegradable mini-implants that can be filled with antigen followed by a lyophilization process. After subcutaneous delivery, the implant dissolves and the antigen releases. Antigenicity of IPV when formulated in Bioneedles was assessed, both directly upon preparation and after elevated stability testing. Further, we evaluated the immunogenicity of IPV-filled Bioneedles in rats and the residence time at the site of administration.

Mucosal tissues are attractive administration and target sites for vaccination due to their large surface and immunological competence. In chapter 6, the characteristics of and approaches for sublingual and buccal vaccine delivery are described and compared with other mucosal vaccine delivery routes. Besides, this chapter highlights promising developments in the search for vaccine formulations, including adjuvants and suitable dosage forms, which are likely critical for the design of a successful sublingual or buccal vaccine.

Chapter 7 describes the potential of polio vaccination via mucosal surfaces using IPV based on the attenuated Sabin strains. It was investigated whether intranasal or sublingual vaccination with sIPV is able to elicit functional systemic immunity (serum) as well as local immune responses at different mucosal sites. The need of an adjuvant for polio vaccination via mucosal routes was examined as well by testing sIPV in combination with the mucosal

adjuvant cholera toxin. For the induction of protective immunity upon sIPV vaccination under the tongue, the development of novel oral dosage forms that facilitate antigen uptake by the oral mucosa may be required. In **chapter 8** the possibility to make polymer-based films containing trivalent sIPV suitable for oromucosal vaccination was evaluated. Different film forming polymers were selected from literature and tested in combination with excipients that stabilize the antigen during the drying process in order to obtain a sIPV-containing oral film formulation with minimal loss of antigenicity.

Chapter 9 summarizes the results and conclusions of this thesis. Moreover, all different aspects of this thesis are discussed and perspectives of improved formulations and alternative delivery strategies for polio vaccination are given.

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Expert Review of Vaccines, 2016 Aug; 15(8)

Alternative administration routes and delivery strategies for polio vaccines

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ABSTRACT

Global polio eradication is closer than ever. Replacement of the live attenuated oral poliovirus vaccine (OPV) by inactivated poliovirus vaccine (IPV) is recommended to achieve complete eradication. Limited global production capacity and relatively high IPV costs compared to OPV, spur the need for improved polio vaccines. The target product profile of these vaccines includes not only dose sparing but also high stability, which is important for stockpiling, and easy application important for (emergency) vaccination campaigns.

In this review, the current status of alternative polio vaccine delivery strategies is given. Furthermore, we discuss the feasibility of these strategies by highlighting challenges, hurdles to overcome, and formulation issues relevant for optimal vaccine delivery.

INTRODUCTION

Poliomyelitis is an infectious disease caused by poliovirus, an enterovirus belonging to the Picornaviridae family. After infection by one of the three serotypes, the virus multiplies in the intestine from where it can invade the nervous system and cause paralysis.

The only way to combat poliomyelitis is by prevention through vaccination. Most industrialized countries use trivalent inactivated poliovirus vaccine (IPV, based on the wild polio 'Salk' strains) in their pediatric vaccination programs. IPV may be formulated as a combination vaccine with other antigens, such as diphtheria/tetanus/(acellular) pertussis (DTP), *Haemophilus influenzae* type B (Hib) and hepatitis B surface antigen. In developing countries, the live attenuated oral poliovirus vaccine (OPV, based on Sabin strains) has been the vaccine of choice because of a number of advantages as compared to IPV. These include the induction of stronger mucosal immunity, ability to interrupt wild poliovirus circulation in areas of intense fecal-oral transmission, immunization of close contacts through secondary spread, affordability and ease of administration. However, the use of OPV comes with a rare, but serious adverse effects, i.e. reversion to virulence resulting in vaccine-associated paralytic polio (VAPP). Circulating reverted vaccine viruses (circulating vaccine-derived polioviruses, cVDPV), may have similar transmissibility and neurovirulence as wild poliovirus and can cause new polio outbreaks.

The eradication of polio is one of the top global health priorities. Efforts to eradicate polio should focus on both wild polioviruses as well as vaccine-derived viruses. Therefore, the Global Polio Eradication Initiative (GPEI) has defined an endgame strategy that includes a phased withdrawal of OPV and the worldwide inclusion of IPV into all routine immunization programs [1]. Besides the short term changes in current immunization procedures, more affordable, more effective and safer forms of the existing polio vaccines are needed [2]. The target product profile of the ideal polio vaccine may differ depending on the eradication phase (Table 1). In the development of new polio vaccine delivery systems, the intended use is an important consideration.In the short term, the worldwide switch to injected IPV at the expense of OPV will occur, which brings some challenges:

1. IPV is injected and so has the disadvantages of needles and syringes, like risk of needle stick injuries, potential re-use of needles and, as a result, complicated waste management.

2. IPV is, compared to OPV, considerably more expensive. The downstream processing is more complex since the parenteral version is extensively purified. Besides, unlike

OPV, the inactivated virus is not able to replicate in the host. The dose needed to confer protection is about ten times higher.

3. In general, IPV does not induce mucosal intestinal immunity (polio-specific secretory IgA antibodies), which is crucial to provoke a strong herd immunity effect and to interrupt poliovirus transmission in developing countries. Although IPV can prevent poliovirus outbreaks and provide herd protection to some extent, IPV is probably less effective to stop transmission of poliovirus.

A disadvantage of both OPV and IPV is that they need a cold-chain for their storage and logistics. For use in emergency vaccinations or post-eradication stockpiling this is undesirable. Stockpiling after eradication and cessation of routine polio vaccination is important in case of re-emergence of the virus.

The GPEI is pursuing some priority approaches to make IPV more affordable for low-income countries, like dose-sparing strategies using adjuvants and the introduction of IPV based on Sabin strains, instead of wild type poliovirus (Salk) strains [2]. An overview of different approaches that are currently under development, including their strengths and weaknesses, is given in **figure 1**.

Table 1 The ideal polio vaccine is not able to revert to virulence, is stable during storage, affordable and easy to produce, and induces sterilizing immunity (i.e., interrupts virus transmission). .
The relevance of this ideal target product profile depends on the polio status worldwide (i.e., current phase with OPV/IPV in use, after complete OPV cessation, post-eradication or, eventually, without routine polio vaccination), but also on the aim (i.e., routine immunization program versus outbreak control campaigns)

Polio Status	OPV/IPV in use		IPV only used		Post polio eradication		Post vaccination
Purpose	Routine vaccination	Outbreak control	Routine vaccination	Outbreak control	Routine vaccination	Outbreak control	Outbreak control
Product attribute							
No reversion to virulence	-	-	+	+	++	++	++
Transmission interrupting	0	++	0	+	-	++	++
Stable	0	+	+	+	+	++	++
Affordable	+	0	++	0	++	0	0
Easy to administer	0	++	0	++	++	+	+
Easy to produce	-	-	-	-	-	++	++
Safe to produce	+	+	+	+	++	++	++

- less important; 0 neutral; + important; ++ very important
IPV: inactivated poliovirus vaccine; OPV: oral poliovirus vaccine

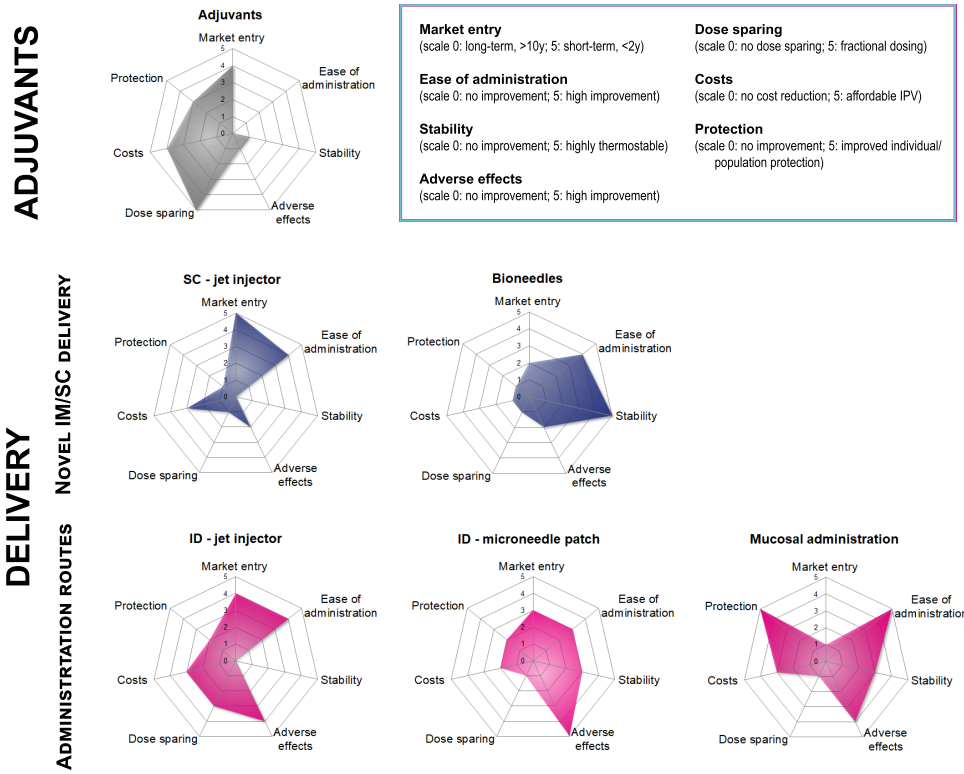

Figure 1 An overview of different approaches, which are currenlty under development, to make inactivated poliovirus vaccine (IPV) more affordable for low-income countries.

The current review will focus on IPV delivery approaches by highlighting recent developments of alternative administration methods for IPV. The use of dermal delivery of polio vaccines, like jet injection and microneedle approaches, are discussed. Mucosal delivery and the potential of new mucosal delivery routes for IPV are described. Finally, future perspectives, including the potential of improved vaccine formulations, the use of adjuvants and promising delivery technologies, are given.

NOVEL INTRAMUSCULAR AND SUBCUTANEOUS ADMINISTRATION

Most of high-income countries use Salk IPV, which is administered intramuscularly, in their routine vaccination programs, often in combination with other antigens. Alternative delivery approaches are developed for polio vaccination. These vaccine delivery strategies could address issues, like safety, needle phobia and vaccine stability.

Needle-free (intramuscular/subcutaneous) jet injection

An option to facilitate intramuscular (IM) or subcutaneous (SC) delivery is the use of needle-free jet injectors. Jet injectors use high-pressure to inject the fluid into or through the skin, either subcutaneously or intramuscularly. Injection depth depends on nozzle design, pressure and pressure profile during injection. Initial studies aimed at demonstrating non-inferiority or superiority of intramuscular jet-injections over the needle-based intramuscular vaccinations. The study of Lipson *et al*. demonstrated that IPV vaccinations using the press-o-jet [3] induced similar responses in children as the needle approach [4]. In general, the use of jet injectors could provide a solution for safety issues accompanied with needles, reduces the amount of waste, and increase the immunization speed. However, after the introduction of the devices concerns about blood-borne infection were raised. The use of multi-dose systems with the same nozzle, occasionally lead to transmission of for example Hepatitis B between recipients. Studies demonstrated the infectious potential of several jet injectors [5,6]. Today, safe jet injection systems are available using disposable parts facing the vaccine recipient. Current systems in use for IM and SC administration of vaccines are the Biojector2000 (Bioject Medical Technologies Inc, Tigard, OR, United States), ZetaJet (Bioject Medical Technologies Inc, Tigard, OR, United States) and PharmaJet (PharmaJet, Golden, CO, United States).

Soonawala *et al*. compared the PharmaJet with conventional needle-injection (one IPV dose) in healthy adults in a phase 1 study [7]. Vaccination with the jet injector was less painful (87% no pain) than vaccination with needle and syringe (60% no pain), but caused more adverse effects at the site of administration, like transient erythema and swelling. Moreover, IM jet injector vaccination resulted in similar geometric mean virus-neutralizing antibody titers as induced after IM injection using needle and syringe. These data demonstrated that the technique itself could help to improve acceptability by reducing the pain sensation [7]. Intradermal jet-injection further reduced pain and increased immunogenicity as further described in section 3.2. Since subjects had background immunity against polio in this phase 1 study, further clinical testing (phase 2 and 3) is needed to assess immunogenicity.

A recently completed trial in a large cohort of infants in Gambia addresses safety and immunogenicity of IPV given concomitantly with other vaccines (Measles, Rubella and Yellow Fever) (study nr. NCT01847872). One of the aims of this trial was to compare the performance of a jet injector with that of needle and syringe for IM and intradermal (ID) delivery. Seroconversion levels, adverse events, cellular immune responses and virus shedding after OPV ‘challenge’ are measured to quantify the type of response induced and the potential presence of mucosal immunity. The results have not been published yet.

Bioneedles

Bioneedles are dissolvable implants (12 mm) made from biodegradable polymers that can be filled with antigen and are injected subcutaneously by air pressure. The included vaccine formulation is in a solid state. Using a solid formulation could diminish the dependence on the cold-chain. Furthermore, the Bioneedles could eliminate needle-stick accidents and do not create sharp waste.

IPV formulated in Bioneedles showed improved thermostability compared with liquid IPV [8]. Storage of the IPV-filled Bioneedles at 45°C for one week led to a reduction of antigenicity between 20-50% for the different serotypes compared to 80 to 100% antigen loss for the standard liquid form. Storage of the liquid vaccine at 60°C for one hour did lead to complete loss of antigen compared to 20-30% reduction for the antigen in Bioneedles. Furthermore, vaccination of rats with IPV Bioneedles induced comparable levels of virus-neutralizing antibodies to the IM administered conventional IPV vaccine. These data demonstrate that the Bioneedle polio vaccine has similar immunogenic properties and better resistance to higher temperatures compared to current liquid IPV. The implementation of the thermostable vaccine in biodegradable needles could help to stimulate the expansion of IPV usage to developing countries, because of its improved thermostability, which is required in remote areas, and potential to vaccinate relatively fast.

Table 2 Preclinical assessment of other administration routes than conventional intramuscular (IM) or subcutaneous (SC) injection for polio vaccination

Delivery method	IPV dose (DU) T1;T2;T3	Adjuvant	Animal model	Immune response	Ref
Novel IM/SC delivery					
Bioneedle	2.7;0.6;2.1	PagL[a]	Rats	Inferior VN titers for type 3 after prime immunization for Bioneedle-group. Similar VN titers for all serotypes after booster regime.	[8]
Intradermal					
ID injection	(unclear)	CAF01[b]	Mice	Similar (type 1 and 3) or superior VN titers after ID vaccination plus adjuvant and superior IgG titers compared to IM injection. No mucosal IgA (feces)	[39]
ID injection	1-10; 1-10; 1-10	dmLT	Mice	Similar VN titers and superior polio-specific IgG titers compared to IM vaccination. Prolonged systemic immunity (VN) Mucosal type 1 and 2-specfic IgA titers (feces and PP)	[40]
Microneedles (hollow)	5/15;NA;NA	NA	Rats	Polio-specific IgG and VN titers similar to IM and ID vaccination using needle/ syringe	[77]
Microneedles (hollow; Micronjet600)	2-40; 0,4-8; 1,6-32 (5-100% of shd)	NA	Rats	Superior VN titers for 40% ID with 40% IM and when comparing 40% ID with 100% IM (49 days)	[35]
Microneedles (coated)	47;9;38	NA	Monkeys	VN titers similar to IM injection for type 1 and 2, but inferior VN titers for type 3	[38]
Microneedles (coated)	45;NA;NA	TMC	Rats	Inferior polio-specific systemic IgG titers compared to IM or ID injection	[34]
Mucosal (sublingual)					
Thermoresponsive gels	1.34;0.3;1.1 6.5;1.5;5.5	dmLT	Mice	No immune responses detected without adjuvant. Inferior VN titers or systemic Ig titers compared to IM injection. Superior mucosal IgA titers (feces, saliva) for SL vaccination (+adjuvant) compared to IM injection	[60]

Abbreviations used:
DU: D-antigen unit; dmLT: double mutant heat-labile toxin; ID: intradermal; IM: intramuscular; IPV: inactivated poliovirus vaccine; NA: not applicated; SL: sublingual; T1/2/3: serotype 1/2/3; TMC: trimethyl chitosan; VN: virus-neutralizing

[a] *PagL LPS is a LPS-derivate obtained through expression of the Bordetella bronchiseptica PagL gene in Neisseria meningitides LPS.*
[b] *CAF01 is an adjuvant composed of cationic liposomes DDA (dimethyldioctadecylammonium) and TDB (trehalose 6,6′-dibehenate).*

DERMAL IMMUNIZATION

The dermis and epidermis of the human skin are rich in antigen-presenting cells (APC) and therefore are attractive sites for vaccine delivery. The skin's structural and cellular composition enables it to function as a physical and immunological barrier, suggesting that delivery of vaccines to the dermal layers, rather than IM or SC vaccine delivery, could be more efficient and induce protective immune responses with smaller amounts of vaccine antigen [9].

Dendritic cells (DCs) are APCs that serve to efficiently amplify innate and adaptive immune responses. In the normal human skin two distinct populations of immature DCs are found, each within a specific layer, i.e., Langerhans cells (LCs) in the epidermis, and dermal DCs in the deeper skin layers [10]. However, the skin is equipped with an impressive barrier, the stratum corneum, which makes it almost impossible to induce an immune response through dermal vaccination without disrupting this first defense line. Therefore, effective, safe, and convenient methods to achieve disruption of the stratum corneum are needed [11].

The intradermal (ID) delivery methods that are currently available can be roughly classified into three categories: administration by (i) needle and syringe; (ii) jet injectors; and (iii) microneedles [12]. An overview of these different ID delivery methods tested in combination with IPV is given in table 2 (preclinical) and table 3 (clinical).

Traditional needle-based dermal vaccination

The traditional needle-based ID vaccines rely on a single needle inserted shallow into the skin (the 'Mantoux' technique, originally used as diagnostic for tuberculosis) or needle(s) especially designed to penetrate only into the dermis (i.e., bifurcated needles, multipuncture systems). They have been used extensively in the past for various vaccination programs, like those for smallpox, and some are studied for the use in the polio immunization programs. However, ID injection methods using needles and syringes require considerable expertise and are, therefore, not ideal for routine vaccinations.

Although IPV is given intramuscularly, the initial experiments of Jonas Salk anticipated its use via the ID route. In 1953, Salk demonstrated the immunogenicity of IPV administrated both intramuscularly and intradermally [13]. Despite these and more promising results in the mid-1950s [13-16], the ID route was only in Denmark the most used route for IPV vaccination at that time [16,17].

With the purpose of developing a more affordable IPV for the lower-income countries and increase its use in the post-eradication era, different studies investigated ID polio vaccination [18]. After development and licensure of the enhanced-potency IPV, which was responsible for highly improved seroconversion rates for all three serotypes due to its higher content of poliovirus antigen [19], three trials using ID administration of the IPV have been conducted in India since the early 1990s. Those proof-of-concept studies established the immunogenicity of a fractional (one-fifth) IPV dose delivered ID (via Mantoux injection) in subjects who had been previously immunized [20], or had never been immunized against polio [21]. The trial among 69 Indian infants demonstrated that two or three fractional doses ID were equivalent in terms of seroconversion to two full doses of IPV delivered IM or five doses of OPV (based on historical data). All infants who had no pre-existing maternal antibodies seroconverted to all serotypes [22]. In none of these studies, however, a comparator IM group was included. Therefore, a randomized controlled trial was conducted in the Philippines, to compare the primary and booster immunogenicity of IPV by ID injection (one-fifth dose) with the IM route (full dose). These data demonstrated non-inferiority of fractional dosing by the ID route, and thus confirmed the validity of this IPV vaccination strategy [23].

The bifurcated needle and multipuncture system were introduced to improve ID delivery of vaccines by limiting the penetration depth, which ensures dermal delivery. The bifurcated needle consists of a needle that branches out into two solid needle points. Formulations are administered via multiple punctures at a local area. To our knowledge, no research has been conducted with this type of needle administering IPV.

The multipuncture system was deployed for Bacillus Calmette-Guerin (anti-tuberculosis) vaccines. The vaccine administration is a two-step process. First, the skin is penetrated using a device with multiple needles. Secondly, the vaccine is applied evenly on the punctured area. A large clinical trial conducted in Cuba included the multipuncture system as a control in a comparative study for three needle-free delivery devices for polio vaccination. In this study, inferior immune responses (defined as seroconversion and increase in virus-neutralizing titers) following fractional-IPV dose administered via the ID route compared with full-dose IPV administered IM were reported [24].

Considered together, the trials in the Philippines (Mantoux injection) [23] and Cuba (multipuncture system) [24] provided inconclusive results after ID delivery of IPV. Moreover, a problem for traditional needle-based methods is their inaccuracy, the need of well-trained personal for administration and low patient compliance. To be able to completely use the

potential of the skin as a vaccination site, less invasive systems are examined in animal and human trials.

Jet injector

While several studies confirm the observations of Salk about the potential of dose sparing by ID injection, usage of needle ID injections on a large scale is not foreseeable given that the need for skilled personnel, which is a major limitation for large-scale campaigns, and the safety and disposal concerns related to the use of needles remain. To overcome these problems and increase the affordability of IPV, needle-free devices for ID injection, which can be manually reset and used by volunteers if necessary, have been developed [25].

Needle-free jet injector systems are used for IM and SC administration (see section Needle-free jet injection), but are also studied as ID delivery devices (e.g., Biojector 2000, PharmaJet). Furthermore, the ID Pen injector (Bioject Medical Technologies Inc., Tigard, OR, United States) and the PharmaJet Tropis (PharmaJet, Golden, CO, United States) are especially designed for the ID delivery of vaccines. Several clinical trials with polio vaccines are already conducted to compare the different injector systems to IM (conventional) hypodermic needles or to each other (Table 3).

The Biojector 2000, a disposable syringe jet injector for ID delivery, has been evaluated by WHO sponsored studies in Cuba and Oman, and compared to IM delivery using conventional syringe and needle. Two different IPV vaccines and two different immunization schedules were evaluated. Target groups were infants in both studies. The primary objective of these trials was to demonstrate non-inferiority of fractional (one-fifth) dose in terms of seroconversion for the ID route compared to the full dose via the IM route. Non-inferiority could not be demonstrated in the Cuban study; significantly lower seroconversion rates (ID: 52.9%, 85.0%, and 69.0% versus IM: 89.3%, 95.5%, and 98.9% for serotypes 1, 2, and 3, respectively) and significant lower median antibody titers were induced in the ID arm after three doses of IPV [26]. In the Omani study, similar levels of seroconversion for serotypes 1 and 3 were after ID delivery of fractional doses and after IM vaccination of the full dose were detected. Serotype 2 showed a statistically significant different, although small, reduction in seroconversion rate after ID delivery (ID: 95.7% vs. IM: 100%). For all serotypes, the median antibody titers were significantly lower in the fractional dose group [27], but it remains unclear whether the differences have practical implications since any detectable titer of neutralizing antibody against poliovirus would be expected to prevent against paralytic disease [28]. Maternal

antibodies may interfere with IPV vaccination at very young age [29,30], Administration of fractional doses of IPV is unlikely to serve as an optimal antigen-sparing strategy when given at the standard ages of 6, 10, and 14 weeks, rather than a schedule in which the first dose is administered at 2 months of age [26,27].

Dermal IPV vaccination with the PharmaJet device has been evaluated in clinical trials in India and The Netherlands [7,27]. The study in The Netherlands was performed administering vaccine to young adults, whereas the study in India was conducted with infants. For the Indian study, the ID administered fractional dose was less effective than full-dose IM administration in seroconverting seronegative infants and in increasing antibody titers in seropositive children. This result is in contrast with the very small differences found between fractional and full-dose in a three-dose schedule conducted in Oman (as described above) [27], which might be related to the device and/or geographic differences. Unfortunately, as with most studies mainly aiming at non-inferiority of ID fractional dose IPV delivery, the study was not designed to evaluate the impact of ID-administration solely, e.g., by including a group that receives fractional dose by IM injection as performed in the Dutch study [7].

More recently, in another WHO-sponsored clinical trial in Cuba the performance of three jet injectors, i.e., Biojector2000, Bioject ID Pen injector and PharmaJet Tropis, was evaluated and the immune response induced by a ID administered fractional dose with that induced by full-dose IPV given via the IM route [24]. Children between 12 and 20 months of age, who had previously received two doses of OPV, received a single dose of IPV either full-dose IPV via IM injection or fractional dose given via the ID route using one of the jet injectors or via 'Mantoux' needle and syringe. Whereas the Indian study reported excellent immunogenicity of fractional IPV when administered in a three-dose schedule with appropriate age and interval between doses [27], the results from recent Cuban study were more comparable with to those from the boosting study in India were fractional IPV also induced significantly lower immune responses than full-dose IPV [24]. Nevertheless, at the end of the study, the seropositivity rates were similar for both ID jet injector, 'Mantoux' needle and syringe (ID), and conventional IM-injection groups [24].

The fractional dose strategy might be suitable as a substitute to full-dose IPV when given at the correct interval. The newly developed jet injector (Tropis Needle-Free Injector from PharmaJet) would facilitate the administration of a fractional dose when given ID [24]. The use of jet injectors may solve two existing problems of IPV by being safer to administer and decreasing costs by using less antigen. In addition, it has been hypothesized that ID delivery

could improve protection against infection in the gut, since it may stimulate IgA mucosal immunity [31]. However, further investigations are needed to assess whether the lower immunogenicity of fractional IPV is sufficient to provide adequate protection and whether potential loss of immunogenicity is worth the cost savings.

Microneedle approaches

Another approach for ID vaccine delivery makes use of microneedle arrays that can penetrate the stratum corneum. These arrays are designed to disrupt the stratum corneum and target Langerhans cells in the epidermis, but are minimally invasive, since the nerves in the underlying tissue are not reached, and therefore caused no pain and only minimal irritation [32]. Different microneedle strategies are being exploited, i.e., the straightforward methods by pre-treating the skin with solid microneedles followed by application of a vaccine containing patch on the pretreated skin surface or using hollow microneedles to inject the vaccine into the epidermis. More recent strategies include the use of dissolvable microneedles or antigen-coated microneedle arrays. Microneedle technologies are in preclinical or early clinical development and the optimal microneedle strategy (material, shape) to deliver a vaccine into the skin has not yet been established [33].

Since ID administration of IPV has been shown to have potential, a microneedle approach appears to be a useful delivery method for IPV vaccination. To guarantee the stability and immunogenicity of a dermal polio vaccine by using coated or dissolvable microneedles, the development of a solid IPV formulation is required, which is a major hurdle to overcome. The problem of the often low loading capacity of microneedle arrays may be solved by adjuvants (see section Intradermal adjuvants).

A preclinical study focusing on the production and usage of a single hollow microneedle for IPV has been performed by Van der Maaden *et al.* [34]. Immunization of rats with 5 D-antigen units (DU) of IPV serotype 1 at a depth of 300µm led to similar systemic IgG levels and virus neutralization titers as compared to intramuscular and needle-based intradermal injections [34]. One other micro-injector system has been used for IPV vaccination: the MicronJet600 (NanoPass Technologies Ltd., Rehovot, Israel). The performance of the MicronJet600 in rats dosed with a different fraction of the human IPV dose was studied by Kouiavskaia *et al*. [35]. The response rate of animals immunized with 20 or 40% of the human dose at 35 days was equal for IM and ID injections. The 5% dose ID led to almost double the response rate compared to intramuscular injections. In addition, the neutralizing virus titer for type

1 and 3 after 35 days and 40% of the full-dose was higher than full -dose administered intramuscularly. These results confirmed the potential of using fractional doses during ID vaccination programs. To study the performance of the MicronJet600 in humans, a large cohort of infants in Bangladesh was vaccinated with a fractional IPV-dose. No adverse events were reported among participants within 30 minutes after vaccine administration. None of the adverse events reported during follow-up were attributed to the MicronJet600-device. When compared with full-dose IPV given via IM injection, the fractional IPV dose given ID by microneedles failed the non-inferiority test for all serotypes for seroconversion observed with 1 or 2 doses [36]. In this study, the assessment of the microneedle device was limited to safety and injection quality. Therefore, the comparison of immune responses induced by IPV administered by MicronJet600 with standard needle and syringe for ID administration was not possible.

Apart from hollow microneedles, also solid needles for skin pretreatment, biodegradable needles and coated needles [37] are under development (Table 2). The usage and action of both the solid and hollow needle arrays are similar to the single microneedle system. First of all, they require the use of a delivery device like a pressure-based applicator to actually puncture the skin. Secondly, the vaccine is introduced from an external source, via a syringe into the hollow needle or with a patch applying the vaccine onto the punctured skin. The biodegradable and coated needles are manufactured with the vaccine in or on top of the needles and forced into the skin followed by release of their content. Van der Maaden *et al*. developed a protocol for the production of alternating layers of IPV and N-trimethyl chitosan on microneedle arrays. Using a one-layer coating technique, Edens *et al*. were able to create an IPV-coated microneedle array that induced comparable neutralizing antibody titers as IM injections [38].

Intradermal adjuvants

The necessity of adjuvants to stimulate systemic as well as mucosal immunity has been reported in the preclinical dermal immunization studies for Hepatitis B, HIV, Diphtheria, Cholera and ETEC diarrheal antigens. Two adjuvants have been studied in combination with ID administration of IPV; CAF01 and dmLT (Table 2).

CAF01 is a liposomal formulation composed of the cationic lipid DDA (dimethyldioctadecylammonium) and TDB (trehalose-6,6-dibehenate). Dietrich *et al*. reported that IPV mixed with CAF01 and administered to mice via ID injection, was able to

induce superior polio-specific serum IgG levels and virus-neutralizing titers compared to the non-adjuvated vaccine [39]. No mucosal immunity (IgA in feces) was detected after ID administration alone. However, simultaneous priming of CAF01 adjuvated IPV at an ID and IM site followed by IM boosting induced significant levels of fecal IgA, without compromising serum virus-neutralizing titers [39].

Another study investigated the use of genetically detoxified E. coli heat-labile toxin (dmLT) as adjuvant for IPV administered via IM or ID injection in mice [40]. Intradermal vaccination with a fractional IPV-dose combined with dmLT as adjuvant, elicited serum virus-neutralizing antibody titers similar to those obtained by non-adjuvated IPV given via IM injection leading to a five-fold dose sparing. The duration of the systemic antibody responses was prolonged for the mice vaccinated with IPV adjuvated with dmLT either via IM or ID delivery. Moreover, dmLT enhanced mucosal immunity as defined by fecal and intestinal polio-specific IgA secretion, when mixed with IPV and given IM or ID [39].

Table 3 Clinical trials investigating the intradermal (ID) route for vaccination with inactivated poliovirus vaccine (IPV).

Study (year)	Vaccine	Age	N	Route	Administration	Volume (mL)	IPV dose (DU) T1;T2;T3	Schedule	Seroconversion (%)			Ref
									Type 1	Type 2	Type 3	
India (1998)	Imovax Polio	6, 14 w or 6, 10, 14 w	69	ID	Mantoux injection	0.1	8;1.6;6.4	2 doses	90	70	97	[22]
								3 doses	90	80	98	
Oman (2010)	Poliorix	2, 4, 6 mo	186	IM	Needle & syringe	0.5	40;8;32	1 dose	22	32	45	[27]
								2 doses	88	86	92	
								3 doses	100	100	100	
			187	ID	Jet injector (Biojector 2000)	0.1	8;1.6;6.4	1 dose	10	17	9	
								2 doses	70	72	72	
								3 doses	97	96	98	
Cuba (2010)	SSI, Denmark	6, 10, 14 w	177	IM	Needle & syringe	0.5	40;8;32	1 dose	19	36	42	[26]
								2 doses	63	76	93	
								3 doses	89	96	99	
			187	ID	Jet injector (Biojector 2000)	0.1	8;1.6;6.4	1 dose	5	19	8	
								2 doses	21	55	43	
								3 doses	53	85	69	
India (2012)	IPV1: Poliorix IPV2: Panacea	6-9 mo	183	IM (IPV1)	Needle & syringe	0.5	40;8;32	1 dose	100	89	95	[78]
			169	IM (IPV2)	Needle & syringe	0.5	40;8;32		100	100	94	
			168	ID (IPV1)	Jet injector (Pharmajet)	0.1	8;1.6;6.4		100	89	70	

Philippines (2012)	Imovax Polio	6, 10, 14 w	111	IM	Needle & syringe	0.5	40;8;32	2 doses	98	98	100	[23]
			113	ID	Mantoux injection	0.1	8;1.6;6.4	2 doses	99	95	96	
Cuba (2013)	NVI[1], Netherlands	4, 8 mo	153	IM	Needle & syringe	0.5	40;8;32	1 dose	46	63	32	[79]
								2 doses	100	100	99	
			157	ID	Jet injector (Biojector 2000)	0.1	8;1.6;6.4	1 dose	17	47	15	
								2 doses	94	98	93	
Netherlands (2013)	NVI[1], Netherlands	Young adults (20-25 y)	30	IM	Needle & syringe	0.5	40;8;32	1 dose	NA (4.1)[2]	NA (2.8)	NA (5.7)	[7]
			30	IM	Jet injector (Pharmajet)	0.5	40;8;32	1 dose	NA (3.8)	NA (2.3)	NA (5.6)	
			30	IM	Needle & syringe	0.1	8;1.6;6.4	1 dose	NA (1.9)	NA (1.1)	NA (3.9)	
			30	ID	Jet injector (Pharmajet)	0.1	8;1.6;6.4	1 dose	NA (3.7)	NA (2.4)	NA (4.4)	
Cuba (2015)	Imovax Polio	12-20 mo	146	IM	Needle & syringe	0.5	40;8;32	1 dose	90	88	97	[24]
			134	ID	Mantoux injection	0.1	8;1.6;6.4	1 dose	49	49	79	
			145	ID	Jet injector (Biojector 2000)	0.1	8;1.6;6.4	1 dose	59	52	82	
			153	ID	Jet injector (Pen Biojet)	0.1	8;1.6;6.4	1 dose	40	23	45	
			151	ID	Jet injector (Pharmajet Tropis)	0.1	8;1.6;6.4	1 dose	54	55	74	
Bangladesh (2015)	NVI[1], Netherlands	6, 14 w	162	IM	Needle & syringe	0.5	40;8;32	1 dose	35	36	34	[36]
								2 dose	95	91	97	
			164	ID	Microneedles (MicronJet 600)	0.1	8;1.6;6.4	1 dose	13	19	14	
								2 dose	88	81	89	

Abbreviations:
ID: intradermal; IM: intramuscular; mo: months; NA: not applicable; Nr: number of participants; T1/2/3: serotype 1/2/3; W: weeks; Y: years

Seroconversion was defined as a percentage of the participants that either conversed from seronegative to seropositive or showed a fourfold increase in antibody titers.
[1] Netherlands Vaccine Institute (NVI), now Bilthoven Biologicals
[2] Not applicable, because of high background immunity. Instead, increase in antibody titer (IU/mL) relative to mean background one year after immunization is shown.

MUCOSAL IMMUNIZATION

Efficacy of mucosal vaccination

Although parenteral vaccination in some instances can provide protection against mucosal infections, in most cases and especially in naïve subjects, e.g., newborns and young infants, a mucosal vaccine delivery route is needed for effective immunization [41]. Despite the practical advantages of mucosal vaccine delivery over injectable vaccines, only relatively few vaccines for human use are licensed: oral vaccine against cholera, typhoid, rotavirus, and polio, and a nasal vaccine against influenza [42]. With the exception of the cholera vaccines, which have a very strong intrinsic immuno-potentiating capacity [43], all these vaccines are live attenuated vaccines. They effectively induce both systemic (serum) and local mucosal immune responses, superior protection against re-infection, persistence of immunological memory, better herd immunity (because of secondary spread and mucosal immunity) and are easy to administer [44]. For vaccination against polio, polio-specific mucosal immunity in the gut is a powerful protecting and transmission inhibiting mechanism as we know from OPV. To date marketed mucosal vaccines are administered via the mucosa where protection is required. This is in contrast to vaccination strategies that are under development in order to generate mucosal immunity at distant effector sites.

OPV

The only marketed needle-free polio vaccine is the live attenuated oral vaccine, OPV. The success of the live-attenuated OPV is attributed to the capability of the virus to replicate in the intestine, and thus generate an increasing antigen load that elicits both strong systemic (serum IgG) and mucosal (local secretory IgA (sIgA)) antibody responses [45,46] and long-term persistence of neutralizing antibodies against poliovirus [47,48]. The mucosal sIgA confers protection from poliovirus entry and multiplication in the intestine [49].

Although OPV is the most effective vaccine in endemic and high-risk areas to interrupt wild poliovirus transmission, the estimated number of polio cases caused by OPV now likely exceeds those related to wild polioviruses [50]. Wild type 2 poliovirus has not been detected since 1999 and the last case of wild type 3 was reported in November 2012. For that reason, the Endgame Strategy aims for global cessation of type 2 OPV by switching from trivalent to bivalent OPV in routine immunization programs [50]. Such bivalent vaccines (type 1 and 3) are more immunogenic than trivalent OPV [51] and nearly as effective as the monovalent OPV formulations, especially in young children receiving their first polio immunization [52-

55]. However, the risks of VAPP and VDPVs by reversion of the Sabin strains to a pathogenic strain still remain, and thus the global eradication of polio by using these OPVs is impossible.

As a result, OPV cessation and replacement by IPV is highly recommended and supported by the GPEI. Substitution of OPV by a similar (low) dose oral inactivated poliovirus vaccine is unlikely to succeed. Instead, the development of live poliovirus strains with stable attenuation properties seems more feasible [56-58]. It is expected that some of these approaches will be clinically tested in the coming years.

Novel oral mucosal vaccine delivery – sublingual and buccal route

Sublingual vaccine delivery has gained significant attention during the past few years, as shown by the numerous preclinical studies published in the last decade [59].

The use of the sublingual administration route for IPV has been studied preclinically by White *et al*. [60]. They compared the IM administration with sublingual administration using a thermoresponsive gel (TRG) delivery system. These TRG systems are liquid at room temperatures and become solid in warmer environments, like the mouth [61,62]. The solid gel has high mucosal adhesion properties and ensures slow release and potentially minimal loss because of swallowing. The effect of dmLT as oral mucosal adjuvant was investigated in this study as well. Sublingual administration of IPV without dmLT or as liquid (instead of the TRG delivery system) was not able to induce any immune response in mice. Sublingual administered IPV as TRG in combination with dmLT led to serum virus-neutralizing titers and systemic Ig levels, nevertheless significantly lower than when IPV alone is administered via the IM route. However, the TRG-formulation containing IPV plus dmLT induced systemic and mucosal IgA production not seen via IM vaccination. The mucosal immunity as measured by IgA in salivary samples, improved with an increasing IPV dose [60].

To our knowledge the buccal route has not yet been studied with IPV. Whether OPV administration leads to some sublingual or buccal delivery or even replication is not known. In order to achieve successful vaccination via the sublingual or buccal route, enhanced vaccine formulations are essential to target these mucosal inductive sites. It is expected that mucosal adjuvants and muco-adhesive agents to prolong contact with the oral mucosa are needed [63].

Nasal vaccination and other novel mucosal vaccination routes

Intranasal vaccination can avoid degradation of vaccine antigen by digestive enzymes, low pH and strong dilution. As a result nasal vaccination may require smaller doses of antigen when compared to oral immunization [64]. However, for nasal vaccination also to date no vaccine is on the market on the basis of inactivated pathogens or subunits/proteins. A risk of intranasal immunization is the possible deposition of antigen or adjuvant in the central nervous system through the olfactory bulbs and olfactory nerves, which can cause adverse effects like temporary facial paralysis (Bell´s palsy) [65,66]. This has been seen with a marketed virosomal influenza vaccine that was adjuvated by heat labile enterotoxin of E.Coli (LT) and has been withdrawn from the market due to this side effect. Also, wheezing may occur in young children after intranasal vaccination. To date no efforts have been published that address nasal vaccination with polio vaccine formulations since this administration route induces mucosal immune responses mainly in the respiratory and reproductive tract mucosae. Moreover, immunization via the nose is often efficient for inducing systemic immune responses, but not for eliciting intestinal immunity in humans [67].

Other routes that are investigated for mucosal vaccination against infectious diseases include pulmonary, vaginal and rectal routes [11]. However, like nasal vaccination these routes are not yet explored and/or disclosed for use in polio vaccination. Amongst others, this might be related to the fact that these routes are not first choice because of ease of accessibility, acceptance by the public or technical challenges.

Mucosal adjuvants

The necessity of adjuvants to stimulate has been reported in the preclinical mucosal immunization studies for several antigens as reported elsewhere. In contrast to OPV and its potentially live virus successors, mucosal polio vaccination based on IPV is expected to require adjuvants in order to induce sufficient systemic as well as mucosal immunity. Although there is a broad preclinical experience on adjuvants for several antigens as reported elsewhere [11], only limited studies have shown the use of adjuvants for mucosal IPV delivery (Table 2). Current experience is limited to the use of dmLT in combination with TRG as described above.

EXPERT COMMENTARY

Due to the cessation of OPV and the possible eradication of polio there is a need for better and affordable IPV. Ideally, a new generation of IPV should be administered through alternative (needle-free) delivery routes, provide mucosal immunity, be safe to manufacture, have a long shelf-life, be stable outside the cold-chain, and be affordable for low-income countries.

Important variables for the development of improved IPV are the route of administration, the selection of adjuvants, the vaccine formulation and the use of (non-invasive) delivery methods [11]. The use of jet injectors is probably the fastest way to introduce needle-free IPV vaccines, but apart from injection safety it may not lead to dose sparing. Another promising and relatively short-term solution in the context of parenteral IPV vaccination is probably the replacement of the needle and syringe with a biodegradable implant as vaccine carrier. Biodegradable implants could both reduce the safety risks related to conventional injection and carries the vaccine in the (more thermostable) solid form, which might minimize the dependence on the cold-chain.

Mucosal vaccine delivery, like the sublingual and buccal routes, has the potential to elicit local immune responses at the point of virus entry, but often induces in the absence of an adjuvant tolerance or low-to-undetectable immune responses [68]. Therefore, efforts on mucosal vaccine design should focus on (i) overcoming physiological barriers at mucosal routes, (ii) targeting local APCs for appropriate processing of the antigens that lead to specific T and B cell activation, and (iii) controlling the kinetics of antigen and adjuvant presentation to promote long-lived, protective adaptive immune memory responses [44].

Different adjuvants have already proven their potential for (Sabin) IPV though via the parenteral route. However, limited data is available on preclinical evaluation of adjuvants for mucosal vaccination. The E. coli heat labile toxin with 2 mutant (dmLT) has proven its potential for IPV delivery via the mucosal route (sublingual) preclinically and an ongoing clinical phase 1 study in healthy subjects should proof its safety via the sublingual route (study no. NCT02052934). This could be therefore an interesting adjuvant for further development for polio vaccination via the oral mucosa.

Special attention should be given to restrictions related to the final target population for polio vaccination: infants. The delivery method and the delivery device and formulation should be suitable for application in infants. For example sublingual tablets are not suitable

for infants since they may give risk of choking. Improved ways of delivery to the buccal and/or sublingual mucosa are under way. These include sticking formulations, like fluids that jellify upon contact with the mucosa (temperature) or thin films that can be applied below the tongue. Advantage of these formulations is that they prolong the contact time with the mucosa and thereby may decrease the dose needed for induction of immunity.

Dermal delivery might be a more suitable alternative for vaccination of infants. A disadvantage of dermal delivery is that in general no mucosal immunity is elicited by this route. However, for certain vaccine adjuvant, like dmLT, combinations there is evidence that ID vaccination may also have the potential of inducing mucosal immunity [69-72]. New approaches, such as biodegradable or coated microneedles, hold promise for dermal delivery since they also may contribute to the stability of the vaccine.

FIVE-YEAR VIEW

In the next five years, the phased withdrawal of OPV and inclusion of IPV into all global routine immunization programs will create a market for non-invasive delivery of polio vaccines, although it is not clear how large this market will be since IPV demand in the post-eradication era is uncertain. However, several new approaches for IPV delivery are underway. In addition, potentially safer OPV vaccines may be introduced to have a role during polio outbreaks.

Since more research groups have access to (Sabin) IPV via support from organizations, such as BMGF and/or (new) sIPV producers, more efforts to develop alternative administration methods for IPV are expected the coming years. This will result in an increase of preclinical studies evaluating use of new ways of delivery, which in five years may reach the clinical development phase.

Furthermore, it is expected that other novel approaches, such as heterogeneous prime-boost schedules, e.g., priming with OPV and follow-up vaccinations with intramuscular or dermal administered IPV will get attention the coming years. IPV has been shown to boost mucosal immunity among recipients who have earlier received OPV [73,74]. Further clinical studies on heterogeneous prime-boost vaccination schedules, but also of other administration strategies, mucosal immunity will be more and better addressed by modern techniques [75].

Finally, the design of administration methods that have the potential to give improved

thermostability of the vaccine will evolve, such as solid dosage forms for sublingual delivery [59], dissolvable microneedle patches and biodegradable mini-implants (e.g., Bioneedles). These approaches would be favorable to reach remote areas in developing countries for which proper logistics are not available.

One of the main challenges for future introduction of newly administered IPV vaccines is the acceptance by the (final) stakeholders, which include (local) governments shaping their immunization programs, global vaccine procurement organizations like UNICEF, but also key opinion leaders, vaccine producers and vaccine recipients. To this extent, BMGF, PATH and WHO are working as part of the Global Vaccine Action Plan (GVAP) on a method to address total system cost-effectiveness [76]. A total system cost-effectiveness evaluation is a holistic evaluation of trade-offs between price and deliverability that potentially can guide target product profiles and incentive structures that are most representative of what countries need to efficiently achieve maximum immunization coverage [76]. For instance, this involves improved effectiveness of the vaccine for example by mucosal immunity, advantages of logistics without use a cold-chain, but also use of less trained health-care personal and the costs of the vaccine. These types of approaches may on the long term yield insights that for example a thermostable IPV delivered by sublingual patches may come out favorable for total system effectiveness and costs, while the primary cost price of the novel vaccine might be relative high.

KEY ISSUES

- To date the potential of alternative IPV delivery has not been explored comprehensively. Emphasis is on dermal delivery and jet injection without the use of adjuvants.
- IPV has been shown to boost mucosal immunity among recipients who have earlier received oral poliovirus vaccine (OPV), indicating that heterologous vaccination strategies hold promise including those with new(ly developed) delivery methods.
- Ideally, the new generation of IPV vaccines after global OPV cessation, should induce mucosal immunity already after prime immunization in order to stop the transmission of polioviruses in high-risk areas.
- Future (pre)clinical studies have to evaluate mucosal immunity more extensively.
- Costs for novel ways of IPV delivery have to be approached comprehensively in order to calculate cost-effectiveness and warrant the product cost price, which are required for market introduction.

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Development of thermostable lyophilized inactivated polio vaccine

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ABSTRACT

The aim of the current study was to develop a dried inactivated polio vaccine (IPV) formulation with minimal loss during the drying process and improved stability when compared with the conventional liquid IPV.

Extensive excipient screening was combined with the use of a Design of Experiment (DoE) approach in order to achieve optimal results with high probability. Although it was shown earlier that the lyophilization of a trivalent IPV while conserving its antigenicity is challenging, we were able to develop a formulation that showed minimal loss of potency during drying and subsequent storage at higher temperatures.

This study showed the potential of a highly stable and safe lyophilized polio vaccine, which might be used in developing countries without the need of a cold-chain.

INTRODUCTION

Poliomyelitis is a highly infectious disease, which mainly affects young children. The disease, caused by any one of three serotypes of poliovirus (type 1, type 2 or type 3) has no specific treatment, but can be prevented through vaccination.

Currently, the live attenuated oral poliomyelitis vaccine (Sabin OPV) is the vaccine of choice to prevent polio outbreaks and stop transmission of wild polioviruses, especially in the remaining endemic countries. However, a major concern is the ability of OPV to revert to a form that can cause paralysis, so-called vaccine-associated paralytic poliomyelitis (VAPP). Permanent use of OPV would continue to generate circulating vaccine-derived polioviruses (cVDPVs) that will inevitably lead to new outbreaks [1]. Therefore, the new endgame strategy of the Global Polio Eradication Initiative (GPEI) includes the introduction of an inactivated polio vaccine (IPV) into all routine immunization programs followed by phased withdrawal of OPV [2]. In most of the high-income countries, IPV based on Salk strains is already the present preferred way to eliminate the risk of VAPP and cVDPVs.

To achieve global polio eradication, an (improved) IPV must be efficacious, inexpensive, safe to manufacture, and easy to administer [3]. The feasibility of current IPV in developing countries is limited, because IPV is more expensive than OPV and is administered through injections only. In order to extent the availability of IPV, the World Health Organization (WHO) and the Institute for Translational Vaccinology (Intravacc) in the Netherlands have developed a non-commercial IPV for technology transfer to developing countries [4]. Because the containment of the wild-type Salk poliovirus during production might be an issue, especially in developing countries, the new vaccine will be based on the traditional Sabin OPV strains (sIPV). For reduction in costs, Intravacc is developing sIPV formulations that show dose sparing by using an adjuvant [5] and/or other immunization routes [6, 7].

Vaccine delivery encompasses both administration of the vaccine formulation to specific sites and delivery of the antigen to and activation of relevant cells of the immune system [8]. Since alternative delivery methods and improved vaccine formulations have the potential to make vaccine delivery easier and safer [9, 10], several alternative vaccine delivery methods are currently being developed. However, the focus in vaccine development has been on optimization of the immunological properties, while stability issues are minimally addressed. Most vaccines, IPV included, are insufficiently stable to allow them to be purified, transported and stored at unrefrigerated conditions [11, 12].

One way to improve the storage stability of (s)IPV might be conversion into the dry state as is known to improve the stability of biopharmaceuticals [13]. An increased shelf life is not only of use for the final product for use within three months to two years, but also for stockpiling (1-10 or more years of storage). Particularly after polio eradication, a stockpile of polio vaccines is required to anticipate the potential risk of new polio outbreaks caused by circulating VDPV (even after OPV cessation) [14, 15] or bioterrorism attacks. In order to achieve an optimal vaccine stockpile, various issues need to be considered. The shelf life is paramount, because a delayed expiration time will reduce the stockpile costs [16]. Moreover, storage of dried materials at ambient temperature, including concomitant costs (e.g., reformulation costs), is cost effective compared with storage options at low temperatures. In addition, the ability to develop solid antigen formulations is crucial for new vaccine delivery routes including dermal delivery by coated or dissolving microneedles, parenteral delivery by powders or dissolvable needles, and pulmonary delivery of powders [8]. Technologies for producing dried biologicals include vacuum drying, air-drying, coating, spray (freeze-)drying and foam-drying [17-19]. One of the oldest and commonly used techniques is freeze-drying, also called lyophilization. However, during the lyophilization process the proteinaceous vaccine is subjected to freezing and drying stress by which its activity can be lost. Therefore, cryoprotective and lyoprotective agents are required. Many compounds, such as sugars, polymers, amino acids and surfactants, have been shown to improve the stability of biopharmaceuticals during lyophilization and subsequent storage [20].

Aim of the current study is to design IPV in the dry state with maintenance of the potency. A potency indicating parameter is D-antigenicity, which can be determined in ELISA using specific antibodies as stated in the European Pharmacopeia. Lyophilization of polio vaccines has been shown to be challenging since earlier studies failed to obtain a stable product with preservation of all three serotypes [21-23]. We describe the development of an IPV formulation by selecting excipients that [i] minimize potency loss upon drying and subsequent reconstitution, and [ii] increase the stability of IPV at elevated temperatures. Extensive excipient screening was combined with the use of a Design of Experiments (DoE) approach in order to achieve optimal results with high probability.

MATERIALS AND METHODS

Materials

Trivalent IPV, containing the inactivated Mahoney strain for type 1, MEF for type 2 and Saukett for type 3, was obtained from the process development department of Intravacc (Bilthoven, The Netherlands). The ten times concentrated trivalent bulk used in this study was determined at a nominal concentration of 400-80-320 DU/mL by ELISA as described [24].

The excipients sucrose, D-sorbitol, D-trehalose dihydrate, mannitol, L-glutamic monosodium salt monohydrate (MSG), glycine, myo-inositol, magnesiumchloride hexahydrate, lithium chloride and ovalbumin were all purchased from Sigma (St. Louis, MO). Peptone (vegetable) and dextran (6 kDa, from Leuconostoc ssp) were from Fluka (Buchs, Switzerland) and sodium chloride was from Merck (Darmstadt, Germany). To prepare 10 mM McIlvaine buffer, 10 mM citric acid (Sigma-Aldrich, St. Louis, MO) was added to 10 mM disodium hydrogen phosphate dehydrate (Na2HPO4) (Fluka, Buchs, Switzerland) in a ratio of 1:6 resulting in a pH-value of 7.0. All excipients used were of reagent quality or higher grade.

Methods

Dialysis

Unless otherwise indicated, the trivalent IPV bulk material was dialyzed against 10 mM McIlvaine buffer (pH 7.0) using a 10 kDa molecular weight cut-off, low-binding regenerated cellulose membrane dialysis cassette (Slide-A-Lyzer®, Pierce, Thermo Scientific, Rockford, IL) to replace the buffer components of the IPV bulk (M199 medium).

Formulations

All excipients were dissolved in McIlvaine buffer at a double concentration of the indicated end concentration. The dialyzed IPV was mixed 1:1 by volume with the formulation to be tested. Subsequently, 2 mL neutral glass injection vials (Müller + Müller, Holzminden, Germany) were filled with 0.2 mL of the IPV-excipient mixtures and half-closed with 13 mm pre-dried (overnight at 90°C) rubber stoppers (type V9250 from Helvoet Pharma, Alken, Belgium).

Drying processes

The filled (0.2 mL/vial) and half-stopped vials were loaded into a pilot freeze dryer (freeze-drying unit sublimator 2-3-3, Zirbus) at a shelf temperature of -50°C, or at a shelf of 4°C and subsequently frozen to -50°C by reducing the shelf temperature at a rate of 1°C/min. These different processes will be denoted as fast and slow freezing, respectively. The vials were kept at a temperature of -50°C for two hours. For the primary drying phase, the shelf temperature was increased at a rate of 0.2°C/h to -45°C (while decreasing the chamber pressure to 0.045 mbar) followed by drying for 3h. The secondary drying phase was performed by further increasing the shelf temperature at 0.02°C/min to 25°C while decreasing the chamber pressure to 0.01 mbar, followed by 24h drying at 25°C. At the end of the cycle, the vials were closed under vacuum, sealed with alu-caps and kept at 4°C until analysis.

In literature, different vacuum drying processes are described [25-27]. The vacuum drying process used in current study was slightly adapted, due to the characteristics of IPV. Briefly, the vials were loaded at shelves of 15°C and kept at that temperature for 10 minutes. The chamber pressure was reduced to 1 mbar in different ramping steps of 15 minutes and starting at a 25 mbar chamber pressure. The temperature was decreased to -10°C for one hour at 0.05 mbar and for one hour at 0.03 mbar so that freezing of the formulations was prevented since product temperature was kept above the ice-nucleation temperature of the formulations. Subsequently, shelf temperature was increased at 0.05°C/min to 30°C. At the end of the cycle, the vials were closed under vacuum, sealed with alu-caps and kept at 4°C until analysis.

Design of Experiments (DoE)

The Design of Experiments models for D-antigen recoveries measured by ELISA were evaluated in Modde 9.1 software (Umetrics AB, Umea, Sweden) to establish the stability profiles of the lyophilized IPV. In a first pilot experiment, the effect of common used stabilizers, i.e., sucrose (0-20% w/v), trehalose (0-20% w/v), mannitol (0-10% w/v), dextran (0-10% w/v) and NaCl (0-63 mM), was determined using a D-optimal design containing of 22 different formulations and three replicates of the center point (**supplemental data, table S1**). For the screening of some excipients, a full factorial DoE was performed around sorbitol, $MgCl_2$, monosodium glutamate (MSG) and mannitol, all within the concentration range from 0 to 10% w/v. In this full factorial design 24 formulations were tested and three replicates of the center point (**Table 2**). For the optimization experiment a central composite circumscribed

(CCC) design was used around sorbitol (8 to 12% w/v), $MgCl_2$ (5 to 12% w/v) and MSG (5 to 12% w/v). The CCC design consisted of eight corner experiments, six axial experiments and three replicated center points (Table 3). The models were fitted using partial least squares (PLS) regression and subsequent optimized by deleting non-significant terms leading to a model with the best model performance parameters, i.e., goodness of fit (R^2), goodness of prediction (Q^2), model validity and reproducibility.

D-antigen ELISA

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to quantify D-antigen units (DU) of the lyophilized polio vaccine formulations as described by Ten Have *et al*. (2012) [28]. Briefly, microtiter plates were coated with serotype-specific bovine anti-polio serum (Bilthoven Biologicals, Bilthoven, The Netherlands). After washing dilutions of IPV-formulations were added (in duplicate). After a 30 minutes incubation at 37°C under gentle shaking, plates were washed and a mixture of serotype-specific anti-poliovirus monoclonal antibody (mab 3-4-E4 (type 1), 3-14-4 (type 2), 1-12-9 (type 3); all from Intravacc, Bilthoven, The Netherlands) and HRP-labeled anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) was added and incubated for 30 minutes at 37°C while shaking. Subsequently, plates were washed followed by addition of ELISA HighLight signal reagent (ZomerBloemen BV, Zeist, The Netherlands). Chemiluminescence was measured for 10-15 minutes by using a luminometer (Berthold Centro LB960). The signal at maximum intensity was used to calculate D-antigen content relative to the reference standard. Unless otherwise indicated, D-antigen recovery values were shown as normalized values for liquid formulations prior to lyophilization.

Dynamic Light Scattering (DLS)

Particle size measurements were performed using a Zetasizer Nano-ZS system (Malvern Instruments, Malvern, UK). DLS measurements were done in triplicate with 0.5 mL liquid (undialyzed) IPV bulk at an operating temperature of 25°C. Homogeneity of the size distribution was reflected in the polydispersity index (PdI), which ranges between 0.0 (fully homogeneous size distribution) and 1.0 (random size distribution).

Moisture content analysis

The water content was determined using a Karl Fischer coulometric titrimeter (Model CA-06 Moisture meter, Mitsubishi). The samples were weighted, subsequently reconstituted in

the Karl-Fischer reagent, Hydrana Coulomat A (Fluka, Buchs, Switzerland), and injected into the titration vessel. Each vial was measured in triplicate. The empty vials were weighted and the water content was calculated based on the water content measured by the titrimeter, the weight of the lyophilized product in the vial, the reconstitution volume of the reagent, titration volume and the water content of the blank titration.

Differential Scanning Calorimetry (DSC)

The thermodynamic behavior of the formulations was determined by differential scanning calorimetry (DSC). Aluminum DSC pans were filled with the liquid formulations and subjected to a controlled temperature program in a differential scanning calorimeter (DSC Q100, TA Instruments). The samples were cooled to -70°C at a rate of 10°C/min, kept isothermal for 2 min, and subsequent heated from 0°C to 20°C at a heating rate of 10°C/min. The sample chamber was purged with nitrogen gas (50 mL/min). The glass transition temperatures (Tg') were determined as the midpoint of the discontinuities in the heat flow curves using thermal analysis software (Universal Analysis 2000, TA Instruments).

RESULTS & DISCUSSION

Common lyoprotectants

The first design of experiment approach was based on the most commonly used lyoprotectants, i.e., sucrose, trehalose and mannitol, in combination with dextran, with and without NaCl (**Table S1, supplemental data**). These excipients are known to provide physical and biochemical stabilization as well as appropriate structural properties to the cake structure, during and after lyophilization [29-32].

A partial least squares (PLS) regression model was fitted and optimized per serotype, which resulted in valid models to predict the D-antigen recoveries directly after lyophilization according to the model performance parameters. For serotype 1, 2 and 3, the Q^2 values were, respectively, 0.650, 0.592 and 0.671, while the R^2 values were 0.905, 0.873 and 0.929. The effects of the different stabilizers, after optimization (excluding non-significant parameters) on the D-antigen recovery after lyophilization are presented in **figure 1A**.

Without the addition of stabilizers, D-antigen recoveries of type 1, 2 and 3 after lyophilization were 9, 11 and 2%, respectively (**Table S1, supplemental data**). Both sucrose and mannitol are able to stabilize all serotypes to a certain extent during the lyophilization process (**Figure**

1A). Dextran has a negative effect on type 1 and 3 during the lyophilization process, whereas the addition of NaCl has no significant effect on the D-antigen recovery, independent of serotype (Figure 1A). Best results, with recoveries of approximately 55%, 85% and 50% for serotype 1, 2 and 3 respectively, were obtained with formulations containing sucrose and/or trehalose in combination with mannitol (Figure 1B). Among the three serotypes, type 2 was the least affected during lyophilization, resulting in a maximum recovery of 85% after lyophilization. This serotype is known to be the most stable at higher temperatures [33].

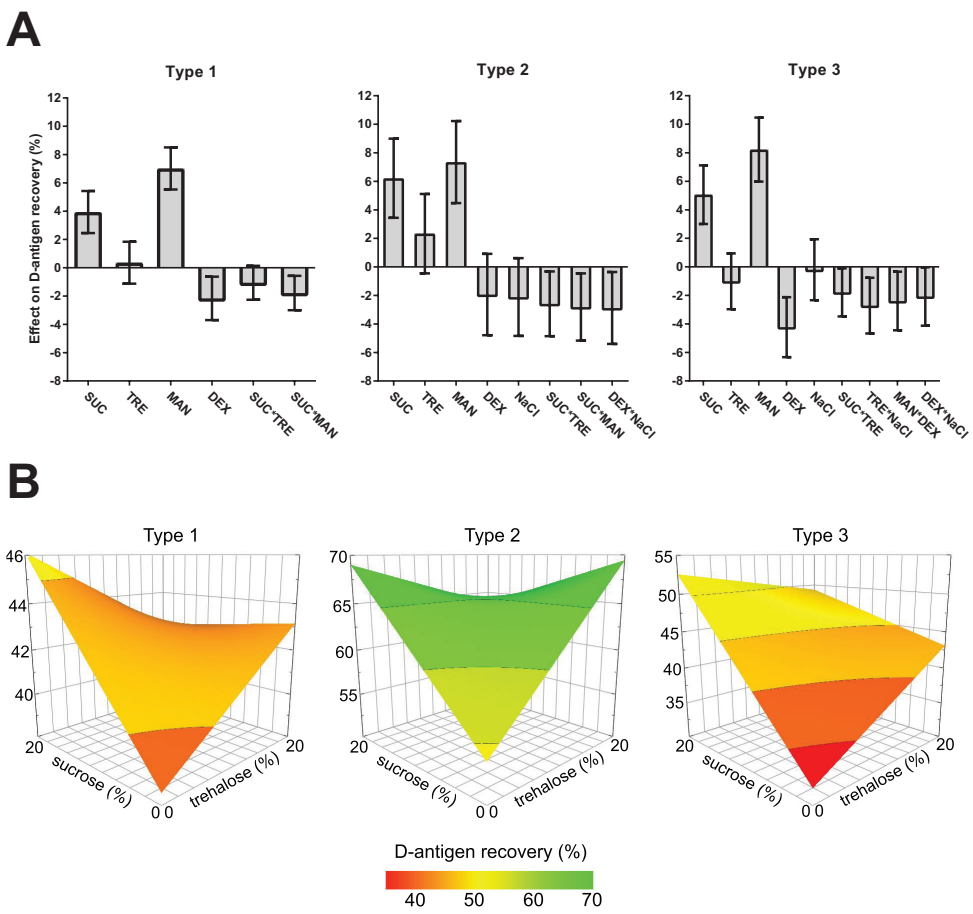

Figure 1 Stabilizing potential of the excipients sucrose (SUC), trehalose (TRE), mannitol (MAN), dextran (DEX) and NaCl on D-antigen recovery directly after lyophilization. Main and interaction effects that contribute (per serotype) to the best model, according to the model performance parameters (Q^2=0.650, R^2=0.905 (type 1); Q^2=0.592, R^2=0.873 (type 2); Q^2=0.671, R^2=0.929 (type 3)), are depicted in coefficient plots (A). Surface response plots of the D-antigen recovery for each serotype based on formulations containing sucrose and trehalose in combination with 10% mannitol (without dextran or NaCl) (B).

This first experiment illustrated the complexity of lyophilizing a trivalent polio vaccine retaining its antigenicity and displayed that each serotype may behave different within the same formulations. It has been reported earlier that lyophilization of polio vaccines is challenging. For example, Nagel *et al*. revealed that with the excipients sorbitol and peptone relative potencies of 70% for type 1 and 50% for type 2 could be obtained after lyophilization of IPV, while all activity of type 3 was destroyed [22]. In addition, Pollard *et al*. exhibited that lyophilization of the wild type poliovirus resulted in almost complete inactivation, although it was not determined whether D-antigenicity was also negatively affected [34].

Drying methods

Literature indicates that vacuum-drying, a drying process without a freezing step, can be used to stabilize IPV [35]. With the use of disaccharides sucrose and trehalose as stabilizing agents, it is feasible to obtain a dried (or highly viscous) polio vaccine by vacuum-drying without affecting its potency. As a result, we decided to investigate the impact of different drying processes on the integrity of IPV. Trehalose and sucrose based IPV formulations were vacuum dried and compared with the same formulations that underwent different lyophilization processes. One lyophilization process starting with a slow freezing step (cooling of shelves from 4°C to -50°C, at 1°C/min) and one with a fast freezing step (shelves pre-cooled to -50°C as has been used in the pilot experiment). The conventional trivalent IPV formulation, without

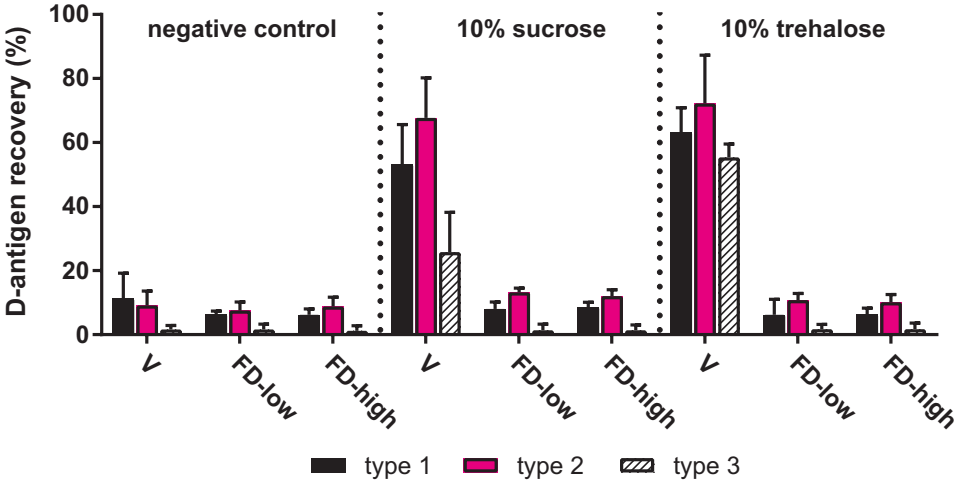

Figure 2 D-antigen recovery of dried IPV using different drying methods, i.e., vacuum drying (V) or lyophilization with low (FD-low) or high freezing rate (FD-high). Common used stabilizing sugars sucrose (10% w/v) and trehalose (10% w/v) were compared with the formulation without additives (negative control). Mean values (n=3) and SD are shown.

Table 1 Formulations tested in an excipient screening experiment (S). D-antigen recoveries were determined directly after lyophilization.

	Sugars	Polyols	Amino acids	Proteins	Other	D-antigen recovery (%)		
						T1	T2	T3
S0	-	-	-	-	-	12	17	2
S1 *	-	7% sorbitol 7% mannitol	2% MSG 2% glycine	7% ovalbumin	-	66	89	73
S2 *		7% sorbitol 7% mannitol	2% MSG 2% glycine	7% ovalbumin	10 mM EDTA	66	81	77
S3 *	-	7% sorbitol 7% mannitol	2% MSG 2% glycine	-	-	73	95	74
S4	3% sucrose 3% dextran 3% myo-inositol	-	-	3% ovalbumin	-	27	58	12
S5	5% sucrose	5% sorbitol 5% mannitol	2% glycine 3% lysine 3% arginine			15	49	5
S6 *	5% sucrose	5% sorbitol 5% mannitol	3% MSG 2% glycine 3% lysine	-	-	65	86	76
S7	5% sucrose 5% trehalose		3% lysine 3% alanine			34	67	36
S8	5% sucrose 5% trehalose		3% lysine 3% alanine		0.01% Tween80	38	82	43
S9	5% sucrose		3% lysine 3% alanine		3% Ca- lactobionate	36	36	24
S10	5% sucrose		3% lysine 3% alanine		3% rec. gelatin	45	72	46
S11 *	-	5% sorbitol	-	5% peptone	2% $MgCl_2$	80	79	75
S12 *	-	5% sorbitol	-	5% peptone	1% LiCl	86	100	87
S13	5% sucrose 5% trehalose	-	-	5% peptone	-	29	62	26

** Formulation selected for stability testing (as shown in figure 3)*

any additives, showed recoveries <15% for all serotypes after vacuum drying or lyophilization (Figure 2). The vacuum drying process yielded highly viscous IPV formulations with a water content of respectively 9% or 12% for the formulations containing sucrose or trehalose, whereas lyophilization yielded formulations with a water content of <1%. While more than 55% of the antigen is intact after vacuum drying of IPV containing 10% trehalose, both lyophilization processes resulted in almost complete loss of D-antigenicity. These results depict both one of the main disadvantages of vacuum drying as well as the opportunity to dry without freezing stresses. The high moisture content is caused by the relatively low specific surface area during vacuum drying, which results in an extremely slow secondary drying when compared to lyophilization. As a result, the risk of sugar crystallization and/or phase

separation in the rubbery state exists [13].

The poor recoveries depicted in figure 2 suggest that neither trehalose nor sucrose alone was able to protect IPV against both the freezing and drying stresses during lyophilization. Although the different freezing rates of the lyophilization processes did not show significant differences in D-antigenicity, particle size measurements after freeze thawing revealed differences in IPV particle size that were dependent on freezing rate. Slowly frozen IPV had a size of 44.7 ± 2.1 nm with a PdI of 0.485 ± 0.062, whereas fast frozen IPV remains at a particle size of 38.7 ± 1.1 nm with a PdI of 0.166 ± 0.009 (similar to IPV bulk prior to freeze thawing). Thus, IPV appeared to be most resistant to freeze thawing when a fast freezing rate was applied, which induced less aggregation than slow freezing. For that reason, a fast freezing step was selected for optimization of the IPV formulation for lyophilization.

Extensive excipient screening

In order to obtain an IPV formulation that is suitable for lyophilization we performed a more extensive excipient screening. The selection of excipients for the screening (Table 1) was based on findings from literature [20, 22, 36-40] and earlier unpublished data.

In general, the formulations containing sorbitol showed high recoveries directly after lyophilization. Especially formulations containing sorbitol, mannitol and monosodium glutamate (MSG) stabilized the IPV during the process of freezing and subsequent dehydration with D-antigen recoveries of >65% for all serotypes (Table 1; S1-S3, S6). Another notable formulation is the combination of sorbitol, peptone and the salts LiCl or $MgCl_2$ (Table 1; S11 and S12) indicating that this combination of excipients is able to protect the IPV during lyophilization. The best formulations, which showed D-antigen recoveries of >60% directly after lyophilization for all serotypes, were selected and subsequently tested for stability (Figure 3). In general, the tested formulations so far showed disastrous recoveries after incubation at higher temperatures (Figure 3B-C) and even after incubation for a month at ambient temperature, a large drop in antigenicity was observed (Figure 3D).

The appropriate performance of sorbitol combined with mannitol and MSG indicates that the presence of polyols in combination with MSG stabilizes the IPV during lyophilization in a similar way as the disaccharides sucrose and/or trehalose (combined with mannitol) did as shown in the pilot study. In earlier lyophilization studies, sorbitol was used as excipient in combination with peptone [22], which showed again to be a valuable combination in this experiment, whether or not in the presence of a salt like $MgCl_2$ or LiCl. Peptone-

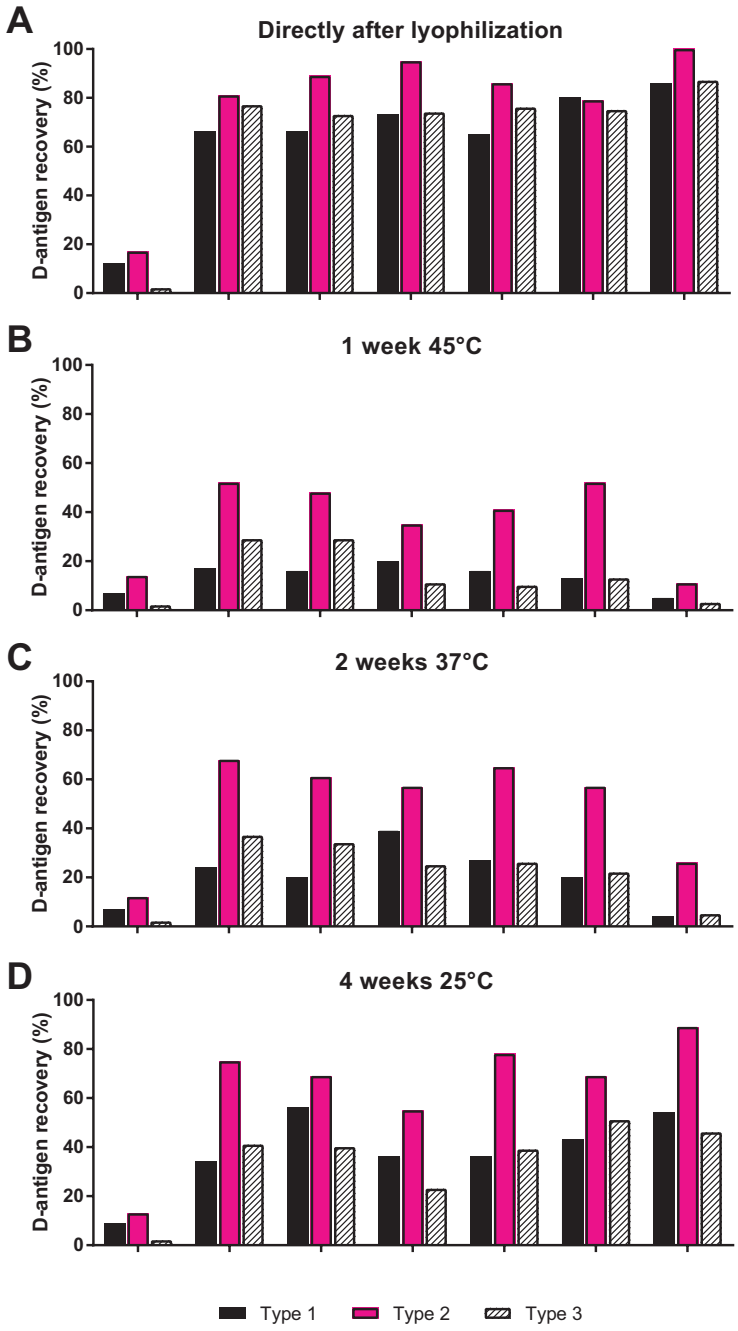

Figure 3 D-antigen recoveries of the best formulations from the screening experiment based on recoveries directly after lyophilization (>60% for all serotypes). Panel A shows the D-antigen recoveries directly after lyophilization. Panel B, C and D show the recoveries after incubation for one week at 45°C, two weeks at 37°C, and 4 weeks at room temperature, respectively. The formulations are described in table 1.

containing lyophilized IPV formulations, however, showed limited storage stability at elevated temperatures (**Figure 3B-C**). Furthermore, peptone is poorly defined and heterogeneous in composition; therefore, it was chosen to exclude peptone further in the formulation design. $MgCl_2$'s stabilizing potential has been described for fluid oral polio vaccine. As a result, a number of manufacturers use $MgCl_2$ to stabilize their OPV (39). Thus, $MgCl_2$ could also be a critical additive in a dried IPV formulation.

Table 2 Excipients sorbitol, magnesium chloride ($MgCl_2$), monosodium glutamate (MSG) and mannitol (all 0-10% w/v) examined in a full factorial design (D). Glass transition temperature (Tg') of the liquid formulation before lyophilization and residual moisture content (RMC) of the dried cake were determined.

	Sorbitol	$MgCl_2$	MSG	Mannitol	Tg' (°C)	RMC (%)
D1	-	-	-	-	n.d.	0.2
D2	10%	-	-	-	-43.4	2.4
D3	-	10%	-	-	n.d.	45.9
D4	10%	10%	-	-	n.d.	20.6
D5	-	-	10%	-	-47.0	7.4
D6	10%	-	10%	-	-41.2	1.8
D7	-	10%	10%	-	-58.6	4.0
D8	10%	10%	10%	-	-49.6	7.6
D9	-	-	-	10%	-34.6	1.1
D10	10%	-	-	10%	-40.5	0.3
D11	-	10%	-	10%	n.d.	16.1
D12	10%	10%	-	10%	-51.6	17.1
D13	-	-	10%	10%	-41.0	2.8
D14	10%	-	10%	10%	-39.7	2.3
D15	-	10%	10%	10%	-51.2	8.2
D16	10%	10%	10%	10%	-48.2	9.7
D17	5%	5%	5%	5%	-48.1	5.8
D18	5%	5%	5%	5%	-47.7	17.7
D19	5%	5%	5%	5%	-47.7	9.7

Design of experiments – full factorial design

Based on the results of our study so far, we selected the most promising excipients to screen them further using a DoE approach and get more insight in the IPV stabilizing potential of these additives. Therefore, a full factorial design was performed around the excipients sorbitol, MSG, $MgCl_2$ and mannitol (all in the concentration range of 0-10% w/v) (Table 2). The mixture of sorbitol, mannitol and MSG has already shown its capacity to provide valuable protection during the lyophilization process. In addition, mannitol was selected as a common bulking agent due to its excellent cake-forming property and the option to apply annealing when needed [41, 42]. As mentioned above, $MgCl_2$ is already a proven stabilizer for OPV and therefore we examined its contribution in a dried IPV formulation as well.

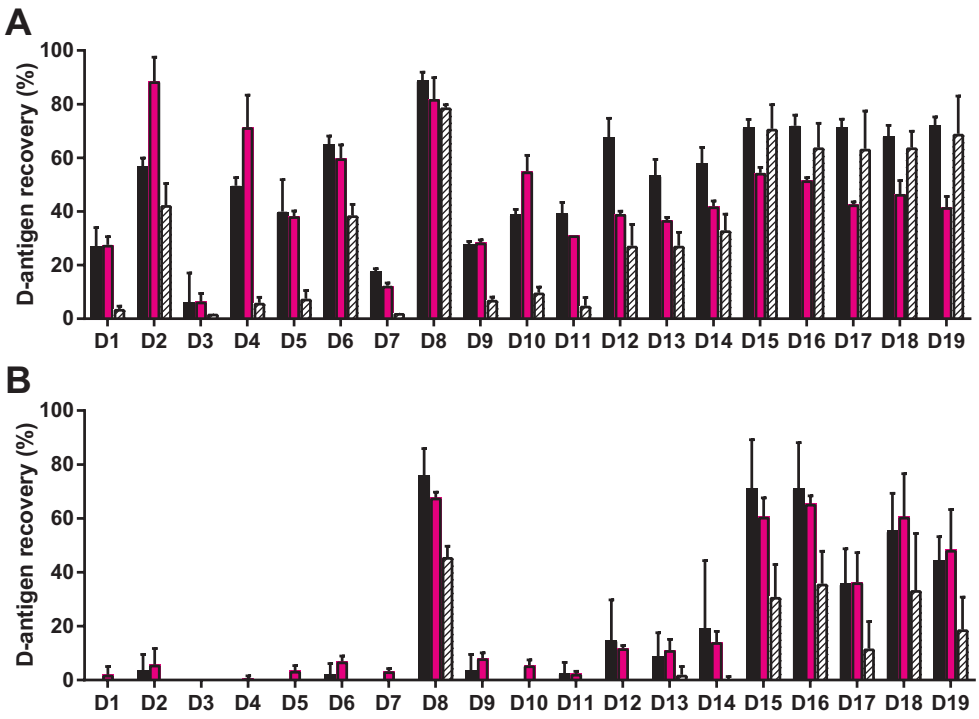

Figure 4 The stabilizing effect of sorbitol, magnesium chloride ($MgCl_2$), monosodium glutamate (MSG) and mannitol was investigated in a screening experiment using a DOE approach. Mean D-antigen recoveries and standard deviations (n=3) directly after lyophilization (A) and after incubation for one week at 45°C (B) are shown.

Figure 4A shows that sorbitol seemed to be an important excipient for the stabilization of both type 1 and 2 during lyophilization with recoveries up to 89% (Formulation D2 and D8). D-antigen recoveries after one week accelerated stability testing clearly illustrate the benefit of

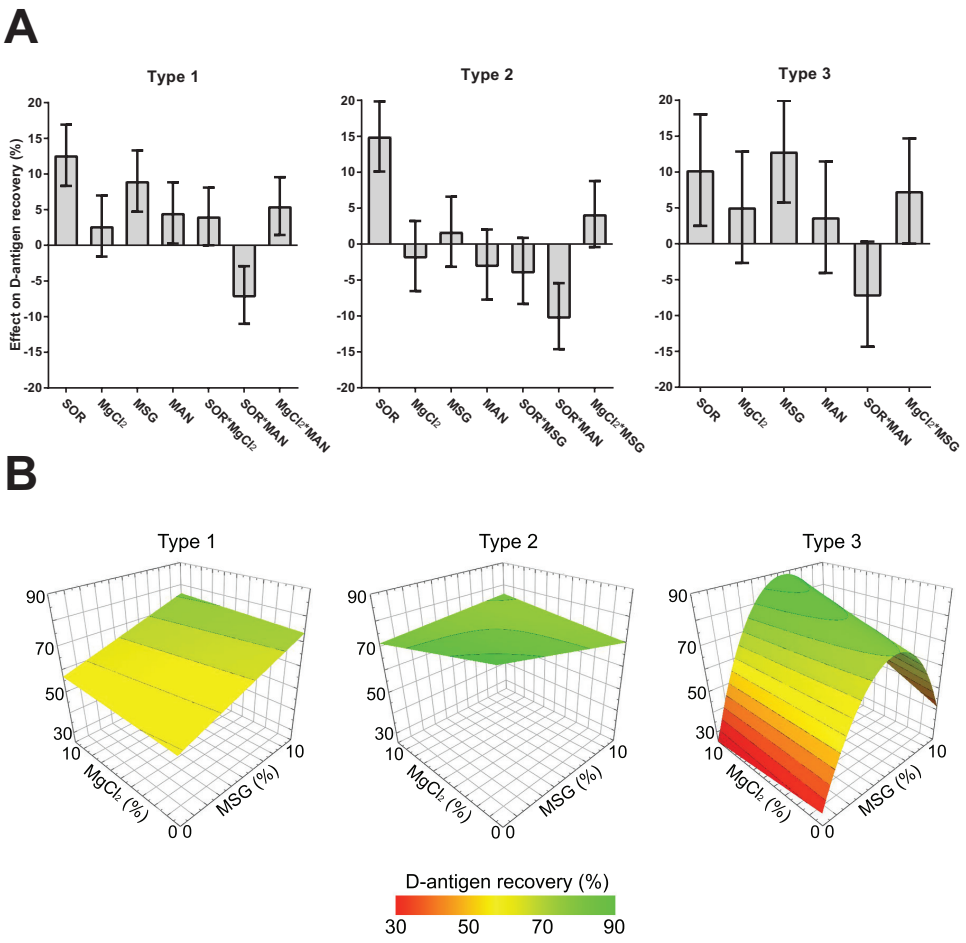

Figure 5 Stabilizing potential of the excipients sorbitol (SOR), $MgCl_2$, monosodium glutamate (MSG) and mannitol (MAN) on the D-antigen recovery directly after lyophilization. Main and interaction effects that contribute (per serotype) to the best fitted model, according to their model performance parameters (Q^2=0.685, R^2=0.923 (type 1); Q^2=0.577, R^2=0.877 (type 2); Q^2=0.575, R^2=0.824 (type 3)) are shown in coefficient plots (A). Surface response plots of the D-antigen recovery for each serotype based on formulations containing MSG and $MgCl_2$ in combination with 10% sorbitol (without mannitol) (B).

sorbitol, as well as $MgCl_2$ and MSG in the formulation (**Figure 4A**, formulation D8). Regression models (R^2>0.65 and Q^2>0.5) of these data confirm the findings that are described above. The stabilizing effects of the excipients on D-antigen recovery directly after lyophilization are depicted per serotype in coefficient plots after model optimization (**Figure 5A**). For all serotypes, there is a main effect of sorbitol, whereas MSG showed to be significant beneficial for the D-antigen recovery of type 1 and 3. However, sorbitol showed an interaction with mannitol that negatively affected IPV recovery after lyophilization, implying that the addition of mannitol to the IPV formulation for lyophilization is not desired. Accelerated stability

testing revealed that $MgCl_2$ and MSG are important stabilizers for all serotypes, indicated by significant main effects and interaction between these excipients. This means that the addition of $MgCl_2$ or MSG (10% w/v) results in an increase of 7-13% in D-antigen recovery, while the combination of these excipients would boost the D-antigenicity with 6-10%. The inclusion of $MgCl_2$ to the IPV formulation increases the residual water content (Table 2) after lyophilization significantly. This excipient is also responsible for reducing the glass transition temperatures (Tg') of the formulation before drying. The sorbitol and MSG interaction factor is able to increase the Tg' significantly (data not shown). As such, formulations might be optimized for Tg' by increasing the sorbitol-MSG content in order to be capable to lyophilize at higher temperatures resulting in shorter process time.

Again, it was observed that there are some divergences between the serotypes regarding their preference for stabilizers during lyophilization, whereas all serotypes seemed to prefer the

Table 3 Sorbitol (8-12% w/v), magnesium chloride ($MgCl_2$: 5-12% w/v) and monosodium glutamate (MSG: 5-12% w/v) tested in a central composite circumscribed design. D-antigen recoveries were determined per serotype directly after lyophilization and after accelerated stability testing (one week at 45°C).

	Sorbitol	$MgCl_2$	MSG	D-antigen recovery (%) Directly after lyophilization			D-antigen recovery (%) Stability 45°C		
				T1	T2	T3	T1	T2	T3
O1	8%	5%	5%	98.6	96.5	85.7	91.9	97.0	55.2
O2	12%	5%	5%	88.9	88.7	65.2	87.6	91.6	73.7
O3	8%	12%	5%	76.0	82.4	33.6	62.5	49.2	12.2
O4	12%	12%	5%	89.4	91.7	59.1	95.8	90.8	48.2
O5	8%	5%	12%	90.6	81.9	70.7	86.0	81.4	57.5
O6	12%	5%	12%	79.3	82.5	62.1	80.2	77.0	39.4
O7	8%	12%	12%	84.9	86.7	64.6	92.0	84.6	63.5
O8	12%	12%	12%	87.9	82.5	73.0	90.3	86.7	69.8
O9	6.6%	8.5%	8.5%	90.0	99.3	84.8	93.3	90.6	73.0
O10	13.4%	8.5%	8.5%	86.2	85.3	67.4	86.1	85.3	69.3
O11	10%	2.6%	8.5%	77.5	72.4	47.4	74.4	60.7	29.9
O12	10%	14.4%	8.5%	89.1	84.6	63.6	91.7	72.7	40.0
O13	10%	8.5%	2.6%	98.1	85.2	49.2	93.0	83.3	42.2
O14	10%	8.5%	14.4%	84.7	70.3	66.3	74.8	81.2	35.5
O15	10%	8.5%	8.5%	86.6	88.4	68.2	90.1	91.1	67.3
O16	10%	8.5%	8.5%	92.2	90.8	75.1	93.5	89.1	69.2
O17	10%	8.5%	8.5%	96.0	98.3	76.7	91.7	94.0	57.0

presence of MSG and $MgCl_2$ during stability testing. The surface response plots demonstrate that for type 1, the highest DU recoveries after lyophilization were found in a formulation containing 10% sorbitol in the presence of the highest amounts of MSG, regardless the $MgCl_2$ concentration, whereas both additives have no significant effect on type 2 (Figure 5B). Though, type 3 showed to be most delicate for small differences in excipient concentrations with maximal D-antigen recoveries of more than 80% with a formulation containing 10% sorbitol, 5-10% $MgCl_2$ and 4-8% MSG.

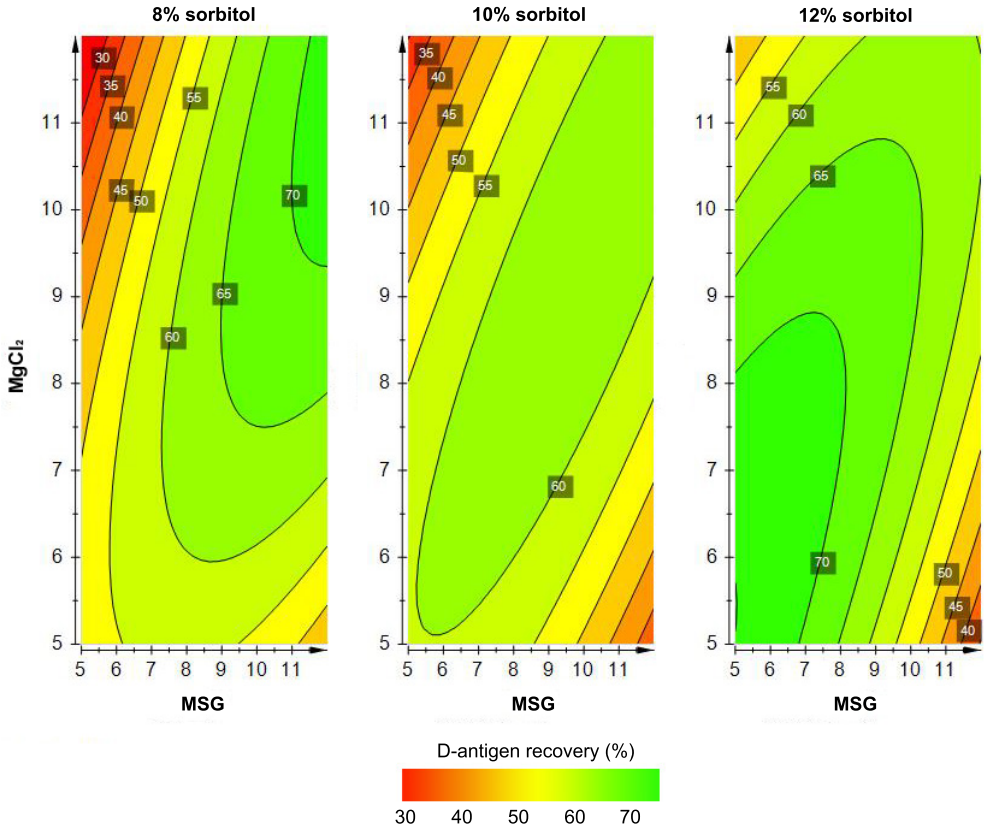

Figure 6 Contour plots show the effect of MSG (5-12% w/v) and $MgCl_2$ (5-12% w/v) in combination with 8% (A), 10% (B) or 12% w/v sorbitol (C) on the D-antigen recovery after lyophilization and subsequent accelerated stability testing for serotype 3.

Optimization

With the purpose to optimize the formulation, a response surface methodology design was implemented. Hence, a central composite circumscribed (CCC) [43] study set-up with the factors sorbitol (8-12% w/v), MSG (8-12% w/v) and $MgCl_2$ (5-10% w/v) was designed (Table 3) and D-antigen recovery was determined directly after lyophilization. An extensive stability study was included here as well to test whether the dried IPV formulation has an improved stability when compared with the conventional liquid IPV.

Within the design of the study, all formulations resulted in comparable recoveries after lyophilization and subsequent storage (Table 3). This indicates the formulation robustness of the formulation based on sorbitol (8-12% w/v), MSG (8-12% w/v) and $MgCl_2$ (5-10% w/v). Due to these small differences in D-antigen recovery after lyophilization and stability assessment between the tested formulations, it was not possible to obtain a valid model to describe the data. With all formulations, satisfying stabilization was achieved for serotypes 1 and 2, which showed recoveries of between 75% and 100% directly after lyophilization and only a small loss (0% to maximal 15%) during accelerated stability testing (Table 3). As mentioned above, type 3 showed to be the determining aspect in the decision for the final concentrations of the three excipients in our IPV formulation. Therefore, we decided to focus on the stability data of this serotype. PLS regression describes the accelerated stability data (one-week incubation at 45°C) well with high values for model validity and reproducibility (respectively 0.67 and 0.86). However, the predicting power of the model is limited (Q^2 = 0.43 instead of Q^2 >0.5). Despite this limitation, we requested the contour plots (Figure 6) to get an indication of the important parameters to stabilize serotype 3. It seems that the best D-antigen recoveries (>70%) were obtained with the lowest sorbitol (8% w/v) concentration in combination with the highest concentrations of both $MgCl_2$ and MSG (>10% w/v) or the highest sorbitol (12% w/v) content combined with relatively low amounts of $MgCl_2$ and MSG (<8% w/v) (Figure 6).

This experiment showed the robustness of the formulation since many formulations containing sorbitol, $MgCl_2$ and MSG in the tested concentrations showed high recoveries directly after lyophilization and preserved antigenicity during accelerated stability. Based on excellent D-antigen recoveries after lyophilization and subsequent stability testing (Table 3), the formulation containing 10% (w/v) sorbitol, 8.5% (w/v) $MgCl_2$ and 8.5% (w/v) MSG was selected for additional extensive stability testing up to 24 weeks at 25°C and 37°C, and up to one month at 45°C. Both the conventional liquid IPV and lyophilized IPV formulation remain

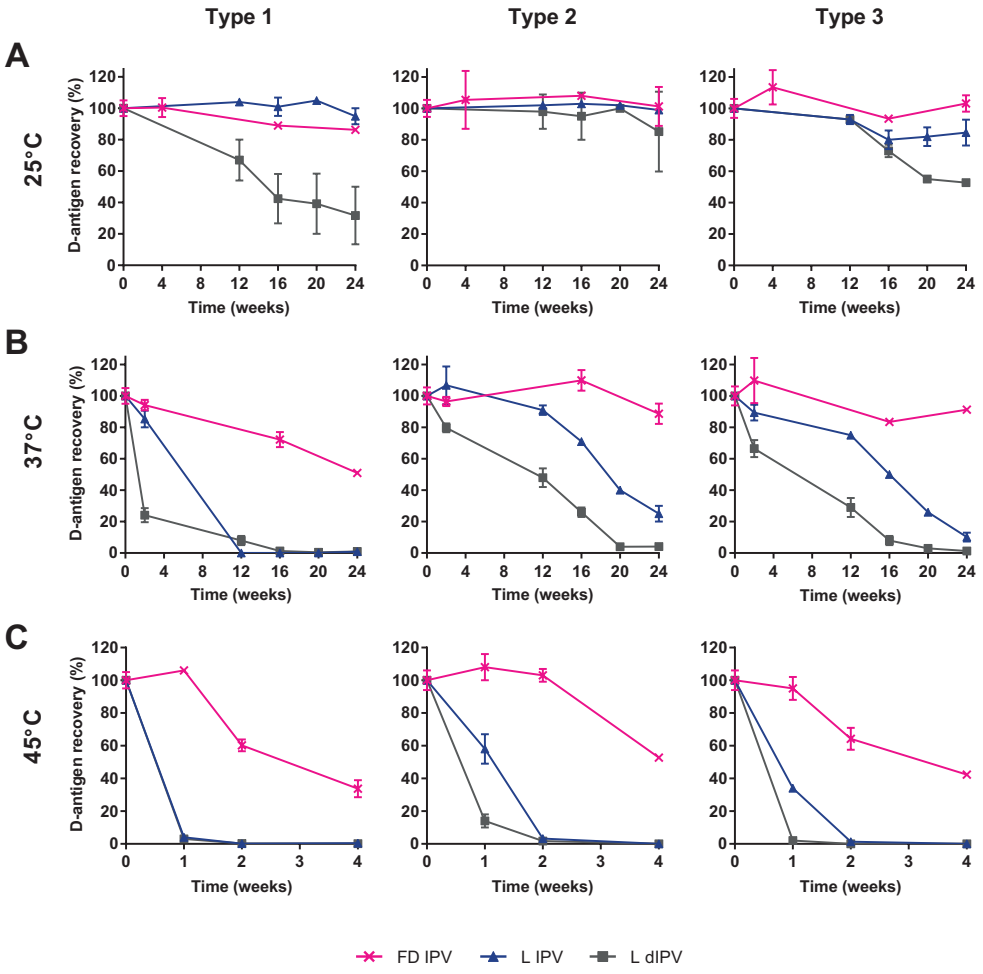

Figure 7 Stability testing of a lyophilized trivalent IPV formulation containing 10% (w/v) sorbitol, 8.5% (w/v) $MgCl_2$ and 8.5% (w/v) MSG at 25°C (A), 37°C (B) or up to 4 weeks at 45°C (C). The freezedried formulation (FD IPV, pink lines) was compared with the conventional liquid IPV (L IPV, blue lines) and a liquid IPV dialyzed against McIlvaine buffer (L dIPV, grey lines), which is the buffer of choice during lyophilization.

stable during long-term incubation at ambient temperature (Figure 7A). However, at elevated temperatures, the lyophilized IPV formulation revealed its improved stability profile. Despite its relatively high residual moisture content, which was determined at 4.5 ± 0.9%, minimal loss was observed for the dried IPV after storage at temperatures above room temperature where the liquid IPV has lost its antigenicity completely (Figure 7B and 7C).

The current study shows the feasibility to convert IPV into the dry state using lyophilization. The focus here was on development and optimization of a dried IPV formulation. However,

the lyophilization process needs optimization as well, since the RMC of our lyophilized IPV formulation exceeds the limit of 3% water content from the European Pharmacopoeia. This high residual water content is probably due to the presence of $MgCl_2$, a hexahydrate with strongly bound water, in the IPV formulation. The duration of the secondary drying step dictates the residual moisture level in a lyophilized product [20], so probably prolongation of this drying step and raising the end temperature of the lyophilization process could decrease the RMC and thus could possibly improve the final product.

CONCLUSION

The aim of the study was to develop a dried IPV formulation with minimal loss during the drying process and improved stability when compared with the conventional liquid IPV, which could allow distribution and storage under unrefrigerated conditions. Extensive screening of a large number of excipients combined with a DoE approach yielded a lyophilized IPV formulation with remaining antigenicity for all serotypes when kept at ambient or even higher temperatures.

Although further improvement and research is still possible, this study showed the potential of a highly stable and safe lyophilized polio vaccine, which could be distributed in developing countries without the need of a cold-chain transport.

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SUPPLEMENTAL DATA

Table S1 Commonly used excipients tested using a design of experiments approach. D-antigen recoveries were determined per serotype by ELISA directly after lyophilization.

	Sucrose	Trehalose	Mannitol	Dextran	NaCl (mM)	D-antigen recovery (%)		
						T1	T2	T3
P1	-	-	-	-	-	8.5	11.3	1.8
P2	20%	20%	-	-	-	30.3	58.0	22.5
P3	20%	-	10%	-	-	45.3	68.3	46.4
P4	-	20%	10%	-	-	44.4	68.5	40.5
P5 *	11.4%	11.4%	5.7%	5.7%	-	30.5	51.1	29.9
P6	-	-	6.7%	10%	-	25.9	54.1	8.9
P7	-	6.7%	10%	10%	-	31.6	62.2	16.3
P8 *	13.3%	13.3%	2.2%	6.7%	-	33.9	57.8	25.9
P9	6.7%	-	10%	10%	-	34.9	58.1	27.5
P10	10%	10%	-	5%	-	22.2	45.0	8.2
P11	10%	10%	5%	-	-	35.5	62.7	34.9
P12	20%	-	-	-	63	32.8	59.3	33.3
P13	-	20%	-	-	63	21.7	47.6	7.1
P14	-	-	10%	-	63	39.8	57.9	44.2
P15	-	-	-	10%	63	8.2	15.1	4.0
P16	10%	-	5%	5%	31.5	31.9	56.7	31.5
P17	10%	10%	5%	5%	63	29.2	49.6	25.2
P18	16%	16%	8%	-	50	42.3	64.6	42.0
P19 *	13.3%	13.3%	-	6.7%	41.7	22.7	43.7	9.9
P20 *	11.4%	-	5.7%	5.7%	35.7	33.1	56.3	32.9
P21	-	16%	8%	8%	50	25.9	49.1	16.2
P22 *	13.3%	6.7%	3.3%	3.3%	41.7	29.2	52.2	27.2
P23	10%	10%	5%	5%	31.5	29.2	49.6	25.2
P24	10%	10%	5%	5%	31.5	30.3	52.8	27.2
P25	10%	10%	5%	5%	31.5	34.0	58.1	28.1

** Concentrations differ from design due to maximal solubility of the formulation.*

4

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Incompatibility of lyophilized inactivated polio vaccine with liquid pentavalent whole-cell-pertussis-containing vaccine

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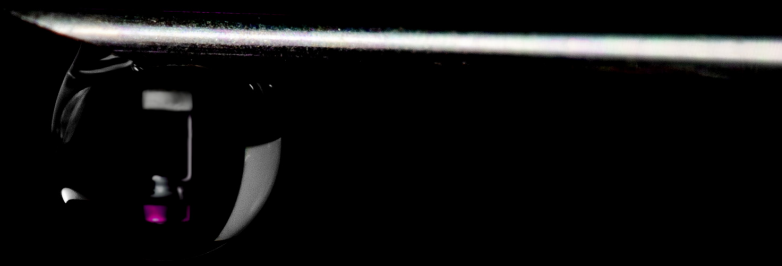


ABSTRACT

A hexavalent vaccine containing diphtheria toxoid, tetanus toxoid, whole cell pertussis, Haemophilius influenza type B, hepatitis B and inactivated polio vaccine (IPV) may: i) increase the efficiency of vaccination campaigns, ii) reduce the number of injections thereby reducing needle-stick injuries, and iii) ensure better protection against pertussis as compared to vaccines containing acellular pertussis antigens. An approach to obtain a hexavalent vaccine might be reconstituting lyophilized polio vaccine (IPV-LYO) with liquid pentavalent vaccine just before intramuscular delivery. The potential limitations of this approach were investigated including thermostability of IPV as measured by D-antigen ELISA and rat potency, the compatibility of fluid and lyophilized IPV in combination with thimerosal and thimerosal containing hexavalent vaccine.

The rat potency of polio type 3 in IPV-LYO was 2 to 3-fold lower than standardized on the D-antigen content, suggesting an alteration of the polio type 3 D-antigen particle by lyophilization. Type 1 and 2 had unaffected antigenicity/immunogenicity ratios. Alteration of type 3 D-antigen could be detected by showing reduced thermostability at 45°C compared to type 3 in non-lyophilized liquid controls.

Reconstituting IPV-LYO in the presence of thimerosal (TM) resulted in a fast temperature dependent loss of polio type 1-3 D-antigen. The presence of 0.005% TM reduced the D-antigen content by ~20% (polio type 2/3) and ~60% (polio type 1) in 6 hours at 25°C, which are WHO open vial policy conditions. At 37°C, D-antigen was diminished even faster, suggesting that very fast, i.e., immediately after preparation, intramuscular delivery of the conceived hexavalent vaccine would not be a feasible option. Use of the TM-scavenger, L-cysteine, to bind TM (or mercury containing TM degradation products), resulted in a hexavalent vaccine mixture in which polio D-antigen was more stable.

INTRODUCTION

Combination vaccines are very successful, especially for delivery in children. The inclusion of multiple vaccine antigens in a single formulation reduces the number of injections, facilitates inclusion of new vaccines and increases coverage of routine pediatric immunization programs. For example, the use of pentavalent vaccine combining diphtheria-tetanus-pertussis (DTP), Haemophilus influenzae type B (Hib) and hepatitis B (HBV) antigens has raised the coverage of Hib and hepatitis B in the poorest developing (Gavi-supported) countries [1].

One of the challenges for an IPV-containing hexavalent vaccine is the presence of the preservative thimerosal (TM). TM negatively affects the antigenicity and immunogenicity of IPV [2] and is used in the production process of whole cell pertussis (wP) vaccine as an inactivating agent as well as a preservative [3]. Hence, pentavalent vaccine contains trace amounts of TM (<0.01 % (w/v)).

Currently, the globally marketed IPV-containing hexavalent pediatric combination vaccines (Infanrix Hexa® (GSK) and Hexaxim® (Sanofi Pasteur)) contain an acellular pertussis (aP) component, which is devoid of TM. The use of wP in hexavalent vaccines intended for developing countries is important because of the lower costs and emerging doubts about the long-term effectiveness of aP vaccines. Unfortunately, no hexavalent combinations with wP (without TM) are licensed or in late-stage development [1].

The aim of this study is to investigate whether IPV-LYO, as previously developed [4], could be used in combination with a wP-containing pentavalent vaccine to generate a concept hexavalent vaccine, for example, for use in developing countries. By reconstituting IPV-LYO with pentavalent vaccine no substantial change in total volume is anticipated, likely the same injection volume for IM-injection may be used. This study addresses the (thermo)stability of IPV-LYO with respect to both D-antigenicity and immunogenicity (rat potency) and shows D-antigenicity data on IPV-LYO reconstituted with a pentavalent vaccine (DTwP-Hib-HBV).

MATERIALS AND METHODS

Materials

The IPV used in this study is a ten times concentrated trivalent bulk containing the inactivated Mahoney (type 1), MEF (type 2) and Saukett (type 3) strains at a nominal D-antigen content (expressed in D-units, DU) of 400-80-320 DU/mL (for types 1, 2 and 3, respectively) and produced under cGMP conditions according to a routine production process [5]. The pentavalent vaccine, Diphtheria, Tetanus, (whole cell) Pertussis, Hepatitis B and *Heamophilus influenza* type b Conjugate Vaccine Adsorbed, was a gift from Serum Institute of India (SII).

D-Sorbitol, magnesium chloride hexahydrate ($MgCl_2.6H_20$) and monosodium glutamate monohydrate were from Sigma-Aldrich (St. Louis, MO). Citric acid (Sigma-Aldrich, St. Louis, MO) and disodium hydrogen phosphate (Fluka, Buchs, Switzerland) were used to prepare McIlvaine buffer. Thimerosal (TM) and L-cysteine were from Sigma-Aldrich (St. Louis, MO). All excipients used were of reagent quality or of a higher grade.

Methods

Formulating IPV

Unless indicated otherwise, the trivalent IPV bulk material was dialyzed against 10 mM McIlvaine buffer (pH 7.0) using a low-binding regenerated cellulose membrane dialysis cassette (Mw cut-off = 10 kDa). The dialyzed IPV was diluted 1:1 with formulation buffer containing: D-sorbitol (20% w/v), $MgCl_2.6H_20$ (17% w/v), and monosodium glutamate monohydrate (17% w/v) in McIlvaine buffer (10 mM, pH 7.0). This formulated IPV was used for the preparation of IPV-LYO. Liquid IPV was prepared by 1:1 dilution of (not dialyzed) trivalent IPV with ultrapure water. This material was used as a control in experiments.

Lyophilization

Injection vials (3 mL, Aluglas BV, Uithoorn, The Netherlands) were filled with 0.2 mL of the formulated IPV and half-stoppered with pre-dried (overnight at 105°C) 13 mm lyophilization stoppers (PH21/50 from Aluglas BV, Uithoorn, The Netherlands). Vials were loaded on precooled shelves (-50°C) and the solidified material was subsequently lyophilized. Primary drying was done at 0.045 mbar and -45°C for 26 hours. Secondary drying was done at a pressure of 0.01 mbar and shelf temperature that increased from -45°C to 25°C in 13.3 hours. Thereafter, both shelf temperature (25°C) and pressure (0.01 mbar) were kept constant for 24

hours. After lyophilization, vials were closed under vacuum, sealed with alu-caps and stored for stability testing.

Stability testing

For stability studies, liquid IPV (0.2 mL in stoppered and capped 3 mL injection vials) and IPV-LYO (in stoppered and capped 3 mL vials) were incubated at 2-8, 25, 37, and 45 °C. After various periods of time, vials were taken for analysis.

Effect of thimerosal

The effect of thimerosal (TM) on liquid IPV was studied by diluting trivalent IPV 10-fold with a solution of TM in ultrapure water. IPV-LYO was reconstituted either with ultrapure water (0.5 mL), 0.5 mL TM solution, or 0.5 mL pentavalent SII-vaccine containing 0.005% (w/v) TM. Final TM concentrations were 0.005 and 0.01% (w/v).

The possible neutralizing effect of L-cysteine on TM was investigated by pre-incubating pentavalent vaccine for one hour with 0.05% (w/v) L-cysteine or ultrapure water as negative control. Subsequently, IPV-LYO was reconstituted with the pre-incubated pentavalent vaccines or with ultrapure water as a control. D-antigen recoveries were determined directly after mixing or after subsequent storage at 37°C for 24h.

Analysis

D-antigen ELISA

The D-antigen ELISA was performed as described elsewhere [6]. Microtiter plates were coated with serotype-specific bovine anti-polio serum (Bilthoven Biologicals, Bilthoven, The Netherlands). After washing, dilutions of IPV (reconstituted IPV-LYO or liquid) were added. After an incubation period of 30 minutes at 37°C under gentle shaking, plates were washed. Subsequently, a mixture of serotype-specific anti-poliovirus monoclonal antibody (3-4E4, 3-14-4 and 1-12-9 for type 1, 2 and 3, respectively) and HRP-labeled anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) was added and plates were incubated for 30 min. at 37°C while shaking. Subsequently, plates were washed followed by addition of ELISA HighLight signal reagent (ZomerBloemen BV, Zeist, The Netherlands). Chemiluminescence was measured for 10-15 min by using a luminometer (Berthold Centro LB960). The signal at maximum intensity was used to calculate the D-antigen content relative to the reference standard.

Biosensor analysis

Antigenicity was also measured using a Biacore T200 (GE Healthcare, Hoevelaken, The Netherlands), equipped with an anti-polio biosensor as described elsewhere [7]. Goat anti-mouse IgG Fc-specific (Thermo Fisher Scientific Inc, Waltham, MA), antibodies were covalently immobilized on the dextran layer of a CM3 sensorchip (GE Healthcare, Hoevelaken, The Netherlands) by primary amine coupling, following the manufacturers recommendations (GE Healthcare, Hoevelaken, The Netherlands). Serotype-specific monoclonal antibodies (3-4E4 (antigenic site 1, type 1), 3-14-4 (antigenic site 1, type 2), HYB300-06 (antigenic site 1, type 3) and 1-12-9 (antigenic site 2/3/4, type 3) were bound to the sensor, followed by IPV. The sensor chip was regenerated with 10 mM glycine-HCl (pH 1.5). Assay data were analyzed by four-parameter curve fitting using the Biacore T200 evaluation software. Antigenicity was calculated relative to the international reference PU91-01.

Rat potency

Immunogenicity of IPV-LYO was measured in the rat potency test performed as described earlier [7] with the exception that the highest dilution of the vaccine was not included. Animal experiments were conducted in accordance with the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee for Animal Experimentation of Intravacc. RIVM-TOX rats were immunized with four threefold dilutions of reconstituted IPV-LYO, the liquid IPV control, and the reference vaccine (PU91-01). After three weeks, sera were collected and neutralizing antibodies against all three poliovirus types were measured separately by inoculating Vero cells with 100 TCID50 of the wild-type strains (Mahoney, MEF-1 and Saukett) as described previously [8]. Two-fold serial serum dilutions were made and serum/virus mixtures were incubated for three hours at 36°C and 5% CO2 followed by overnight incubation at 5°C. Subsequently, Vero cells were added and after 7 days of incubation at 36°C and 5% CO2 the plates were stained and fixed with crystal violet and the results were read macroscopically. Virus-neutralizing (VN) titers were expressed as the last serum dilution that has an intact monolayer (no signs of cytopathogenic effect). Immunogenicity was expressed in two ways: A) as the relative potency to the reference vaccine using the parallel-line model, and B) as the average virus-neutralizing antibody endpoint titer at the second (1/15 dilution) or third (1/45 dilution) highest vaccine dose.

Moisture content analysis

Residual moisture content was determined using a Karl Fischer Coulometer C30 (Mettler-Toledo, Tiel, The Netherlands) according to the literature [4]. Samples were weighed,

reconstituted in Hydranal Coulomat A (Fluka, Buchs, Switzerland), and injected into the titration vessel. The empty vials were weighed and water content was calculated based on the measured water content, the weight of the lyophilized product in the vial, the reconstitution volume of the reagent, the titration volume, and the water content of the blank titration.

Statistical analysis

For comparative analysis of immunogenicity, data were tested by one-way analysis of variance (ANOVA) followed by a Bonferonni test for multiple comparisons. Probability (p) values <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Immunogenicity of IPV-LYO

Two batches of IPV-LYO were prepared, characterized and used for further experiments. The D-antigen composition, D-antigen recovery, and RMC (Table 1), and thermostability (data not shown) of both batches were comparable. Immediately after lyophilization, the rat potency of IPV-LYO was 0.88, 1.17 and 0.39, respectively for polio type 1, 2 and 3. The rat potency of type 3 was lower than anticipated as based on: A) the D-antigen concentration, and B) historical data of polio containing vaccines (data not shown). Virus-neutralizing (VN) titers of polio type 3 were significantly lower in rats immunized with IPV-LYO if compared to liquid IPV (Figure 1A). This unanticipated result was confirmed with an independent IPV-LYO preparation and throughout subsequent experiments (see paragraph Stability of IPV-LYO).

Table 1 Characterization of two IPV-LYO batches that were used for further stability testing. D-antigen composition (DU/mL), D-antigen recovery and residual moisture content (RMC) of both batches were determined. Mean values and SD are shown (n=3). Rat potency of batch 2 was determined as well. Relative rat potency values and lower and upper limits (95% confidence intervals (CI)) are shown.

IPV-LYO	RMC (%)	Type 1	Type 2	Type 3
Batch 1	8.9 ± 0.4			
Composition (DU/mL)	-	180 ± 16	37 ± 5	138 ± 5
D-antigen recovery (%)	-	83 ± 7	78 ± 10	75 ± 3
Batch 2	8.0 ± 0.3			
Composition (DU/mL)	-	155 ± 2	34 ± 2	126 ± 4
D-antigen recovery (%)	-	80 ± 1	86 ± 4	77 ± 3
Rat potency [95% CI]	-	0.88 [0.58 – 1.32]	1.17 [0.76 – 1.81]	0.39 [0.30 – 0.51]

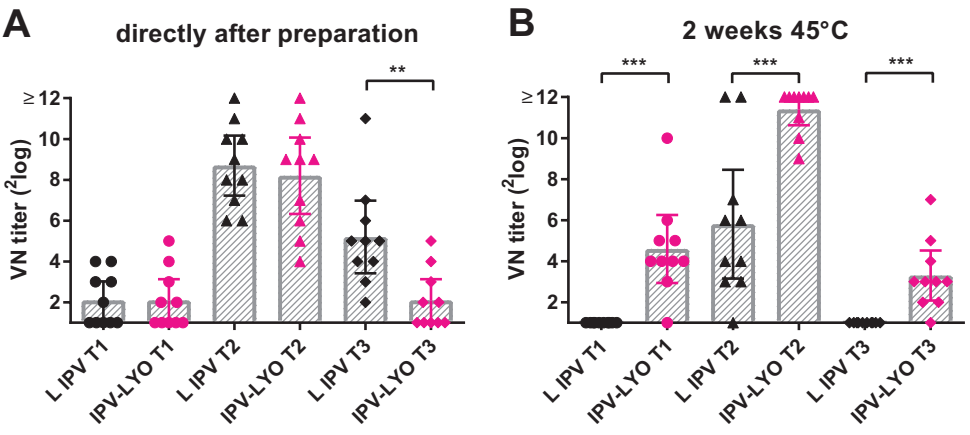


Figure 1 Mean virus-neutralizing (VN) titers of serum from rats (n=10) immunized with 1/45 human dose (panel A) or 1/15 human dose (panel B) of liquid IPV (L IPV, in black) or IPV-LYO (in red) directly after preparation or after subsequent two weeks storage at 45°C. Individual VN titers specific for serotype 1 (circles), 2 (triangles) and 3 (diamonds) were shown. Mean values were depicted as horizontal line and error bars showed 95% confidence interval (CI) values. Asterisks indicate significant differences between groups (*p<0.01, **p<0.001, ***p<0.0001).

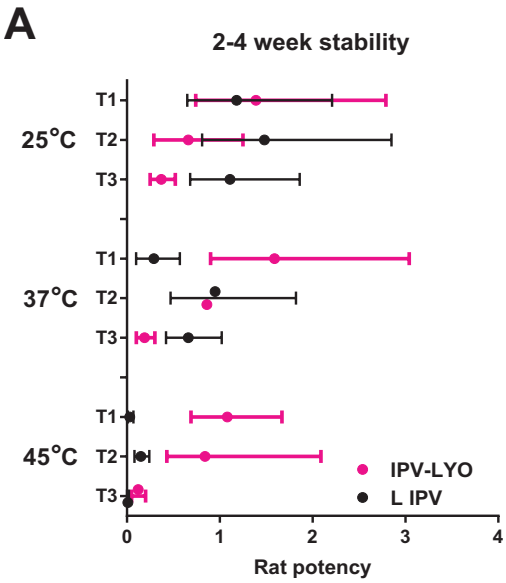
In an earlier study it was demonstrated that disruption of antigenic site 1 diminished the rat potency of polio type 3 [9]. However, Biosensor analysis showed that antigenic site 1 remained intact after freeze-drying, as observed D-antigen concentrations were 130 ± 5 DU/mL (IPV-LYO) and 137 ± 1 DU/mL (liquid IPV).

Stability of IPV-LYO

Accelerated stability testing for two weeks at 45°C, showed significantly higher VN-titers for all three serotypes in IPV-LYO when compared to liquid IPV (Figure 1B), the low average VN-titer of type 3 in IPV-LYO was retained, whereas it was completely nullified in liquid IPV.

In general, at temperatures of 37°C and 45°C, the recovery of type 1-3 D-antigen was lower in liquid IPV than in IPV-LYO (Figure 2). After four weeks storage at 25°C, the rat potency of liquid IPV was approximately 1 for all serotypes, whereas significantly lower potency values were observed after short-term storage at 37°C or 45°C (Figure 2A). In contrast, IPV-LYO maintained its potency after short-term storage at 25-45°C. Even after storage for up to 6 months at 2-8°C or 25°C, no further decrease in potency was observed in case of IPV-LYO (Figure 2B). During 6 months of storage at 37°C, liquid IPV showed no measurable D-antigen recovery and the rat potency was almost completely lost, whereas IPV-LYO still showed D-antigen recoveries of 39±4%, 122±11% and 73±4%, respectively for type 1, 2 and 3, and significantly higher rat potency values than liquid IPV (Figure 2B). The

rat potency of polio type 3 in IPV-LYO was consistently 2 to 3-fold lower than anticipated as based on the recovered D-antigen percentage (Figure 2A and B).



	D-antigen recovery	
	L IPV	IPV-LYO
T1:	124 ± 7%	91 ± 2%
T2:	153 ± 14%	122 ± 10%
T3:	146 ± 7%	105 ± 4%
T1:	20 ± 4%	74 ± 4%
T2:	108 ± 8%	106 ± 13%
T3:	90 ± 7%	94 ± 4%
T1:	2 ± 2%	59 ± 5%
T2:	15 ± 0%	124 ± 17%
T3:	2 ± 2%	80 ± 12%

	D-antigen recovery	
	L IPV	IPV-LYO
T1:	89 ± 10%	94 ± 15%
T2:	91 ± 3%	113 ± 18%
T3:	104 ± 5%	93 ± 4%
T1:	96 ± 13%	80 ± 16%
T2:	182 ± 19%	128 ± 15%
T3:	128 ± 9%	81 ± 8%
T1:	0 ± 0%	39 ± 4%
T2:	0 ± 0%	122 ± 11%
T3:	0 ± 0%	73 ± 4%

Figure 2 Rat potency of liquid IPV (L IPV) and IPV-LYO after incubation for a short period of time; 2 weeks at 45°C or one month at 25°C or 37°C (panel A), or long period of time (6 months at 4°C, 25°C or 37°C) (panel B). The rat potency is calculated based on a theoretical composition of 40, 8, 32 D-antigen for type 1, 2, and 3, respectively.

Thermostability of polio type 3 in reconstituted IPV-LYO

To examine whether IPV-LYO (type 3) after reconstitution was more vulnerable to higher temperatures than liquid IPV, a thermostability experiment was performed using reconstituted IPV-LYO, and both formulated liquid IPV and liquid IPV as controls.

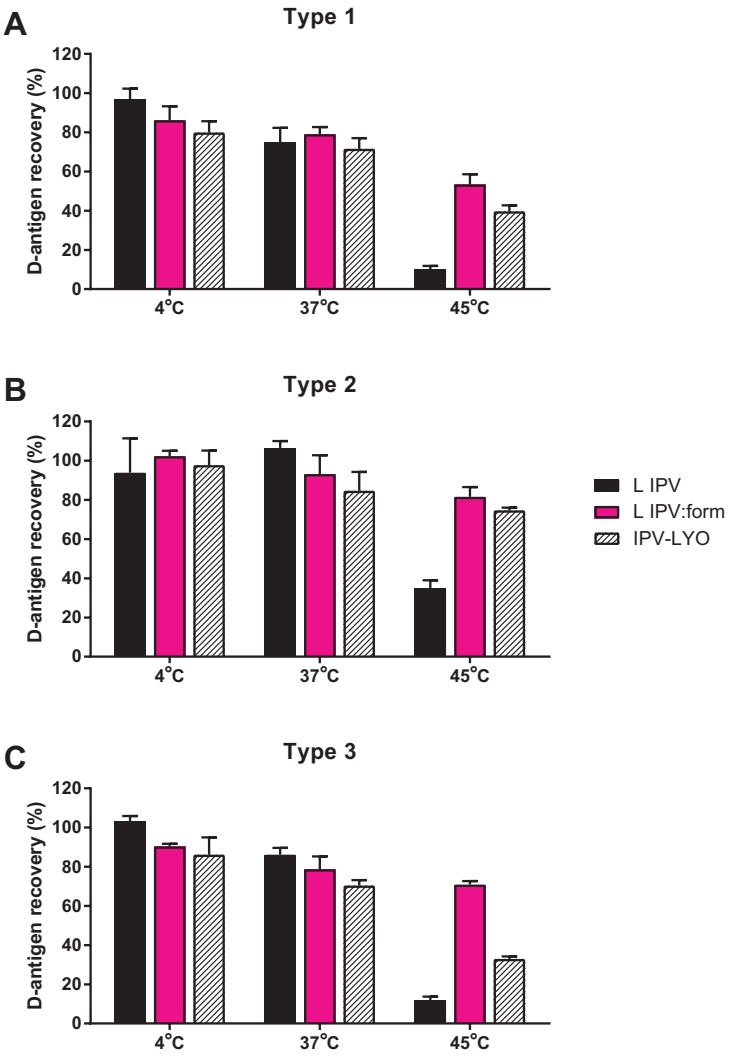


Figure 3 Stability of reconstituted IPV-LYO. Liquid IPV (L IPV, black bars), formulated (liquid) IPV prior to lyophilization (L IPV:form, striped bars), and reconstituted IPV-LYO (red bars) were incubated for 24 hours at 4°C, 37°C or 45°C. Subsequently, D-antigen recoveries were determined by ELISA, specific for type 1 (panel A), type 2 (panel B) and type 3 (panel C), and normalized for D-antigen recoveries directly after lyophilization. Mean values (n=3) and SD are shown.

An incubation period of 24 hours at either 4°C or 37°C did not result in a difference between the samples (Figure 3A-C). However, at 45°C the D-antigen recovery of liquid IPV was clearly lower than the formulated liquid IPV, and reconstituted IPV-LYO. From figure 3C it is clear that freeze-drying and resuspension rendered a type 3 particle characterized by less thermoresistance (recovery 33%) than its formulated counterpart (recovery 71%). Such a difference was not observed in case of polio serotype 1 and 2 (Figure 3A and B). From these findings it is hypothesized that the type 3 particle was altered by the lyophilization process rendering type 3 with a lower thermostability and a lower specific immunogenicity.

Effect of thimerosal containing pentavalent vaccine on IPV-LYO

Reconstitution of IPV-LYO with pentavalent vaccine (0.005% thimerosal, TM) resulted in an evident negative trend in D-antigen recovery (Figure 4). The control, IPV-LYO reconstituted with ultrapure water, showed either no (in case of type 1 and 2) or a minimal loss (in case of type 3) in D-antigen recovery during incubation for 24 hours at 37°C.

The negative effect of TM on polio D-antigen was increased at a higher temperature in the range from 2-8°C to 37°C. After 6 hours at 25°C (relevant conditions for the WHO open vial policy), there was already a marked (and unacceptable) loss of ~20% in case of type 2 and 3 and ~60% in case of type 1.

A similar drop in D-antigenicity was observed when incubating IPV-LYO with 0.005% TM solution (data not shown) indicating that the negative effect on D-antigen recovery of IPV-LYO was most likely caused by the presence of TM.

Effect of pentavalent vaccine on IPV-LYO after pre-incubation with L-cysteine

Low molecular weight thiols are well known for the ability to scavenge mercury and/or mercury containing compounds such as TM [10, 11]. Therefore, pentavalent vaccine was pre-incubated with the TM-scavenger L-cysteine and was used thereafter to reconstitute IPV-LYO. Figure 5A shows a small initial loss of the D-antigen recovery upon reconstituting IPV-LYO with pentavalent vaccine. This initial loss is absent in samples that are reconstituted with purified water or in samples in which TM was scavenged.

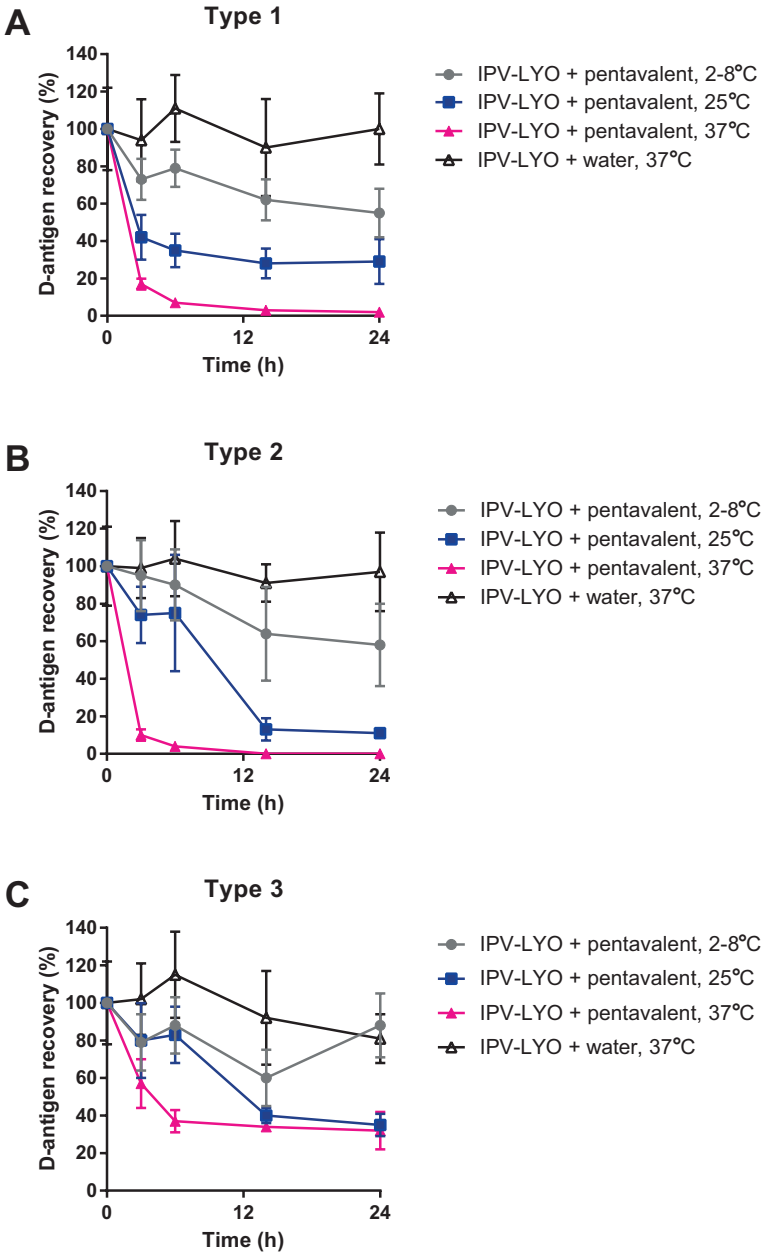


Figure 4 Effect of pentavalent vaccine on IPV-LYO. IPV-LYO was reconstituted with pentavalent vaccine (penta) containing 0.005% of thimerosal at temperatures of 2-8°C (closed circles, grey), 25°C (closed squares, blue) or 37°C (closed triangles, red) for up to 24 hours. Subsequently, D-antigen recoveries were determined by ELISA, specific for type 1 (panel A), type 2 (panel B) and type 3 (panel C). Mean values (n=3) and SD are shown.

After 24 hours at 37°C there is almost no recoverable D-antigen in case pentavalent vaccine was used to reconstitute IPV-LYO (Figure 5B). However, when IPV-LYO was reconstituted with L-cysteine containing pentavalent vaccine, D-antigen could be clearly be recovered, values were 61±7%, 107±2% and 96±4% for type 1, 2 and 3, respectively.

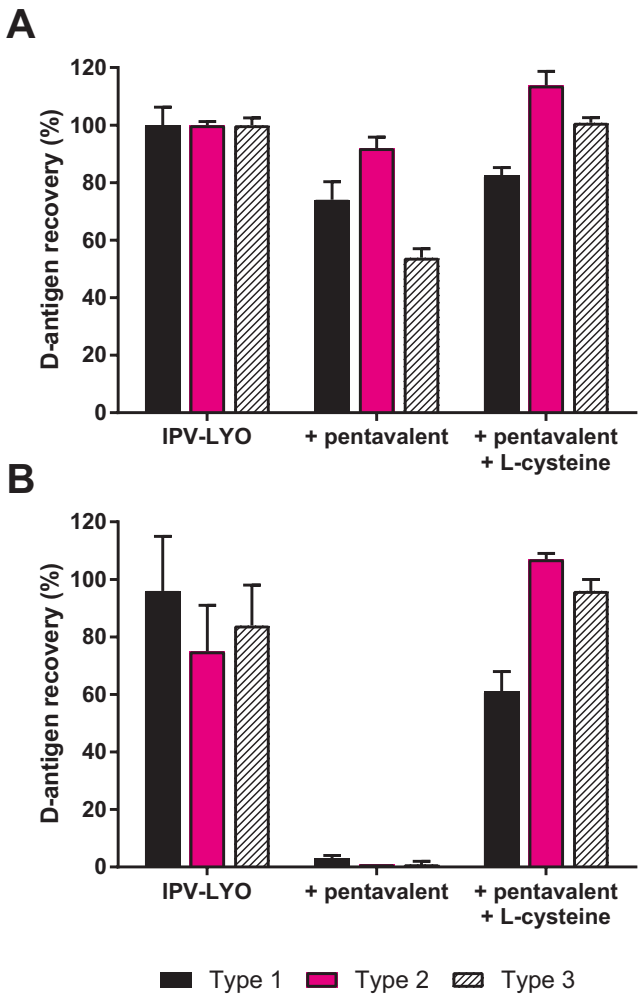


Figure 5 Effect of cysteine on the stability of IPV-LYO mixed with pentavalent vaccine. IPV-LYO was mixed with pentavalent vaccine (penta) pre-incubated for one hour in the absence or presence of L-cysteine (cys). Subsequently, D-antigen recoveries directly after mixing (panel A) and after 24h incubation at 37°C (panel B) were determined by ELISA, specific for type 1 (black bars), type 2 (striped bars) and type 3 (red bars), and normalized for D-antigen recoveries of IPV-LYO prior to mixing and incubation. Mean values (n=3) and SD are shown.

DISCUSSION

Detrimental effect of lyophilization on the rat potency of polio type 3

For release of inactivated polio vaccines two assays are available: an ELISA for determining the D-antigen concentration (antigenicity) and the rat potency test for determining the relative rat potency (immunogenicity). The European Pharmacopoeia (EP) allows to omit the rat potency test and to rely exclusively on the more accurate D-antigen ELISA. Hence, there should be a relationship between the *in vitro* and *in vivo* test, despite the considerable test variation in the rat potency test. Remarkably, in our study the *in vitro* test content was not predictable for the immunogenicity of IPV-LYO type 3 (contrary to the normal *in vitro – in vivo* relationship observed with type 1 and 2). The type 3 rat potency in IPV-LYO was 2 to 3-fold lower than anticipated as based on the measured D-antigen concentration. Earlier studies reported significant lower virus-neutralizing titers against type 3 in rats immunized with lyophilized IPV formulated in dissolvable mini-implants (Bioneedles) [12] or weaker serological responses to IPV type 3 after microneedle patch vaccination of rhesus macaques compared to intramuscular injection [13].

The use of certain site-specific monoclonal antibodies in the ELISA is critical for the antigenicity-immunogenicity relation [14]. In the case of detection of polio serotype 3, it has been recommended to include measurement of antigenic site 1, because disruption of this site strongly diminishes the rat potency [14]. No evidence for antigenic site 1 disruption was observed in this study. However, by performing accelerated stability testing it was revealed that freeze-drying and reconstitution reduced the thermostability of polio type 3 and did not affect the thermostability of type 1 and 2. This observation suggested the existence of an altered type 3 particle formed by freeze-drying, which compared to the formulated control could be characterized by a reduced thermostability and a lower immunogenicity in rats. This altered type 3 particle was not characterized further, but might be an intermediate in the transition from D-antigen to C-antigen stabilized by excipients. Future formulation development and antigen characterization is required to address this. For example, in the development of dried IPV formulations, a proper D-antigen characterization after reconstitution and subsequent stress treatment (e.g., thermal stressing) might give a more reliable prediction of the potency of the dried IPV.

Interestingly, improved thermostability was observed in formulated liquid IPV compared to conventional liquid IPV without extra excipients. This indicates that it is possible to stabilize the polio antigen in the liquid state by the addition of excipients.

Use of IPV-LYO in a hexavalent vaccine: overcoming the detrimental effect of thimerosal

The current study investigated the possible use of lyophilized IPV as a component for mixing with a pentavalent vaccine (DTwP-Hib-HBV) thereby forming a hexavalent mixture. These licensed pentavalent vaccines all contain traces of thimerosal (TM), an organomercury compound known for its antiseptic and antifungal properties. Unfortunately TM may also negatively affect the antigenicity and immunogenicity of IPV [2]. Within hours after reconstitution of IPV-LYO with pentavalent vaccine (containing 0.005% TM), a strong reduction in D-antigenicity was observed. The antigenicity decrease was even more evident when the vaccine was kept at 37 °C showing that also immediate IM-delivery, after mixing IPV-LYO and pentavalent vaccine, is probably not a feasible option to mitigate the negative impact of TM on polio D-antigen, which is consistent with reported findings [2].

Pre-incubation of the TM-containing pentavalent vaccine with L-cysteine reduced the negative effect of TM on D-antigenicity of IPV-LYO after reconstitution with the pentavalent vaccine. The use of L-cysteine for neutralization of TM in a pentavalent vaccine has not been published before. However, the use of L-cysteine as a TM-scavenger is well-known [10, 11, 15]. The use of compounds, which compete with polio D-antigen for binding to TM or stabilize IPV, might offer a potential step towards a TM-resistant IPV formulation, which can be used as a component in a hexavalent vaccine. On the other hand, removing TM completely from the vaccine may be considered as well by, for example, reformulating wP (prior to using it for preparing a pentavalent mixture).

CONCLUSIONS

This research demonstrated a clear difference in rat potency between IPV-LYO and liquid IPV serotype 3. The reduced thermostability of type 3 in IPV-LYO after reconstitution suggested the formation of an altered particle, presumably an D-antigen-intermediate between D-antigen and C-antigen, during the lyophilization process.

Use of IPV-LYO as a component in a hexavalent vaccine by mixing with licensed pentavalent vaccine (DTwP-Hib-HBV) requires neutralization of TM by for example L-cysteine. Improving the vaccine formulation by the inclusion of low molecular weight thiols, which have the ability to bind to TM, could offer a possible solution to neutralize the damaging effect of TM on the polio D-antigen.

Further formulation development is needed, in which screening based on D-antigen thermostability is included, to improve the potency of IPV-LYO type 3.

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Alternative delivery of a thermostable inactivated polio vaccine

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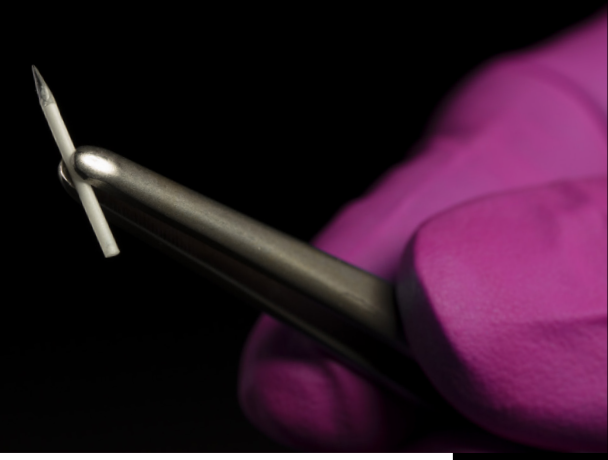

ABSTRACT

In the near future oral polio vaccine (OPV) will be replaced by inactivated polio vaccine (IPV) as part of the eradication program of polio. For that reason, there is a need for substantial amount of safe and more affordable IPV for low-income countries. Bioneedles, which are biodegradable mini-implants, have the potential to deliver vaccines outside the cold-chain and administer them without the use of needles and syringes. In the current study, Bioneedles were filled with IPV, subsequently lyophilized, and antigenic recoveries were determined both directly after IPV-Bioneedle preparation as well as after elevated stability testing. Further, we assessed the immunogenicity of IPV-filled Bioneedles in rats and the residence time at the site of administration.

Trivalent IPV was formulated in Bioneedles with recoveries of 101 ± 10%, 113 ± 18%, and 92 ± 15%, respectively for serotype 1, 2 and 3. IPV in Bioneedles is more resistant to elevated temperatures than liquid IPV: liquid IPV retained less than half of its antigenicity after one day at 45°C and IPV in Bioneedles showed remaining recoveries of 80 ± 10%, 85 ± 4% and 63 ± 4% for the three serotypes. *In vivo* imaging revealed that IPV administered via Bioneedles as well as subcutaneously injected liquid IPV showed a retention time of three days at the site of administration. Finally, an immunogenicity study showed that IPV-filled Bioneedles are able to induce virus neutralizing antibody titers similar to those obtained by liquid intramuscular injection when administered in a booster regime. The addition of LPS-derivate PagL in IPV-filled Bioneedles did not increase immunogenicity compared to IPV-Bioneedles without adjuvant.

The current study demonstrates the preclinical proof of concept of IPV-filled Bioneedles as a syringe-free alternative delivery system. Further preclinical and clinical studies will be required to assess the feasibility whether IPV-Bioneedles show sufficient safety and efficacy, and may contribute to the efforts to eradicate and prevent polio in the future.

INTRODUCTION

Poliomyelitis is caused by any one of three serotypes of poliovirus (type 1, type 2 or type 3) that can be prevented through vaccination. Since the launch of the Global Polio Eradication Initiative (GPEI) in 1988, the global incidence of polio has reduced by more than 99% and the number of countries with endemic polio has decreased from 125 to three. While the live attenuated oral polio vaccine (OPV) is currently the vaccine of choice in developing countries, it is associated with safety concerns, i.e., reversion of the vaccine virus to a form that causes paralysis and the risk of circulating vaccine-derived polioviruses (cVDPVs) [1]. Therefore, replacement of OPV by inactivated polio vaccine (IPV) is the new strategy striving towards global polio eradication. The cost increase is a main issue in replacing OPV with the more expensive IPV. In this context, adjuvants, like the lipopolysaccharide (LPS) derivate PagL, were shown to have dose-sparing capacity for IPV prepared from attenuated Sabin strains [2].

Alternative delivery technologies, via the skin [3, 4] or mucosal sites [5, 6] might further benefit the administration of IPV in the field if they are at least non-inferior in human. These delivery technologies can have advantages, such as easy and fast administration, minimizing/eliminating the risk on needle-stick injuries or reuse of needles and/or minimal generation of waste [7, 8]. Among the alternative delivery technologies that might be used for IPV is the Bioneedle technology, which allows parenteral vaccine administration without the use of needle and syringe. Bioneedles are small hollow mini-implants from biodegradable polymers that can be filled with antigen followed by a lyophilization process. After subcutaneous delivery, the implant dissolves and thereby releases the antigen. Preclinical data with different antigens showed the feasibility of Bioneedles as vaccine delivery system [9-12]. A first phase 1 clinical study with solid Bioneedles (without antigen) revealed good tolerability [13]. Besides, if formulated properly, vaccines in Bioneedles are thermostable, which can diminish the dependence on the cold-chain [10-12].

Recently, we have developed a formulation that stabilizes IPV during lyophilization and subsequent storage at higher temperatures [14]. The aim of current study is to develop a syringe-free administered polio vaccine by using the Bioneedle technology. Therefore, Bioneedles containing lyophilized IPV were developed, thermostability was assessed by D-antigen ELISA, and the immunogenicity in rats was evaluated by determining virus neutralizing (VN) antibody titers. To investigate whether the immune response elicited by IPV-filled Bioneedles could be increased by using an adjuvant, a formulation containing the

LPS-derivate PagL was included in this study. As the kinetics at the site of administration of a dry, encapsulated vaccine may be different compared to fluid injection, we performed a real-time *in vivo* imaging study.

MATERIALS AND METHODS

Materials

The IPV used in this study is a ten times concentrated trivalent bulk at a nominal D-antigen content (expressed in D units, DU) of 400-80-320 DU/mL (for type 1, 2 and 3 respectively, Salk strains) and routinely produced by the Netherlands Vaccine Institute as described previously [15].

D-sorbitol, monosodium glutamate and magnesium chloride hexahydrate were from Sigma (St. Louis, MO). Citric acid (Sigma-Aldrich, St. Louis, MO) and Na2HPO4 (Fluka, Buchs, Switzerland) were used to prepare McIlvaine buffer. All excipients used were of reagent quality or higher grade. IRDye800CW® protein labeling kit used for the *in vivo* imaging study was obtained from LI-COR Biosciences (Lincoln, NE). The adjuvant PagL LPS is obtained through expression of the Bordetella bronchiseptica PagL gene in Neisseria meningitides LPS as described by Arenas *et al*. [16].

Methods

IPV Bioneedle production

Trivalent IPV bulk was concentrated using 10 kDa Amicon Ultra centrifugal filters (Merck Millipore, Billerica, MA) and formulated with 8% (w/v) sorbitol, 5% (w/v) monosodium glutamate and 5% (w/v) magnesium chloride in McIlvaine buffer (10 mM, pH 7.0). For the imaging experiment, IPV was labeled with fluorescent probe IRDye800 CW® according to the accompanying instructions.

Empty Bioneedles (0.9 x 12 mm, made of extruded starch) were obtained from the Bioneedle Technologies Group and filled with 5 µL of the liquid IPV formulation (with or without adjuvant) using a specially designed filling apparatus and immediately frozen on a plate at -50°C. Subsequently, Bioneedles were loaded on a Zirbus freeze-drying unit sublimator 2-3-3 with pre-cooled shelves at -50°C. The lyophilization process was based on

a process used for IPV in vials [14]. In brief, primary drying was performed at -45°C (0.045 mbar) and secondary drying by further increasing the shelf temperature to 25°C followed by a 24 h drying step at 25°C (0.01 mbar). The lyophilized Bioneedles were individually stored in vials, closed under vacuum and sealed with alu-caps.

D-antigen content of the Bioneedles directly after lyophilization and during stability testing at 45°C for four weeks and at 60°C for one week, was determined by a sandwich ELISA as described previously [17]. DU recovery was expressed relative to the liquid formulation prior to lyophilization.

In vivo fluorescence imaging

Animal experiments were conducted in accordance with the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee for Animal Experimentation of Intravacc. Female CD Hairless rats (Crl:CD-Prsshr8, Charles River, Sulzfeld, Germany) received IRDye800CW®-labeled trivalent IPV (40-8-32 DU per dose) via subcutaneous liquid injection (0.1 mL per dose) or Bioneedle administration using a sterilized trocar with mandrin as described previously (5 animals/group, unless other stated) [11]. Scans of animals, positioned in dorsal recumbence, were performed eight times during 72 h after immunization under 2% isofluran/O2 anesthesia using an IVIS Spectrum imaging system (PerkinElmer, Waltham, MA). The threshold was set using a background scan made prior to immunization. The imaging sequences were 760, 780, 800, 820 and 840 nm for emission and 710 nm as excitation. Spectral unmixing was performed to decompose the emitted light into autofluorescence and label-specific fluorescence. The data represent the quantity of the fluorophore at each pixel. Data was analyzed using the Living Imaging software 4.1 from PerkinElmer and GraphPad Prism 6.4 software. The relative fluorescence intensity (percentage of initial fluorescence) at the site of administration was calculated for each animal.

Immunization study

Outbred Wistar rats (HsdCpb:WU, Harlan Laboratories, The Netherlands) received trivalent IPV (2.7-0.6-2.1 DU/dose, 10 animals/group) with or without PagL LPS (1 µg/dose). Liquid formulations were administered intramuscularly (i.m.) or subcutaneously (s.c.) by injection in the hind limb or neck between the ears, respectively, and Bioneedles were implanted as described above. All immunizations were performed under isofluran/O2 anesthesia. Vaccinations were given on days 0 (prime) and 28 (boost), and sera collected prior to immunization on day 0, on day 21 and on day 35. On day 49, animals were sacrificed

under anesthesia by bleeding (heart puncture).

Serum polio-specific IgG and virus neutralizing (VN) antibodies against all three poliovirus serotypes were determined as described earlier [2]. Baseline VN titers reported are based on four animals receiving mock vaccine as negative control.

Statistical analysis

For comparative analysis of immunogenicity, data were tested by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test for multiple comparisons. Probability (p) values <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

RESULTS

IPV-Bioneedle: process and product stability

Bioneedles were filled with a trivalent IPV formulation containing excipients that are known to stabilize the antigen during lyophilization and subsequent storage. Directly after lyophilization, the D-antigen content was determined by dissolving Bioneedles, followed by a D-antigen ELISA. DU recoveries were 101 ± 10%, 113 ± 18% and 92 ± 15%, respectively for type 1, 2 and 3 (n=3).

Product stability was evaluated by incubating Bioneedles for up to one month at 45°C or one week at 60°C. Subsequently, DU recovery was determined and compared to the liquid IPV stored at the same conditions (**Figure 1**). Liquid IPV stored for 24h at 45°C showed recoveries of 10 ± 2%, 47 ± 0% and 18 ± 5% for type 1, 2 and 3, respectively, whereas the IPV Bioneedle maintained its D-antigen content at respectively 80 ± 10%, 85 ± 4% and 63 ± 4% (**Figure 1A**). One-week storage of the liquid IPV formulation at 45°C resulted in complete loss of type 1 D-antigenicity and 80% loss of type 2 and 3. After four weeks also type 2 and 3 were not detectable anymore. In contrast, Bioneedles showed about 60% type 1 recovery and complete type 2 recovery and about 50% type 3 recovery after one week 45°C. After four weeks at 45°C DU recoveries were 27±10% for type 1, 64 ± 15% for type 2, and 18 ± 13% for type 3. IPV Bioneedles containing the PagL adjuvant were also subjected to stability studies and showed DU recoveries that were comparable to those of IPV Bioneedles without PagL (data not shown).

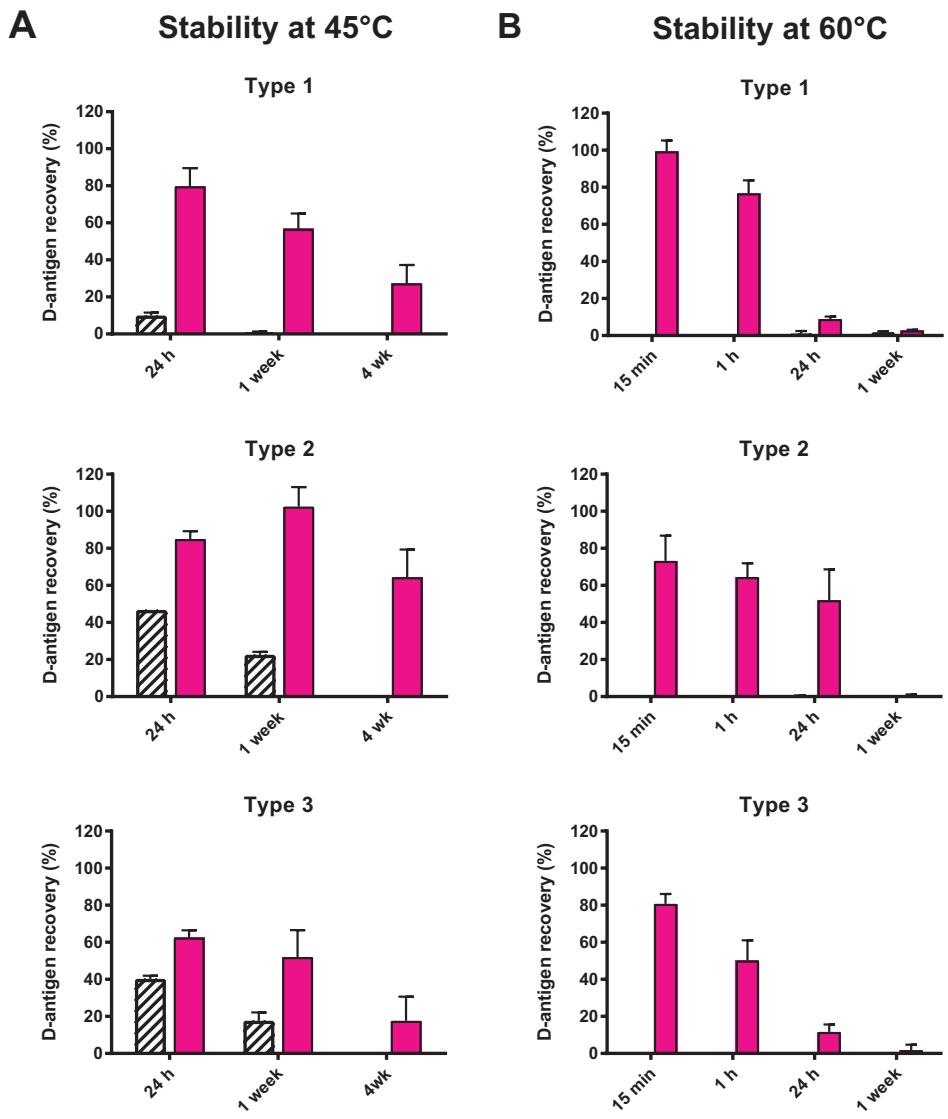

Figure 1 Thermostability of IPV Bioneedles. Liquid IPV formulations and IPV-filled Bioneedles were incubated at 45°C (A) and 60°C (B). Subsequently, for each serotype D-antigen content (D-antigen recovery) was determined by ELISA. Bars represent mean D-antigen recoveries ± SD (n=3) normalized for the D-antigen recovery directly after lyophilization.

At 60°C, the liquid formulation showed complete loss of D-antigenicity after 15 minutes, whereas no loss in DU recovery for type 1 was observed in IPV-filled Bioneedles and remaining recoveries of 73±6% for type 2 and 81 ± 5% for 3 were found (**Figure 1B**). Upon storage for a longer period at 60°C, D-antigen content decreased dramatically showing highest loss for

serotypes 1 and 3. After one week at 60°C, the Bioneedles did not contain any D-antigen anymore.

In order to test whether the formulations used in this study were able to resist real-life, unrefrigerated and thus varying conditions, liquid IPV and IPV-Bioneedles were taken on a three-week trip through Middle Eastern countries. Temperature was tracked during those weeks and DU content was determined afterwards (Figure 2B). The average temperature was determined at 26.0°C with a minimum of 17.0°C and a maximum of 46.5°C. During the trip, the IPV Bioneedles and liquid IPV were four times exposed to a temperature above 40°C (Figure 2A). For type 2 and 3, DU recoveries of 70-80% were found for both the liquid and Bioneedle formulations. However, whereas liquid IPV did not contain type 1 D-antigen, 100% DU recovery was observed in the IPV Bioneedle (Figure 2B).

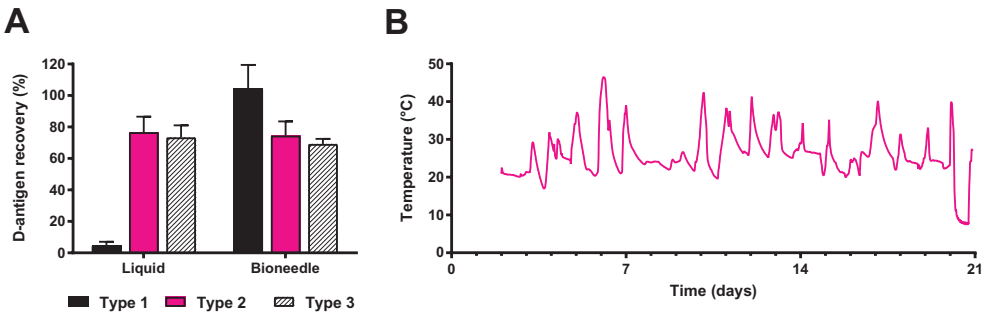

Figure 2 Stability of IPV-Bioneedles outside the cold-chain. Liquid IPV formulations and IPV-filled Bioneedles were taken on a travel through Mideast Asia countries. Temperature was logged for three weeks (A) and, subsequently, D-antigen content (D-antigen recovery) was determined by ELISA (B). Bars represent mean D-antigen recoveries ± SD (n=3) normalized for the D-antigen recovery directly after lyophilization.

Biodistribution study

To determine the kinetics of the antigen release from the Bioneedle and removal from the injection site, a real-time *in vivo* imaging study was performed over the course of time post inoculation. Within five hours after administration, the fluorescent signal from a Bioneedle filled with free IR-dye (not linked to IPV) decreased to background level (Figure 3B). Labeled IPV was removed from the injection site considerably slower than free dye irrespective of the administration method (injection or Bioneedle). Surprisingly, IPV in Bioneedles disappeared with the same kinetics as fluid injection. At 72 h post immunization, labeled IPV was not detectable at the site of administration.

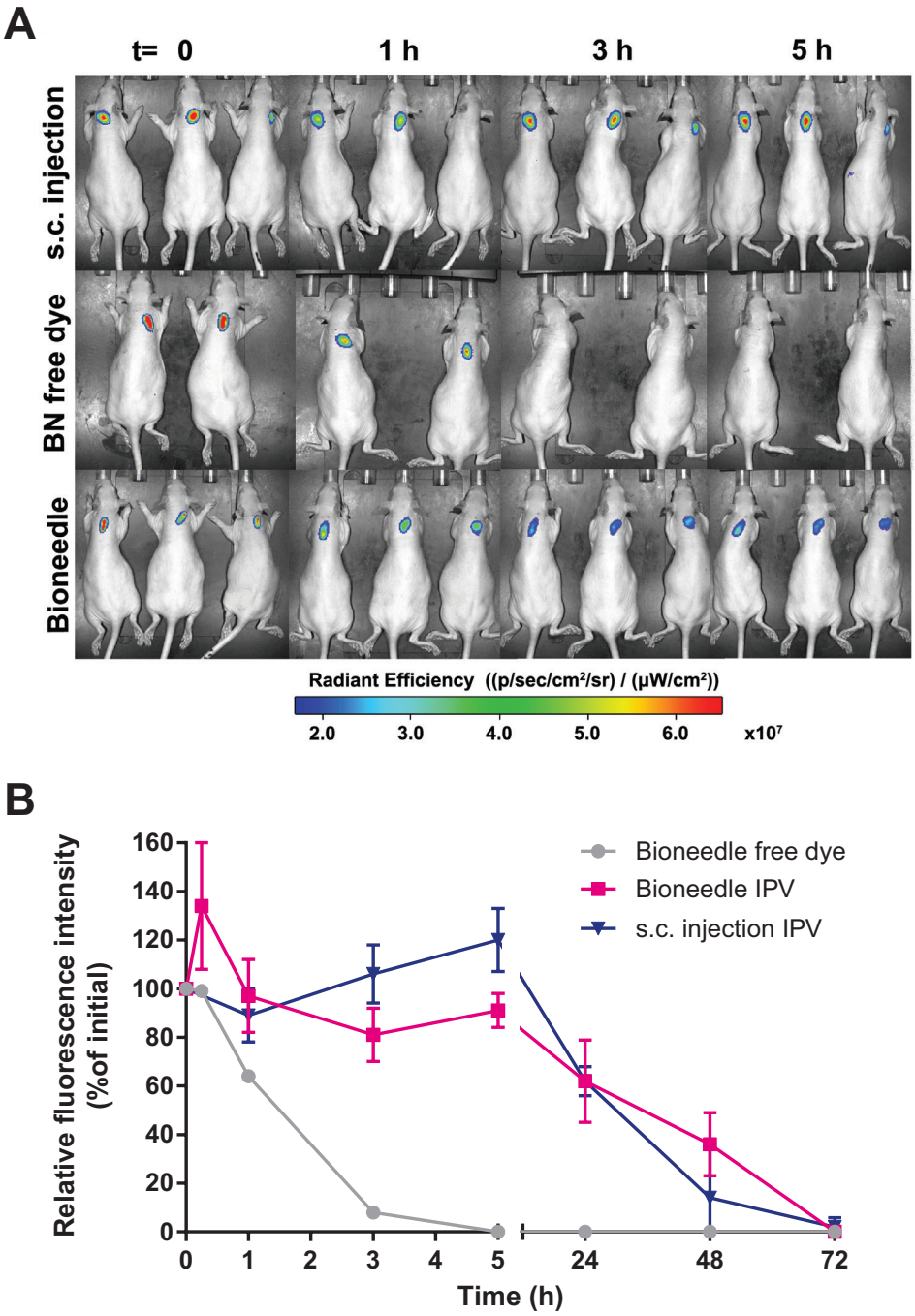

Figure 3 Kinetics of the antigen at the site of administration following IPV immunization either via liquid injection or Bioneedle insertion. Whole-body fluorescence images of nude rats in dorsal recumbence at different time points post immunization by s.c. liquid injection with labeled IPV or Bioneedle (BN) insertion with either labeled IPV or free IR dye. Depicted animals were representative for the whole group (A). The relative fluorescence intensity at the site of administration was quantified (B). Data represent means ± SD from five animals (except for BN free dye group where only two animals were used).

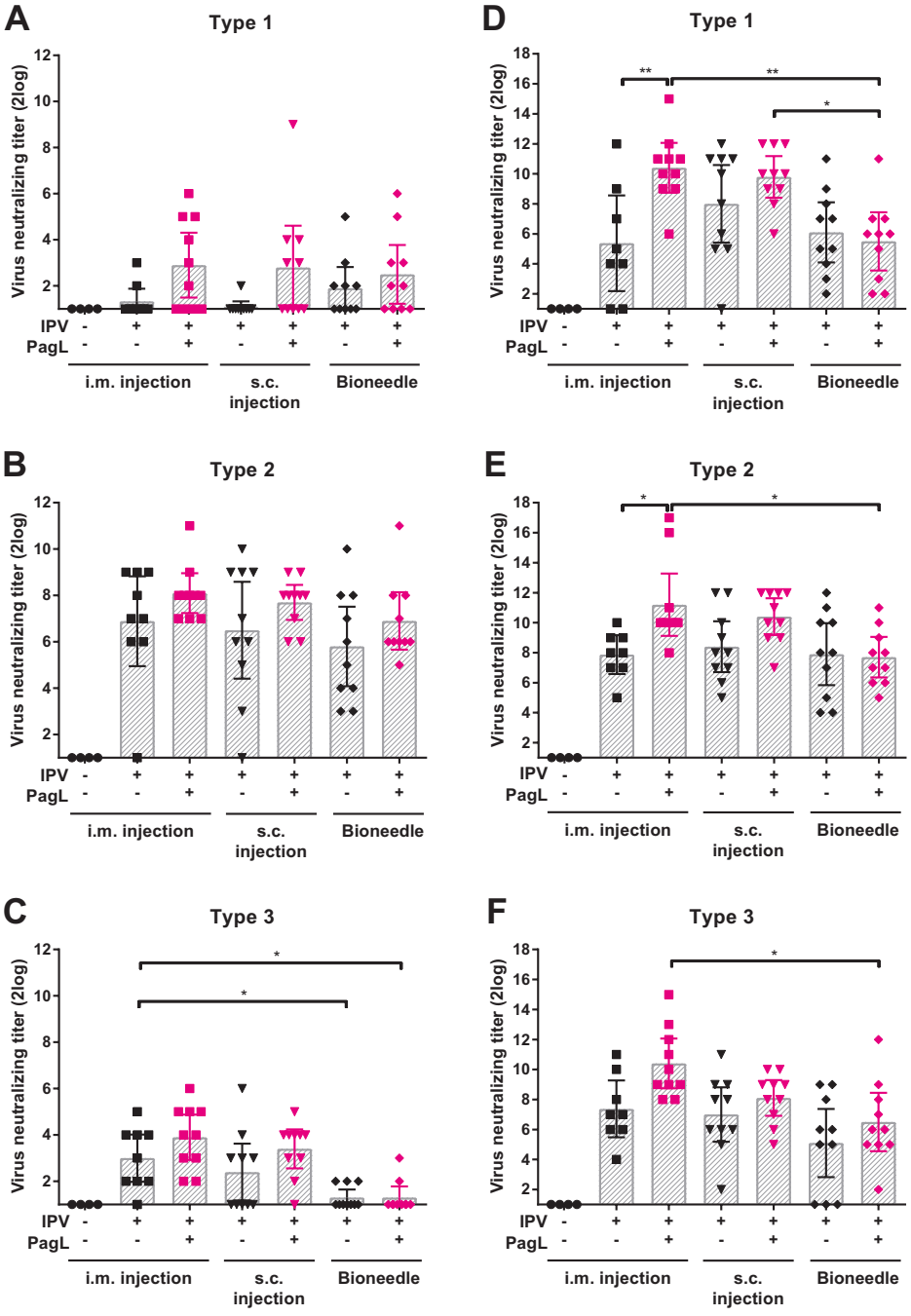

Figure 4 Mean virus neutralizing (VN) antibody titers in serum. Rats (n=10) received IPV in the absence (closed symbols) or presence of PagL LPS (open symbols) as adjuvant. Four animals received mock vaccine as negative control and are the source of the baseline VN titers. Immunizations were given via intramuscular (i.m.) or subcutaneous (s.c.) liquid injection or via subcutaneous Bioneedle insertion and the neutralizing capacity of serum antibodies was determined three weeks after prime (day 21, A-C) or three weeks after booster immunization (day 49, D-F). Asterisks indicate significant differences between groups (* $p<0.01$, ** $p<0.001$).

Immunization study

Wistar rats were immunized with IPV, delivered via either Bioneedles or liquid injection, in a prime-booster regime. Subsequently, immune responses elicited by IPV-filled Bioneedles were compared with those obtained via liquid injection.

After prime immunization, rats that received i.m. liquid injection showed low VN titers and only two of them elicited a detectable response against type 1 (Figure 4A), whereas all animals showed a VN titer against type 2 following i.m. immunization with IPV (Figure 4B). Rats receiving a prime immunization with an IPV-Bioneedle showed VN titers for type 1 and 2 that were similar to those obtained with i.m. liquid injection using syringe and needle. Although prime immunization with an IPV-containing Bioneedle induced significant lower VN titers for type 3 compared to i.m. liquid injection ($p<0.01$), it induced similar VN type 3 titers compared to s.c. liquid injection (Figure 4C). In general, higher numbers of responders were observed in the groups immunized with IPV in the presence of PagL LPS (Figure 4A-C). These responses were only significantly higher after the boost immunization for type 1 and 2.

VN titers in rats were comparable for all serotypes after booster immunization via either IPV-filled Bioneedles or i.m. IPV injection (Figure 4D-F). The route of administration had no effect on the induction of VN antibodies after booster immunization. The addition of PagL LPS showed significant improved VN titers for type 1 and 2 when administered via i.m. injection (Figure 4D-E). Serum IgG titers, which were determined by ELISA, showed similar results for all serotypes compared with the VN titers (data not shown).

DISCUSSION AND CONCLUSIONS

The current proof of concept study demonstrated the potential of an alternative delivered IPV using the Bioneedle technology. Compared with liquid IPV, IPV formulated in Bioneedles showed improved thermostability and similar kinetics at the site of injection as well as comparable immunogenicity when administered in a booster regime. Whereas improved immunogenicity was generated by addition of PagL to liquid IPV, immune responses elicited by IPV-Bioneedles were not potentiated by PagL.

The lyophilization step needed during the production of vaccine-filled Bioneedles is an important characteristic of the Bioneedle technology that might make it an interesting vaccine delivery approach without dependence on the cold-chain [10-12]. However, lyophilization of polio vaccines while maintaining their functionality showed to be challenging

[18]. Excipients are required to protect the antigen against freezing and drying stresses that occur during the lyophilization process. For production of Bioneedles containing vaccine formulations in general, the disaccharide trehalose (5% w/v) was able to fully protect tetanus toxoid [10], hepatitis B surface antigen [11] and the influenza hemagglutinin antigen (HA) during lyophilization [12]. Nevertheless, even 10% (w/v) trehalose or sucrose was not enough to maintain the D-antigen content of IPV during the freezing and subsequent drying steps [14]. The formulation used in IPV-Bioneedles was inferred from an earlier study where a combination of sorbitol, monosodium glutamate (MSG) and magnesium chloride ($MgCl_2$) was able to protect IPV during lyophilization and storage [14]. In order to obtain an appropriate viscosity for the use in Bioneedles, it was required to use the reported optimized formulation at lower amounts of the same excipients (8-5-5% instead of 10-8.5-8.5% sorbitol, MSG and $MgCl_2$, respectively) [14]. This formulation yielded similar D-antigen recoveries as compared with the optimal formulation in vials as described in the lyophilization study [14].

At temperatures above 56°C, within minutes poliovirus and vaccine are converted to C-antigen [19], which are not able to induce VN antibodies [20]. Therefore, accelerated stability was determined above and below this threshold: 45°C and 60°C. The lyophilized formulation used in this study was slightly less stable when compared to the optimal formulation in vials, both in Bioneedles and vials [14]. In contrast, whereas the lyophilized IPV formulation in vials maintained type 1 antigenicity after a one-week incubation at 45°C [14], a significant loss in type 1 D-antigen was observed for the same formulation in Bioneedles. The optimized formulation in vials had a residual moisture content above 3%, the European Pharmacopoeia limit [14]. Unfortunately, we were not able to measure the water content in Bioneedles due to interference of the Bioneedle-material with the assay. Since this formulation was less stable than the optimized lyophilized IPV formulation (in vials), we expect a higher residual moisture content for lyophilized IPV in Bioneedles, potentially resulting in the observed decrease in thermostability when compared to lyophilized IPV in vials. Nevertheless, the lyophilized IPV formulated in Bioneedles was more thermostable than the liquid IPV for all serotypes. Optimization of the IPV-Bioneedle formulation and lyophilization process, e.g., by prolongation of the secondary drying step and thereby reducing the residual moisture content, could probably further increase the stability of IPV-filled Bioneedles.

Earlier, it was suggested that Bioneedles might induce a short-term ‘depot effect’ or alter the kinetics of antigen recognition and processing, which could explain the enhanced immunogenicity of Bioneedles for some antigens [10, 12]. Some vaccine delivery systems are able to prolong the localization period of the antigen at the site of injection, thereby

slowly releasing the antigens at the injection site for a period of up to more than one week [21]. An *in vivo* imaging study was performed using infrared-dye labeled IPV to investigate whether the residence time at and release from the administration site was different between IPV formulated in Bioneedles and subcutaneously injected liquid IPV. Both administration methods (Bioneedle and liquid injection) showed a comparable release from the injection site and a similar distribution pattern over time. Within a few days, the labeled IPV in Bioneedles completely disappeared from the site of administration showing comparable clearance as subcutaneously injected ovalbumin alone in mice [22]. This demonstrated that Bioneedles did not form a depot (at least for IPV and the formulation used) at the site of injection and supports the fact that in general the immunogenicity of Bioneedle formulated antigens seemed to be comparable to injected fluid vaccine, which is an advantage in the licensing process.

Immunogenicity of IPV Bioneedles was evaluated in rats measuring VN capacity of serum, which is a surrogate marker for protection [23]. A booster regime seemed to be essential for a proper immune response similar to that induced by IPV injection in the rat model. The observed low VN titers after prime immunization were not surprising, since other IPV formulations showed also moderate immunogenicity, as indicated by low VN titers and low numbers of responders following a single vaccination (i.m.) [2, 24].

In order to increase the immunogenicity of IPV, PagL was included as adjuvant. Several studies have shown the potential of dose sparing by using adjuvants for IPV based on Salk [24-27] and Sabin strains [2, 28]. PagL LPS is able to enhance the potency of Sabin IPV after both prime and booster immunization (via the i.m. route), serotype 3 being most immunogenic [2]. Since type 3 is the most vulnerable serotype during lyophilization and subsequent storage of dried IPV [14], it was decided to include PagL in Bioneedles to compensate for possible loss of immunogenicity. The addition of PagL in the lyophilized IPV-Bioneedles did not result in improved VN titers at the dose evaluated. However, PagL was able to enhance the immune response against the poliovirus serotypes when administered via liquid injection (36-, 10-, 8-fold VN titer-increase for type 1, 2 and 3, respectively), albeit to a lesser extent as observed for Sabin IPV in a booster-regime (294-, 578- and 2352-fold for type 1, 2 and 3, respectively) [2]. The lack of comparability between wild type (Salk) IPV and Sabin IPV, which is due to their different antigenic and immunogenic properties, has been reported extensively [29-31]. The lack of adjuvant activity in the Bioneedle material may be the result of interaction between adjuvant and Bioneedle. However, another LPS-derivate, LpxL1, was able to enhance the immunogenicity of Bioneedles in combination with hepatitis B vaccine in mice [11]. Further

investigation on the PagL dose, kinetics and routing upon delivery is needed (e.g., by *in vivo* imaging). In the study reported here significant adjuvant effects of PagL included in IPV Bioneedles, at least for the evaluated dose, were not seen.

This study demonstrated the preclinical proof of concept of Bioneedles for IPV. When formulated in Bioneedles, IPV was more thermostable as compared with the liquid IPV. A clinical study in healthy volunteers showed already that solid Bioneedles without antigen were well tolerated [13]. However, several steps should be taken in the further development of this alternative delivery system for polio vaccination, including toxicity and dose finding studies. Those (pre-) clinical studies, using an approved applicator for Bioneedle administration, should prove the practical use, safety, and efficacy of Bioneedles for human vaccination, and their usefulness to strive for polio eradication and in the period thereafter.

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Conflict of interests: Gijsbert van de Wijdeven (GW) is founder of Bioneedle Technology Group (BTG) and the Bioneedle technology is proprietary to BTG. GW provided the empty Bioneedles. Intravacc did the study design, preparation of the final formulations, product characterization, immunogenicity studies and data processing. There were no restrictions on the use of generated data.

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Buccal and sublingual vaccine delivery

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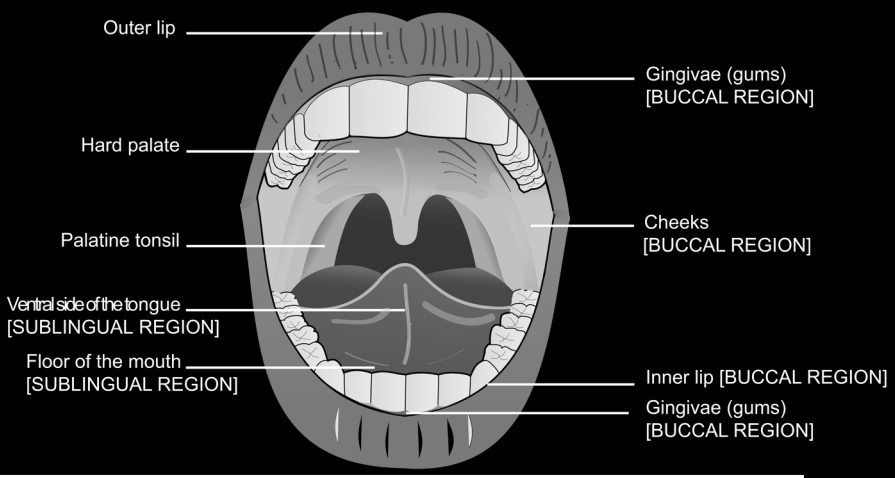
ABSTRACT



Because of their large surface area and immunological competence, mucosal tissues are attractive administration and target sites for vaccination. An important characteristic of mucosal vaccination is its ability to elicit local immune responses, which act against infection at the site of pathogen entry. However, mucosal surfaces are endowed with potent and sophisticated tolerance mechanisms to prevent the immune system from overreacting to the many environmental antigens. Hence, mucosal vaccination may suppress the immune system instead of induce a protective immune response. Therefore, mucosal adjuvants and/or special antigen delivery systems as well as appropriate dosage forms are required in order to develop potent mucosal vaccines.

Whereas oral, nasal and pulmonary vaccine delivery strategies have been described extensively, the sublingual and buccal routes have received considerably less attention. In this review, the characteristics of and approaches for sublingual and buccal vaccine delivery are described and compared with other mucosal vaccine delivery sites. We discuss recent progress and highlight promising developments in the search for vaccine formulations, including adjuvants and suitable dosage forms, which are likely critical for designing a successful sublingual or buccal vaccine. Finally, we outline the challenges, hurdles to overcome and formulation issues relevant for sublingual or buccal vaccine delivery.

INTRODUCTION

Among public health interventions, vaccination is by far the most effective strategy in maintaining population health and combating infectious diseases, especially in developing countries and disaster areas. Vaccination saves millions of lives every year, while bringing numerous social and economic benefits [1]. Since the vast majority of pathogens infect their host through the mucosa, an ideal vaccine should induce protective immunity at mucosal sites in order to act as a first line of defense against infections. However, most of the vaccines currently in use are administered via injection, e.g., via the subcutaneous or intramuscular route. This generally induces poor mucosal immunity, whereas vaccines administered via mucosal routes have proven to be effective for the induction of both systemic and local immunity [2]. Additionally, mucosal immunization makes vaccine delivery easier and safer than parenteral administration routes, is very suitable for mass immunization during pandemic situations, and improves acceptability especially among children [3, 4].

Despite the advantages mentioned above, there are currently only few mucosal vaccines for human use on the market [5]. The reason for this is that mucosal vaccination poses several challenges, such as immune regulation and tolerance, as well as overcoming fast removal of the vaccine by body fluids and enzymes, as has been reviewed for the oral [3, 6], the nasal [7] and the pulmonary route [8]. Compared to the above-mentioned traditional mucosal routes, sublingual and buccal vaccine administration has received less attention. For many years, these routes have been used for the delivery of low-molecular-weight drugs to the bloodstream. Currently, the only vaccines that are widely being used for delivery via the oral mucosae are therapeutic sublingual allergy vaccines. These vaccines are used for sublingual immunotherapy (SLIT) to treat allergic hypersensitivity. Sublingual delivery of allergens can activate regulatory T cells that can suppress undesired immune reactions [9]. This has resulted in several approved sublingual products for allergy immunotherapy, such as SLITone®, Sublivac®, Grazax®, Oralair®, AllerSlit®forte. Allergy vaccines are reviewed by Valenta *et al*. [10] and are beyond the scope of this review since they are aimed at immune regulation (tolerance) instead of activation of the adaptive immune system.

In this review, we will describe the potential and limitations of the sublingual and buccal mucosae as vaccine delivery sites and the mucosal immune responses that are induced upon sublingual or buccal vaccination. Further, the current status of sublingual and buccal vaccine delivery will be discussed and suitable vaccine antigens and potent adjuvants (immune potentiators and/ or delivery systems) will be highlighted. Appropriate dosage forms

that are required for a successful sublingual or buccal vaccine will also be outlined. Finally, the forthcoming perspectives are given, including the existing research and development gaps in this field and the potential of improved or controlled release vaccine formulations for sublingual and buccal vaccine delivery.

SUBLINGUAL AND BUCCAL MUCOSAL SITES FOR VACCINE DELIVERY

Mucosal vaccine delivery in the mouth can be subdivided into sublingual and buccal delivery. Sublingual delivery occurs via the mucosa of the ventral surface of the tongue and the floor of the mouth under the tongue, whereas buccal delivery occurs via the buccal mucosa, which is located in the cheeks, the gums and the upper and lower inner lips (**Figure 1**). The specific structure and cell composition of the sublingual and buccal regions in the mouth define whether they are more or less suitable for vaccine delivery (as described further).

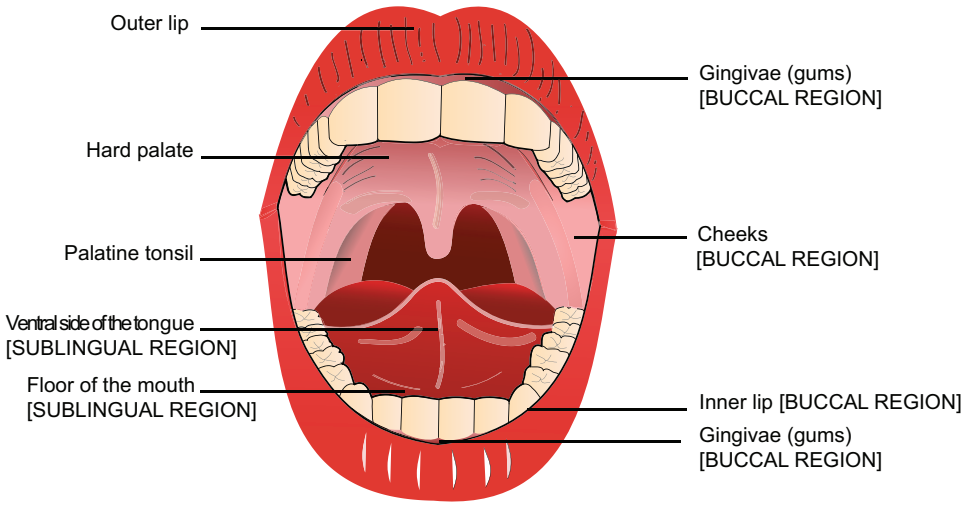


Figure 1 The anatomy of the oral cavity. The sublingual and buccal regions for vaccine delivery are indicated.

Within the oral cavity, some mucosal regions are lined by a keratinized stratified epithelium (gingival, hard palate, outer lips), whereas other regions are lined by a non-keratinized stratified epithelium (**Figure 1**). The epithelium is supported by a basement

membrane, which separates the two major layers of the oral mucosa: the epithelium and the underlying connective tissue or lamina propria. The arrangement of the hard palate and gingival, including the pluristratified keratinized mucosal epithelium and the lamina propria that is anchored onto the periostium of the underlying bone, makes these regions chemically and mechanically resistant to withstand the shearing forces associated with chewing food. The floor of the mouth, the inner surface of the lips and cheeks, and the ventral side of the tongue are covered by a non-keratinized epithelium, rendering these relatively more elastic and pervious than keratinized mucosae, and thus potentially more suitable for drug or antigen delivery.

The epithelium serves as a mechanical barrier protecting underlying tissues and consists of a basal layer, an intermediate layer, and a superficial layer. From the basal to superficial layer the cells become larger, more flattened, more proteinaceous in the form of protein monofilaments, and less viable due to the absence of organelles (**Figure 2**). The compacted, flattened cells of the lower superficial and intermediate layers form the major physical barrier to transport, whereas the intercellular lipids play an important role in the permeability of the mucosa. Besides epithelial cells, the oral mucosal epithelium also contains three other cell types. The basal layer includes Merkel cells, which are endocrine cells associated with nerve fibers that contribute to the overall barrier function of the epithelium. They have also been suggested to play a role in the regenerative processes of oral mucosa [11]. Further, the suprabasal layer contains two types of cells: melanocytes, which produce the pigment melanin and are thus responsible for the color of the mucosa; and Langerhans cells (LCs), which are the most superficial antigen-presenting cells and an important target for the induction of an immune response. Other antigen-presenting cells below the mucosa are the myeloid dendritic cells (mDCs) located along the lamina propria and the plasmacytoid DCs (pDCs) found in the submucosal tissue (**Figure 2**).

Salivary glands, which are located just below the mucosa in the mouth, produce mucin, a major component of the mucus layer on the mucosal surface, and help to promote the production and secretion of saliva. Saliva is needed to moisten and lubricate the mucosae and assists the masticatory process by binding the food bolus prior to and during swallowing. Additionally, salivary secretions protect the oral epithelium from potential harmful substances and regulate the composition of the oral microbial flora by its enzyme activity and by maintaining the oral pH between 5.5 and 7.0 [12].

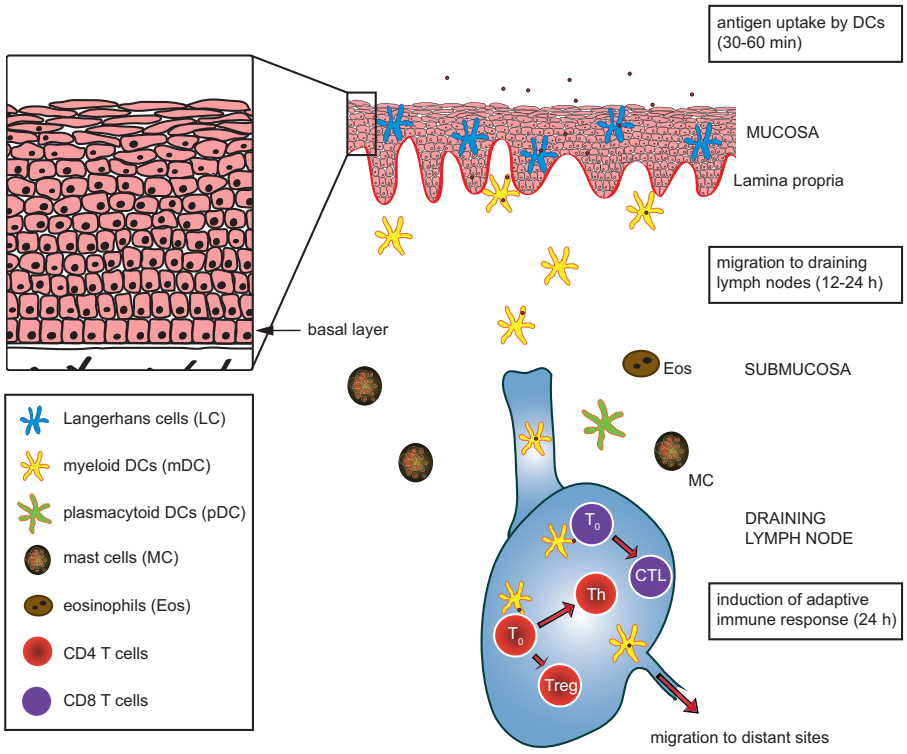


Figure 2 Antigen delivery and antigen presentation following sublingual or buccal vaccination. Upon vaccine delivery, the antigen is likely to be captured by Langerhans cells (LC) within the mucosa itself and myeloid dendritic cells (mDCs) along the lamina propria. Antigen-bearing DCs will migrate to draining lymph nodes where they interact with naïve CD4 and CD8 T cells to support the differentiation into effector T cells (i.e., helper (Th) and cytotoxic T cells (CTL)) and thereby induction of the adaptive immune response.

Implications for mucosal vaccine delivery and comparison with other mucosal sites

Sublingual and buccal mucosae are attractive vaccine delivery sites that may have advantages over other routes, because of their anatomy and physiology. Table 1 shows an overview of characteristics of different sites that have been investigated for vaccine delivery. The dermal delivery site has been included because it contains comparable features with the buccal and sublingual sites, such as the presence of LCs as a main target for the vaccine antigen. However, dermal vaccination has the disadvantage that the impermeable thick keratinized stratum corneum acts as a physiological barrier for the diffusion of antigens to reach LCs after topical administration. As a result, dermal vaccination in general needs chemical disruption and/or microneedle penetration of this wall in order to let antigens reach

the LCs to induce an immune response [13]. In contrast, sublingual and buccal mucosae suitable for vaccine delivery lack keratinized epithelium.

Compared to gastro-intestinal mucosal routes, degradation by gastric fluids and gastrointestinal enzymes is avoided during sublingual or buccal delivery, although some enzymatic activity is present in the mouth.

Most of the mucosal routes have special 'gateways', the so-called microfold (M) cells that are present in the epithelium covering the follicles of mucosal tissues. These M cells take care of the transport of antigens to mucosa-associated lymphoid tissues (MALT). The Peyer's patches (PP), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) are important inductive tissues that generate mucosal immunity upon vaccination via the gastro-intestinal (oral), nasal and pulmonary routes, respectively. In addition, the buccal and sublingual mucosal epithelia are covered with squamous stratified epithelium, whereas the epithelial cells of the small intestine, nasal cavity, trachea and bronchi are covered with columnar epithelium.

Compared to other routes, the sublingual and buccal routes have the potential to induce mucosal immune responses in a broad range of tissues (**Table 1**) as described in more detail in section 2.2 ('**Mucosal immune responses**').

Nagai *et al*. (2014) recently reported the transport of sublingual antigens across sublingual ductal epithelial cells to ductal APCs in mice [14]. Since different studies failed to detect specific sampling cells (or M-like structures) in the sublingual or buccal mucosa[14, 15], it seems most likely that antigens cross the ductal epithelium via paracellular and transcellular pathways [14]. So, probably the efficiency of vaccine delivery via these routes is directly related to the permeability of the mucosal membrane. This permeability is influenced by the thickness and the degree of keratinization of these membranes. The thickness of the human buccal mucosa has been estimated to be in the range of 500-800 μm, whereas the mucosal thickness of the sublingual region is about 100-200 μm [16]. In terms of permeability, the sublingual region is more permeable than the buccal region, which in turn is more permeable than the palatal region (**Figure 1**). Sublingual administration can provide a rapid uptake of macromolecules and thus appears to be an attractive route for dosage forms with a short delivery period.

In the development of sublingual or buccal vaccines, the presence of saliva should be taken into account. Although saliva may be useful for the release of the antigen from

Table 1 Characteristics of different sites for vaccine delivery in humans. Mucosal immune responses (MIR) after immunization by different routes are indicated [82].

	Sublingual	Buccal	Oral / Gastro-intestinal	Intranasal	Pulmonary	Dermal
Estimated surface area (cm^2)	26.5 ± 4.2	50.2 ± 2.9	350000 [a]	160-180	700,000	20,000
Epithelial structure (cell layers)	Stratified squamous, non-keratinized	Stratified squamous, non-keratinized	Simple columnar, non-ciliated [a]	Pseudo-stratified columnar, ciliated	Pseudo-stratified [d] / Simple columnar, ciliated [e]	Stratified squamous, keratinized
Thickness, cell layer	8-12 cells (0.1-0.2 mm)	40-50 cells (0.5-0.8 mm)	Single cell	Single cell	Single cell	Multiple cells (2-3 mm)
Vaccine target	LCs (oral lymphoid foci)	LCs (oral lymphoid foci)	M cells (PP)	M cells (NALT)	M cells (BALT)	LCs
MIR distinct sites:						
Respiratory tract						
Upper	+++	?	-	+++	?	+++
Lower	+++	?	-	+/+++ [b]	++	+++
Gastrointestinal tract						
Stomach	+	?	+	-	?	?
Small intestine	+++	?	+++	-	?	+
Colon	?	?	++	-	?	+
Reproductive tract	+++	+	-	++	++	?
Systemic response	+++	++	+	+++	+	+++

[a] *Based on delivery in the small intestine*
[b] *Strong response by aerosol administration*
[c] *Total surface area lungs*
[d] *Epithelium lining the trachea*
[e] *Epithelium lining the bronchi*

Abbreviations used:

MIR	*mucosal immune response*
LCs	*Langerhans cells*
MALT	*mucosa-associated lymphoid tissue*
PP	*Peyer's patches*
NALT	*nasopharynx-associated lymphoid tissue*
BALT	*bronchus-associated lymphoid tissue*

certain dosage forms, it can be disadvantageous since the salivary composition, pH, and flow rate are variable. Excessive secretion and salivary flow may dilute the antigen or lead to swallowing of the dosage form before the antigen is absorbed through the mucosa, the so-called 'saliva washout' [12]. Moreover, the presence of digestive enzymes could lead to degradation of the antigen.

Since pH is a critical parameter for antigen absorption, the pH values at the different oral mucosal sites should be kept in mind for successful vaccine delivery. For example in adults, the pH of the floor of the mouth is about 6.5, whereas that of the buccal region is about 6.3 [17]. However, factors, such as diet and saliva flow rate, may affect the pH of the oral mucosa.

In section 4 ('**Dosage forms**'), we describe different ways to circumvent the challenging characteristics of sublingual or buccal vaccine delivery.

Mucosal immune responses

Unlike small synthetic drugs, vaccine antigens do not reach the bloodstream after entering the sublingual or buccal mucosa, but are rather captured by antigen-presenting DCs, mainly LCs, in the mucosa. The antigen uptake, process and the presentation of their epitopes to T cells by antigen-presenting cells are required to induce effective adaptive immunity. For example, the ovalbumin antigen crosses the epithelial barrier within 15 to 30 minutes and the uptake by sublingual DCs occurs within 30 to 60 minutes after sublingual administration in mice [18]. Rare pro-inflammatory cells, i.e., histamine-containing mast cells (MC) or eosinophils (Eos) (**Figure 2**), are found in oral tissues, and these cells are mainly spread in the muscular layer beneath the mucosa [18, 19]. Therefore, it is likely that most of the antigen is mainly captured by LCs and other oral DCs in the upper layers prior to reaching pro-inflammatory cells. The relatively high frequency of LCs and low numbers of mast cells in the buccal region [19] make the buccal mucosa an attractive site for vaccine delivery despite its thicker epithelium and lower permeability as compared to the sublingual mucosa.

Antigen-bearing DCs migrate to the lymph nodes draining the sublingual and buccal area where they are able to prime both naïve CD4 and CD8 T cells (**Figure 2**, T0 cells). For example, Eriksson *et al*. reported that a substantial number of DCs leave the buccal epithelium after topical buccal immunization and migrate to draining lymph nodes where they present processed antigen to CD4 T lymphocytes [20]. Another study revealed the buccal epithelium as inductive site of efficient priming of CD8 T lymphocytes [21]. Upon activation, T and B cells leave the site of the initial antigen presentation, enter the circulation and then disperse to

selected mucosal sites, where they differentiate into memory or effector cells. The activation of CD4 T lymphocytes leads to the induction of antigen-specific helper T (Th cells) and/or regulatory T cell (Treg) mediated immune responses (**Figure 2**), whereas CD8 T cells facilitate the induction of cytotoxic T lymphocyte (CTL) responses (**Figure 2**). The tissue destination of these cells appears to be largely determined by site-specific integrins, supposed 'homing receptors', on their surface that will bind to mucosal tissue-specific receptors (addressins) on vascular endothelial cells [22]. Recent work of Hervouet *et al*. demonstrates that antigen-bearing DCs that have captured the antigen in the sublingual mucosa are encountered in distant lymph nodes and spleen following sublingual immunization of mice [23], which suggests that sublingual DCs are capable to enter the blood circulation to seed distant lymphoid organs.

The migration of immune cells from the inductive MALT to distant effector tissues is the cellular basis for the so-called 'common mucosal immune system'. The MALT contains T-cell zones, B-cell enriched areas containing a high frequency of sIgA-positive B cells and a subepithelial area with antigen-presenting cells to induce specific immune responses. As mentioned earlier, the oral mucosa lacks a certain immunological structure as observed in Peyer's patches of the intestine where the antigen is sampled by specialized M cells. However, different studies describe the concept of oral lymphoid foci, the equivalent of the germinal centers observed in other MALT, suggesting that the oral mucosa serves as a site for immune induction. The role of oral DCs in deciding whether to induce adaptive immunity or tolerance, and whether there exist germinal centers in oral lymphoid foci are discussed extensively by others [24, 25]. The distribution of immune cells, particularly the abundant presence of oral LCs, makes the oral mucosa an attractive site for vaccine delivery. Both sublingual and buccal immunization are able to promote mucosal immunity, as well as systemic immunity, against pathogens entering the human body at more distant sites than the mouth (mucosa), such as the respiratory tract or reproductive tract (**Table 1**).

CURRENT STATUS OF SUBLINGUAL AND BUCCAL VACCINE DELIVERY

In the section below, we will describe the use of live (attenuated) pathogens, recombinant (heterologous expression) and inactivated vaccines. Furthermore, we will outline adjuvants, i.e., immune potentiators and/or delivery systems, that have been evaluated for vaccination via the sublingual or buccal route. A summary of the published studies on sublingual and buccal vaccination is given in Table 2.

Live attenuated vaccines

Live attenuated viral vaccines

Sublingual immunization of mice with live attenuated influenza virus (A/PR/8 strain, H1N1) has been found to be safe and effective for inducing protective immune responses in mucosal and systemic compartments. Song *et al*. concluded that the observed protection was mediated by the induction of influenza virus-specific IgG in the serum and secretory IgA (sIgA) in the respiratory mucosa, which limit virus entry and replication in the respiratory tract. A single sublingual dose of A/PR/8 virus prevented lung pathology induced by influenza virus challenge and provided a broad-range cross-protection against different influenza virus subtypes. Thereby, the risk of potential passage of vaccine virus to the olfactory bulb was avoided using the sublingual route since no viral RNA was detected in brains of sublingually vaccinated mice, in contrast to mice that received the same vaccine intranasally [26]. Similarly, sublingual administration of live-attenuated virus lacking the non-structural protein 1 (DeltaNS1) was as protective against influenza virus challenges in mice as intranasal immunization. Sublingual immunization with these DeltaNS1 viruses induced high levels of virus-specific antibodies and stimulated immune cells in mucosa-associated and systemic lymphoid organs [27]. Moreover, the vaccine was well tolerated and did not induce bodyweight loss in sublingually vaccinated mice.

Table 2 Preclinical development of sublingual and buccal vaccins.

Antigen	Adjuvant	*In vivo* model	Dose *(no. of doses)*	Dosage form *(volume)*	Protection	Immune response / characteristics	Ref
Live attenuated vaccines							
Influenza A/PR/8		Mouse	$1x10^2$ pfu $1x10^4$ pfu	L *<7 µl*	+/-	Both systemic and mucosal Abs Dose dependent protection against both homologous and heterosubtypic influenza virus challenge	[26]
deltaNS1 influenza A[1]		Mouse	$2x10^7$ pfu	L *4x5 µl*	+	Protection against challenge with homologous and heterosubtypic influenza virus High levels specific Abs in both mucosal and systemic compartments Stimulated immune cells in mucosa-associated and systemic lymphoid organs	[27]
Heterologous antigen expression – live vaccine carriers							
NP (nucleoprotein) of influenza A/PR/8	rAd5	Mouse	$1x10^7$ pfu $1x10^8$ pfu	L *<5 µl*	-	Single sublingual immunization failed to confer protection by different influenza strains	[28]
HA soluble globular head	rAd5	Mouse	$1x10^8$ pfu	L *10 µl*	+	Complete protection after challenge with lethal dose homologous virus Induction of significant levels of HA-specific mucosal IgA and IgG	[29]
RSV - sFsyn[2]	HDAd2	Mouse	$1x10^8$ pfu	L *5 µl*	+	RSV F protein-specific systemic and mucosal neutralizing Abs RSV-specific IFN-γ producing $CD8^+$ T cell responses in the spleen and lung Effective protection against RSV infection; reduced lung viral titers upon challenge compared with control group	[38]
SARS-S protein (Spike)	rAd	Mouse	$2x10^7$ pfu $1x10^8$ pfu	L *20 µl*	VN	SARS-CoV neutralizing antibodies in serum Airway IgA Induced $CD8^+$ T cells responses in lungs Unlike intranasal vaccination, no redirection of AdV to olfactory bulb	[32]
HIV-Env (Envelope glycoprotein)	rAd5	Mouse	$1x10^8$ pfu	L *10 µl*	n.d.	Serum IgA response Vaginal IgA and IgG	[31]

HIV-Gag	rAd5 rEA	Mouse	1x10^{10} pfu	L *8 µl*	n.d.	Induction of CTL responses in spleen and SMLN Higher innate immune responses and improved T cell responses after co-administration with rEA (TLR agonist)	[30]
SIV-Env/rev SIV-Gag	rAd5	Macaque	1x10^{9} pfu Each *(2x)*	L *0.25 ml*	n.d.	Macrophage targeting in BAL fluid and rectal tissue SIV-specific cellular responses, serum binding Abs and mucosal sIgA	[39]
Ebola ZGP (Zaire glycoprotein)	rAd5	Mouse Guinea pig	1x10^{8} pfu 1x10^{9} pfu	L *10 µl* *40 µl*	+	Induced IFN-y T cells in spleen, BAL, MLN and SMLN Elicited population of effector memory CD8+ cells and strong CTL responses in spleen and SMLN	[33]
Tetanus toxin fragment C (TTFC)	*Bacillus subtilis mLT*	Mouse	1x10^{9} pfu *(3-4x)*	L *10 µl*	+	Tetanus-specific systemic IgG and mucosal sIgA Full protection against lethal toxin challenge in mice immunized with TTFC vegetative cells (without mLT)	[35]
Tetanus toxin fragment C (TTFC)	*Bacillus subtilis mLT*	Pig	1x10^{9} pfu *(4x)*	L *1 ml*	n.d.	Tetanus-specific systemic neutralizing Abs Induction of salivary and vaginal IgA responses	[36]
Streptococcus mutans P1	*Bacillus subtilus*	Mouse	1x10^{8} pfu	L *10 µl*	n.d.	Higher specific IgG titers when compared to group orally immunized with higher dose	[37]
Inactivated vaccines							
Influenza WIV (β-propiolactone-inactivated)		Mouse	20 µg *(1x)* 5 µg (i.m. booster)	L *10 µl*	n.d.	Enhanced HI titers after sublingual priming followed by an intramuscular booster when compared to the intramuscular priming Enhanced lung and nose IgA titers with sublingual priming	[40]
Influenza WIV (formalin-inactivated)		Mouse	20 µg *(2x)*	L *<7 µl*	+	Specific systemic and secretory Ab responses 80% survival	[26]

Antigen	Adjuvant	*In vivo* model	Dose (no. of doses)	Dosage form (volume)	Protection	Immune response / characteristics	Ref
Influenza WIV (formalin-inactivated)	mCTA-LT[3] (5 µg)	Mouse	20 µg *(2x)*	L *<7 µl*	+	100% survival, complete clearance of virus in the lungs (BAL fluid) More IFNy-producing $CD4^+$ and $CD8^+$ T cells in spleens and MLNs than without mCTA-LT More virus-specific CTL-responses than with PBS or killed A/PR/8 alone	[26]
Influenza HA subunit	LTK63[4] (5 µg)	Mouse	10 µg *(3x)*	L	n.d.	Systemic responses (IgG and HI) comparable to intramuscular immunization Influenza-specific Th17 cells and neutralizing mucosal IgA in the nose (comparable to intranasal immunization)	[41]
Influenza A virosome	c-di-GMP[5] (7.5 µg)	Mouse	2 µg *(2x)*	L *7 µl*	n.d.	Induction of systemic and local Abs capable of hemagglutination inhibition Significant adjuvant effect on both systemic and local Ab responses High frequencies of influenza-specific homo- and hetero-subtypic $CD4^+$ Th1 cells Balanced Th1/2 profile and Th17 response after immunization with adjuvanted virosomes	[42]
Influenza 3M2eC protein	CT (2 µg)	Mouse	10 µg	L *15 µl*	+	Both systemic and mucosal Abs Protection against both homologous and heterosubtypic influenza virus challenge	[43]
HPV16L1 VLP		Mouse	5 µg	L *10 µl*	+	Protection against genital challenge with HPV pseudovirions Neutralizing Abs in serum and genital IgG and IgA Abs Neutralizing Abs in cervicovaginal secretions	[44]
HPV16L1 VLP	CT (2 µg)	Mouse	5 µg	L *10 µl*	+	Protection against genital challenge with HPV pseudovirions Higher systemic IgG Ab titers than after sublingual VLP administration without CT Lower vaginal IgG, but higher vaginal IgA Ab responses than intramuscular immunized mice with alum-adjuvanted VLPs Neutralizing Abs in cervicovaginal secretions	[44]

HPV16L1 VLP		Mouse	30 µg 50 µg	L *10 µl*	n.d.	Higher vaginal and salivary sIgA responses when compared to untreated animals Increased number IFN-γ producing CD8+ T cells in spleen	[45]
HPV16L1 VLP	CTB (10 µg)	Mouse	30 µg	L *10 µl*	n.d.	Elevated mucosal sIgA induction after co-treatment with CTB Enhanced production of IL-4 and IFN-γ from stimulated CD4+ T cells Higher number IFN-γ producing CD8+ T cells in spleen and SMLN when compared to HPV16L1 alone	[45]
HPV16L1 VLP	Several adjuvants (see characteristics)	Mouse	30 µg	L *10 µl*	n.d.	No enhanced effects on systemic IgG nor on vaginal and salivary IgA Ab responses (Adjuvants that were used are: Poly I:C (0.1 mg), MPLA (10 µg), Imiquimod (50 µg), L18-MDP (1 µg), Murabutide (10 µg), peptidoglycans (50 µg), Vitamin D3 (0.5 µg), γ-polyglutamic acid (1 mg))	[45]
RSV G protein (Gcf)	CT (2 µg)	Mouse	20 µg	L *15 µl*	+	Strong serum IgG and mucosal IgA responses Protection against RSV challenge without significant lung eosinophilia No adjuvant effect of CT	[46]
Measles virus NP		Mouse	30 µg *(1x)*	L *15 µl*	+/-	Single buccal immunization (injection or topical application) induced antigen-specific CD8 CTLs Rapid recruitment of DCs into the mucosa Protection against lethal challenge following buccal vaccination by injection (100%) or topical administration (40%)	[47]
HIV-1 Pol	CTB CT (1 µg)	Mouse	25 µg *(3x)*	L *5 µl*	n.d.	CTB-Pol conjugate induced IFN-γ producing CD8 T cells Induction of mucosal CTLs in the genital tract after immunization with CTB-Pol mixed with CT, but not with CTB-Pol alone or Pol mixed with CT	[48]
HIV-1 gp41	CT (1 µg)	Mouse	10 µg *(3x)*	L *5 µl*	n.d.	Strong specific IgG and IgA responses in serum and genital secretions after gp41+CT immunization gp41-specific IgA and IgG ASCs in genital tract	[48]

Antigen	Adjuvant	*In vivo* model	Dose (no. of doses)	Dosage form (volume)	Protection	Immune response / characteristics	Ref
HIV-1 CN54gp140 (gp140)	FSL-1 Poly I:C MPLA Pam3CSK4 R848 CpG B (20 µg each)	Mouse	10 µg *(3x)*	L *10 µl*	n.d.	Increased serum IgG and IgA titers when co-administered with Poly I:C Diminished systemic specific Ab responses with MPLA Detected IgA titers in vaginal washes of all animals where antigen was administered with FSL-1, poly I:C, Pam3CSK4 or CpG B	[49]
HIV-1 CN54gp140 (gp140)	Chitosan (100 µg)	Mouse	10 µg *(3x)*	L *10 µl*	n.d.	No significant adjuvant effect of chitosan	[49]
Salmonella proteins (SPP)		Mouse (newborn)	6 / 40 µg *(3+1)*	L *1.2 µl*	-	Slightly enhanced systemic IgG titer and mucosal IgA responses compared to the PBS control group	[50]
Salmonella proteins (SPP)	CT (0.2 / 1 µg) CpG (1 / 10 µg)	Mouse (newborn)	6 / 40 µg *(3+1)*	L *1.2 µl*	+	Enhanced antigen-specific systemic IgG and mucosal sIgA responses in CT or CpG groups compared to mice immunized with SSP alone Protection against intestinal necrosis and higher survival rates for adjuvant groups	[50]
Salmonella proteins (SPP)	CT (1 µg) CpG (10 µg)	Mouse	40 µg *(7x)*	L *10 µl*	+	Significant higher antigen-specific sIgA responses for both groups immunized with SPP alone or together with CT or CpG as adjuvant Higher IFN-γ production in spleen upon SPP-CpG vaccination Higher IL-4, IL-5 and IL-6 production in spleen upon SPP-CT vaccination Protection against intestinal necrosis and higher survival rates for adjuvant groups	[51]
Pneumococcal whole cell (chloroform inactivated)	dmLT	Mouse	10 µg *(3x)*	L *5 µl*	+	Dose-dependent protection in sublingual immunized mice (reduced bacterial load in nasal wash compared to control mice) Induced systemic IL-17A levels upon sublingual vaccination	[52]

Helicobacter pylori lysate	CT (10 µg)	Mouse	500 µg *(2x)*	L *10 µl*	+	Enhanced proliferative responses to *H. pylori* antigens in CMLNs Immune protection against *H. pylori* infection Strong specific serum IgG and IgA titers in stomach and intestine Strong proliferation and IFN-γ and IL-17 production by T cells from spleen and MLNs Increased IFN-γ and IL-17 gene expression in stomach	[53]
Helicobacter pylori lysate	CT (10 µg)	Mouse	500 µg	L *10 µl*	n.d.	Increased expression of chemokines and chemokine receptors known to attract eosinophils, T cells and neutrophils Higher counts of $CD4^+$ T cells, eosinophils, neutrophils and $CD103^+$ DCs in the gastric lamina propria of immunized mice	[54]
Helicobacter pylori lysate	CT (10 µg)	Mouse	400 µg	L *10 µl*		Decrease in bacterial load after challenge when compared to non-immunized control group	[55]
H*elicobacter pylori* lysate	dmLT (10-20 µg)	Mouse	400 µg	L *10 µl*	+/-	Decrease in bacterial load after challenge when compared to non-immunized control group Enhanced *ex vivo* proliferative and cytokine responses from cells from spleen and MLNs after restimulation with *H. pylori* antigens	[55]
Chlamydial major outer membrane protein (MOMP)	CTA1-DD (20 µg)	Mouse	100 µg	L *7 µl*	+/-	Reduction of severity and incidence of genital tract pathology after challenge with Chlamydia muridarum after sublingual vaccination with or without adjuvant 80% of the MOMP-CTA-DD-immunized animals protected in genital tract	[56]
Chlamydial major outer membrane protein (MOMP)	CT-CpG (5 µg CT) (10 µg CpG)	Mouse	100 µg	L *7 µl*	+/-	Reduction of severity and incidence of genital tract pathology after challenge with Chlamydia muridarum after sublingual vaccination with or without adjuvant 60% of the MOMP-CT-CpG-immunized animals protected in genital tract Reduction of duration of vaginal shedding after challenge, but not of the total bacterial burden MOMP-specific IFN-γ, TNF-α and IL-17 cytokine production by lymphocytes isolated from MLNs	[56]

Antigen	Adjuvant	*In vivo* model	Dose (no. of doses)	Dosage form (volume)	Protection	Immune response / characteristics	Ref
Tetanus toxoid (TT)	LT (1 ug)	Mouse	10-20 µg *(4x)*	L *5 µl*	n.d.	Induced systemic TT-specific IgG and mucosal IgA levels after TT-LT immunization when compared to TT alone Long lasting TT-specific ASCs in bone marrow and CD4+ and CD8+ T cells in dLNs and spleen	[57]
Tetanus toxoid (TT)	LTK63 (10 µg)	Mouse	10-20 µg *(4x)*	L *5 µl*	n.d.	Induced systemic TT-specific IgG and mucosal IgA levels after TT-LTK63 immunization when compared to TT alone Long lasting TT-specific ASCs in bone marrow and CD4+ and CD8+ T cells in dLNs and spleen	[57]
Tetanus toxoid (TT)	FSL-1 Poly I:C MPLA Pam3CSK4 R848 CpG B (20 µg each)	Mouse	10 µg *(3x)*	L *10 µl*	n.d.	Significant increase in specific systemic IgG when co-administered with FSL-1, poly I:C, CpG B and an increase in IgA for FSL-1 Detectable IgG titers in vaginal washes of all animals where antigen was administered with FSL-1, poly I:C or CpG B Decreased specific systemic and vaginal IgA responses for TT-MPLA-immunized mice	[49]
Tetanus toxoid (TT)	Chitosan (100 µg)	Mouse	10 µg *(3x)*	L *10 µl*	n.d.	Increase in specific systemic IgG and IgA above TT alone when co-administered with chitosan Increased IgG1/IgG2a ratio relative to TT alone	[49]
Ovalbumin (OVA)	CT (2 µg)	Mouse	200 µg *(3x)*	L *<10 µl*	n.d.	Systemic and mucosal Ab responses Balanced Th1/Th2 cytokine responses Induction of CD8+ T cells in lung tissues and systemic lymphoid organs	[58]
Ovalbumin (OVA)	Ad2F (25 µg) CT (2 µg)	Mouse	25 µg *(3x)*	L *7 µl*	n.d.	Ad2F-delivered OVA was efficiently taken up by DCs and migrated mostly to SMLNs Highest OVA-specific serum IgG, IgA and mucosal IgA titers for OVA-Ad2F+CT-immunized mice Mixed Th-cell response by enhanced IL-4, IL-10, IFN-γ and TNF-α-specific cytokine-forming cells	[59]

Ovalbumin (OVA)	CT (2 µg)	Mouse	200 µg *(3x)*	L *5 µl*	n.d.	OVA-specific IgG and IgA Abs in blood and cervicovaginal secretions IgA ASC in genital mucosa upon sublingual immunization similar to intranasal or vaginal immunization and superior to intragastric vaccination OVA-specific effector CD8-positive CTLs in genital mucosa following sublingual immunization with OVA and CT	[44]
Hcβtre (from Botulinum neurotoxin A)	Ad2F (25 µg) CT (2 µg)	Mouse	25 µg *(5x)*	L *7 µl*	+	100% protection against BoNT/A intoxination for Hcβtre-Ad2F+CT-immunized mice ~60% protection against BoNT/A intoxination for Hcβtre +CT-immunized mice	[59]

Abbreviations used:
Abs antibodies
ASC antibody secreting cells
CTL cytotoxic T cell
HA hemagglutinin
HIV human immunodeficiency virus
(m)CT (mutant) cholera toxin; CTA/B, A/B subunit of CT
(m)LT (mutant) heat labile toxin; LTB, B subunit of LT; dmLT, double mutant LT
MPLA monophosphoryl lipid A
rAd recombinant adenoviral vector
rEA recombinant Eimeria tenella
RSV respiratory syncytial virus
SARS severe acute respiratory syndrome-associated coronavirus
SIV simian immunodeficiency virus
WIV whole inactivated virus

[1] Influenza virus lacking the Nonstructural Protein 1
[2] Vaccine based on helper-dependent adenoviral vector expressing the soluble fusion glycoprotein of RSV
[3] Subunit mutant of cholera toxin (CT) E112K with the pentameric B subunit of LT
[4] Mucosal adjuvant, K63 mutant of LT
[5] Mucosal adjuvant, (3',5')-cyclic dimeric guanylic acid

Recombinant virus based (RNA-) vaccines

Replication-defective adenovirus vectors (rAdV) have been widely explored for the delivery of antigens. Infection by adenoviruses occurs through the airway epithelium and replication takes place in mucosal tissues of the respiratory tract. These characteristics make these vectors suitable for mucosal vaccine delivery. Sublingual immunization with rAdV encoding the conserved influenza nucleoprotein antigen [28] or the soluble globular head of hemagglutinin [29] protected mice against influenza virus infection. Furthermore, sublingual administration of rAd5 vectors encoding HIV proteins induced both significant antigen-specific humoral (serum and mucosal IgG and IgA) [30] and cellular (systemic and mucosal CTL responses) [31] immune responses. Moreover, sublingual vaccination with rAdV encoding a truncated S protein (rAdV-S), which is a major antigenic protein present on severe acute respiratory syndrome-associated coronavirus (SARS-CoV), induced systemic neutralizing antibodies and airway IgA antibody responses in mice. These immune responses were similar to those induced by intranasal administration. It is worth noting that intranasal delivery of rAdV redirected the virus vector to the olfactory bulb, whereas no adenoviral DNA was detected after sublingual delivery [32]. Choi *et al*. reported that both mice and guinea pigs were protected against a lethal Ebola challenge after a single sublingual immunization with an AdV-based vaccine expressing the Zaire glycoprotein in a manner similar to that of traditional intramuscular vaccination [33].

Recombinant bacterial vaccines

With the availability of genetic tools for heterologous gene expression, the concept of live vaccine vehicles has sparked renewed interest, especially for the mucosal routes [34]. Several studies have shown that engineered *Bacillus (B) subtilis* is able to generate systemic and mucosal antibodies against heterologous antigens. *B. subtilis* delivered sublingually and expressing tetanus toxin C-fragment, evoked protective immunity in both mice [35] and piglets [36]. Batista *et al*. reported the use of 'gut-colonizing' *B. subtilis* spores as a new mucosal vaccine delivery platform consisting of two antigen expression strategies. One is active during spore formation, which leads to the display of recombinant adhesins at the spore surface that facilitates adhesion to mucosal surfaces. In addition, the recombinant spores have been shown to germinate after oral delivery resulting in intracellular expression of the antigen. Mice immunized with three doses of *B. subtilis* spores via the sublingual route, developed higher specific serum IgG titers when compared with the mice orally immunized with a nine-fold higher dose of spores of the same strains. Although not proven in this study, the authors speculated that sublingual delivery of these spores will also result in intracellular antigen

expression once they are captured by intraepithelial antigen-presenting cells and germinate [37]. The better immune response to sublingual vaccination might be ascribed to the fact that sublingual delivery has a smaller distribution volume and a less aggressive environment than gastro-intestinal delivery.

Inactivated vaccines

In a study by Cho *et al*., different routes, i.e., intranasal, intravaginal, transdermal, sublingual and intramuscular, were compared in a mouse study using human papillomavirus 16 L1 (HPV16L1) protein vaccine. Among these routes, the intranasal and sublingual routes provided the highest HPV16L1-specific levels of vaginal sIgA and systemic IgG responses that were comparable to those elicited via the intramuscular route [45]. Sublingual vaccination against respiratory syncytial virus (RSV) with a purified G protein fragment (Gcf) without the addition of adjuvants induced strong serum IgG and mucosal IgA responses (similar to intranasal vaccination) in mice. Interestingly, these antibody responses could be elicited by Gcf without the need for an adjuvant. The study demonstrated that the chemotactic activity exhibited by Gcf was necessary to induce protective immunity. Therefore, the authors proposed that Gcf has a self-adjuvanting property [46].

Murugappan *et al*. investigated whether sublingual administration of whole β-propiolactone (BPL)-inactivated influenza virus can prime the immune system for a later intramuscular boost with a heterologous vaccine. Although sublingual priming did not induce any detectable immune responses, it strongly enhanced hemagglutination inhibition (HI) titers against both the homologous as well as the heterologous vaccine after the intramuscular booster. In addition, sublingual priming induced IgA responses in the lung and nose, while intramuscular priming showed higher IgA responses in the lung, but not in the nose [40]. In a study by Song *et al*., a sublingual booster was given, instead of an intramuscular booster, and immune responses were analyzed more extensively. Immunization with formalin-inactivated influenza virus via the sublingual mucosa induced protective immune responses, elevated mucosal sIgA antibody levels, and enhanced virus-specific CTL responses [26]. The different procedures used for virus inactivation affect the membrane fusion properties of the virus to a certain extent, resulting in a less optimal activation of CTLs. Inactivation with formalin severely compromises fusion activity of the virus, while BPL-inactivation reveals preservation of the fusion activity [60]. However, a sublingual booster vaccination with formalin-inactivated influenza induced significant elevated virus-specific CTL responses in mice. Unfortunately, the analysis of CTL activation is lacking in the study using BPL-inactivated influenza.

As with mucosal immunization in general, sublingual (and buccal) vaccination with non-replicative antigens does not induce sIgA and serum IgG responses without the addition of adjuvants. Strong immune potentiators or delivery systems are needed to break mucosal tolerance and facilitate uptake through the oral mucosae. For example, a mucosal adjuvant (cholera toxin, CT) was needed to induce immune responses upon sublingual immunization with a human immunodeficiency virus (HIV) subunit vaccine [48]. Çuburu *et al*. evaluated the sublingual route for vaccine delivery using ovalbumin (OVA) as a model antigen. Mice received three doses of 200 µg OVA and although systemic IgG responses were measurable, mucosal OVA-specific antibody responses were absent after sublingual immunization of OVA alone. Co-administration of a mucosal adjuvant (CT) generated high OVA-specific IgA responses in saliva and nasal wash. These responses were of the same magnitude as those induced by intranasal administration [58].

Recently, sublingual administration of a subunit influenza vaccine was evaluated in mice. After three doses, detectable, but rather low, antigen-specific systemic IgG and HI titers were found after sublingual immunization, whereas mucosal IgA antibodies were below the detection limit. The addition of the mucosal adjuvant LTK63 was needed to those obtained after conventional intramuscular immunization [41]. A *Salmonella* vaccine consisting of sonicated *Salmonella* proteins induced protection only after sublingual immunization in the presence of adjuvants (CpG DNA or CT). This was observed in both adult [51] and neonatal mice [50].

Although mucosal adjuvants improve immune responses upon sublingual immunization using non-replicating/inactivated antigens, in some studies systemic and/or mucosal immunity was obtained with sublingual delivery of non-adjuvanted inactivated (subunit) vaccines [45, 46, 58]. In general, high doses were used and even the size of the antigen seemed to be an issue for successful immunization since small proteins/antigens showed the induction of both systemic and mucosal immune responses upon sublingual administration.

Adjuvants used in buccal and sublingual vaccines

Immune potentiators

Bacterial enterotoxins - The most powerful and hence the best-studied mucosal adjuvants are the bacterial enterotoxins cholera toxin (CT) and the Escherichia coli heat-labile toxin (LT), which have structural and biological similarities.

The adjuvant effect of sublingually administered CT has been documented with a number of antigens, including influenza [43] and HIV [48] subunit vaccines, Salmonella proteins [50, 51] and *Helicobacter pylori* lysates [54, 55] with the latter two as undefined vaccines. LT, when co-administered sublingually with tetanus toxoid (TT) induced higher specific IgG and mucosal IgA antibody titers when compared to TT alone [57]. However, CT and LT cause severe diarrhea in humans and are involved in the clinical occurrences of cholera and enterotoxigenic E. coli enteritis, and therefore not suitable as an oral or sublingual adjuvant for human use [61-63]. Since enterotoxicity is mainly caused by the enzymatically active A-subunit, mutated enterotoxins with reduced toxicity but retained adjuvant properties, have been developed. LT(R192G), also named mLT, showed reduced toxicity in mice, but maintained its adjuvanticity to a level nearly equivalent to that of LT [64, 65]. Building on this mutant, a double mutant of LT, R192G/L211A or dmLT, showed adjuvanticity for a co-administered antigen equivalent to mLT upon oral administration (gastro-intestinal delivery) [66]. Recently, dmLT has been evaluated as an adjuvant for sublingual and buccal vaccination with a whole-cell pneumococcal vaccine that induces protection in mice [52]. In the search for an alternative adjuvant for a *Helicobacter pylori* lysate vaccine, dmLT was compared to CT. Earlier studies have shown that a strong mucosal adjuvant like CT was needed to induce protective immune responses against *Helicobacter pylori* infection. Sublingual immunization with *Helicobacter pylori* lysate and dmLT significantly decreased the bacterial burden after *Helicobacter pylori* infection compared to unimmunized mice and to the same extent as when using CT as adjuvant [55]. Moreover, cellular immune responses that are known to correlate with protection were also fully comparable when using dmLT and CT as adjuvants.

In a study by Cho *et al*., HPV16L1 protein provided both vaginal and salivary sIgA, and serum IgG responses after sublingual administration in mice (150 µg) [45]. Several adjuvants were tested, including the B subunit of cholera toxin (CTB), three toll-like receptor agonists (i.e., Poly(I:C), MPL, imiquimod), three nucleotide-binding oligomerization-domain agonists (L18-MDP, murabutide, PGN), vitamin D3 and poly-gamma-glutamic acid. Among the adjuvants tested, only CTB provided improved mucosal sIgA and systemic IgG induction. Sublingually applied CTB also enhanced the production of IL-4 and IFN-y by stimulated CD4+ T cells from the spleen, as well as the number of IFN-y producing CD8+ T cells that were isolated from the spleen or submandibular lymph node (SMLN). The other adjuvants had no effect on the immune response when compared to the unadjuvanted control [45].

Toll-like receptor (TLR) ligands – Despite the negative results mentioned above, TLR agonists can significantly improve immune responses after sublingual vaccination. A mouse study evaluating TLR agonists (i.e., FSL-1, Poly(I:C), monophosphoryl lipid A (MPLA), Pam3CSK4, R848, cytosine-phosphate-guanosine (CpG)) in different mucosal routes using HIV gp140 and a tetanus toxoid revealed clear differences in immunogenicity [49]. MPLA, a TLR-4 agonist, suppressed systemic responses when administered sublingually, while the responses were enhanced after intranasal or subcutaneous immunisation. CpG, a TLR-9 ligand, evoked enhanced immune responses upon sublingual and intranasal immunization whereas it did not affect the responses after subcutaneous immunization [49]. Another study revealed that sublingual immunization with an Ad5 vector expressing a TLR agonist derived from Eimeriatenella significantly activated NK cells, natural killer T (NKT) cells, B cells, and CD4+ T cells in the spleen. In addition, the number of cells expressing MHC-II increased [30].

Bacterial DNA or synthetic oligodeoxynucleotides (ODNs) containing CpG motifs act as mucosal adjuvants. These TLR 9 ligands induced strong Th1 responses in mice after sublingual delivery of a Salmonella vaccine [67].

Delivery systems

Virus-like particles (VLPs) and virosomes have been evaluated for sublingual delivery. Cho *et al*. showed that sublingual delivery of HPV16L1 VLPs in mice induced systemic IgG and mucosal sIgA responses that were similar to the intranasal route, but significantly higher compared to other delivery (intravaginal, transdermal, intramuscular) routes [45]. In contrast to this study in mice, a clinical trial by Huo *et al*. with Gardasil® (Sanofi Pasteur), which contains the same L1-based VLPs from HPV but is co-administered with aluminumhydroxyphosphate as an adjuvant, showed that sublingual immunization is much less effective than intramuscular immunization [68]. However, this alum adjuvanted VLP formulation is not suitable for sublingual HPV vaccination, which is likely due to the fact that alum adjuvants consist of relatively large (micrometer range) particles that are probably poorly taken up by mucosal epithelial cells. Other adjuvants as well as improved dosage forms (see section 4) may help to increase the immune response to the VLPs.

A sublingual vaccine containing influenza H5N1 virosomes (2 µg HA) in combination with the mucosal adjuvant (3',5')-cyclic diguanylic acid (c-di-GMP) effectively induced local and systemic H5N1-specific humoral and cellular immune responses in mice. The systemic IgG and nasal sIgA antibody levels were lower than those induced by intranasal administration,

but the IgG levels were comparable to those obtained after intramuscular administration, whereas nasal sIgA levels were higher than the levels upon intramuscular administration [42].

DOSAGE FORMS

Optimized dosage forms may improve the performance of sublingual and buccal vaccines substantially. Several dosage forms exist for sublingual and buccal delivery of marketed drugs. These range from droplets, sprays, and orally disintegrating tablets to oral films. However, only a few dosage forms have been used to explore sublingual or buccal delivery of vaccines. Almost all sublingual vaccination studies reported here have been performed by the simple application of droplets of a vaccine under the tongue. There are no studies on the role of potentially crucial variables, like contact time, vaccine viscosity and antigen release kinetics on immunogenicity.

In preclinical studies, vaccine droplets are applied under the tongue on the floor of the mouth and the animal is kept under sedation for less than one hour to allow the vaccine to be taken up (Table 2). For sublingual vaccination of mice 5 µL can be applied sublingually without transfer of the vaccine to the stomach (including a sedation time of 30 minutes) [48, 57].

Sublingual administration of allergens to humans by droplets has been used for years in Europe in sublingual immune therapy (SLIT) against allergies. Typically, in SLIT, droplets of a highly viscous glycerol formulation containing the allergen extract are applied under the tongue. The high viscosity contributes to antigen retention under the tongue and as such facilitates the efficacy of the immune therapy.

In a recent clinical trial, HPV vaccine was applied sublingually to humans. Compared to SLIT with glycerol droplets, a relatively complicated administration protocol was used in this study. In brief, subjects rinsed their mouths with water, the sublingual area was dried and then 0.5 mL of vaccine was applied on the floor of the mouth. Adsorbent pads were applied in the mouth to absorb the saliva during and after the vaccine application. Despite the elaborate administration protocol, in only three of the twelve subjects were virus-neutralizing antibodies induced in serum after three standard doses of HPV vaccine via the sublingual route. These neutralizing antibody titers were still 1000-fold lower than in the intramuscular group. Researchers concluded that alternative delivery systems and adjuvants would be required to enhance and evaluate immune responses following sublingual immunization in humans [68].

The disappointing results of this clinical trial may be related to several factors. First, a significant amount of solution (0.5 mL versus 'a couple of droplets' in SLIT therapy) was applied. Despite the extensive administration protocol, a certain high volume will easily be digested and will follow the gastro-intestinal route. Secondly, the vaccine used in the study (Gardasil®) is a VLP based vaccine containing aluminium hydroxyphosphate sulphate as adjuvant. An alum-adsorbed vaccine has a large particle size (in the range of 1 μm) and thus is not ideal for transfer over membranes. Finally, alum is not known to be a good adjuvant for mucosal vaccination.

Droplets for improved (extended) mucosal retention are currently under development by PATH [69]. Their technology is based on a thermoresponsive gel of undisclosed composition. The vaccine is presented as a liquid solution at room temperature, which enables sublingual delivery with an oral dropper, and transforms into a gel upon contact with the oral mucosa. Typical thermo-responsive gel formulations that gelate at a temperature above 30°C are based on polymers, such as poloxamer and mucoadhesive polysaccharides. The gel matrix enables adherence (retention > 20 min.) to the sublingual mucosa thereby preventing rapid clearance caused by salivation or swallowing and protecting the vaccine antigen from degradation caused by salivary enzymes. Vaccinating mice with a gel formulation containing tetanus toxoid elicits high levels of IgG and IgA in serum as well as in secretions of the mouth, gastrointestinal and reproductive tract. Currently, PATH is evaluating (preclinically) whether the addition of a mucosal adjuvant, dmLT, can contribute to the efficacy of sublingual vaccination using thermo-responsive gel formulations [69].

Thermoresponsive gels - Formulations that are liquid at room temperature (left vial) and turn into a solid gel at body temperature (right vial).

Sprays

Sublingual sprays have been used for years for the sublingual administration of nitroglycerine to patients in order to counter an acute angina pectoris. The most used nitroglycerine sprays are based on a formulation containing the drug substance, ethanol, a small amount of mint oil and a propellant, like tetrafluoroethane, to aerosolize of the formulation. We are not aware of any investigations into direct spraying of the vaccine onto the sublingual or buccal mucosa.

A few studies, however, have applied vaccine sprays into the whole mouth. Immunization of humans with influenza whole inactivated virus (H1N1) without additives, such as an oral spray using a simple nasal spraying device (no use of propellant), did not result in IgA antibody responses in nasal secretions and only resulted in marginally increased IgA antibodies in saliva. Although no stimulation of cytotoxic T cells was shown, an increase in systemic influenza-specific antibodies was found [70].

To what extent the immune response elicited by sublingual vaccination is the result of direct immune activation of the tonsils remains a matter of debate. For example, it has been shown that direct immunization via the tonsils can give rise to adequate immune responses. Oral spray immunization with replication-deficient viral vector vaccines encoding simian immunodeficiency virus (SIV) sprayed directly on the tonsils of rhesus macaques induced cellular and humoral immune responses. Additionally, after SIV challenge viral RNA levels were equally reduced after systemic vaccination and vaccination through the use of the oral mucosal spray [139].

Controlled release formulations

Several oral formulations are already licensed or are in development for drug delivery in the mouth. Based on their drug release kinetics and manufacturing method they can be categorized as: (i) orally disintegrating preparations (tablets and fast-dissolving films) and (ii) extended (slow) release tablets and films. Although their value for sublingual and buccal delivery of antigens has not yet been extensively evaluated, they will be described briefly in the next section since they may play an important role in the development of potent and thermostable sublingual vaccines.

Orally disintegrating preparations

Orally disintegrating tablets for sublingual or buccal delivery are in general relatively small

and porous. Tablets for buccal delivery facilitate fast disintegration and drug or vaccine release without inclusion of a disintegrant. In general, these tablets dissolve within a few seconds after being placed in the mouth without water, making them dried (stable) alternatives for droplets.

Recently, several excipients were studied for their wafer formation abilities (highly porous tablets made by direct compression) with the intention to formulate wafers containing HPV vaccine for sublingual vaccination [71]. A typical fast-disintegrating formulation for the generation of wafers with a diameter of 4 mm that showed a dissolution time of less than 25 seconds (in 3 ml at 37°C) consisted of approximately 65% (w/w) myo-inositol (the placebo HPV powder) and 35% microcrystalline cellulose (MCC) [71].

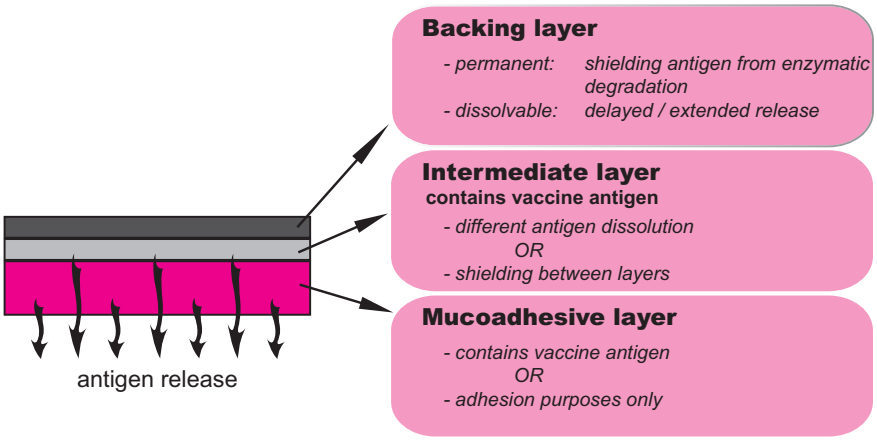
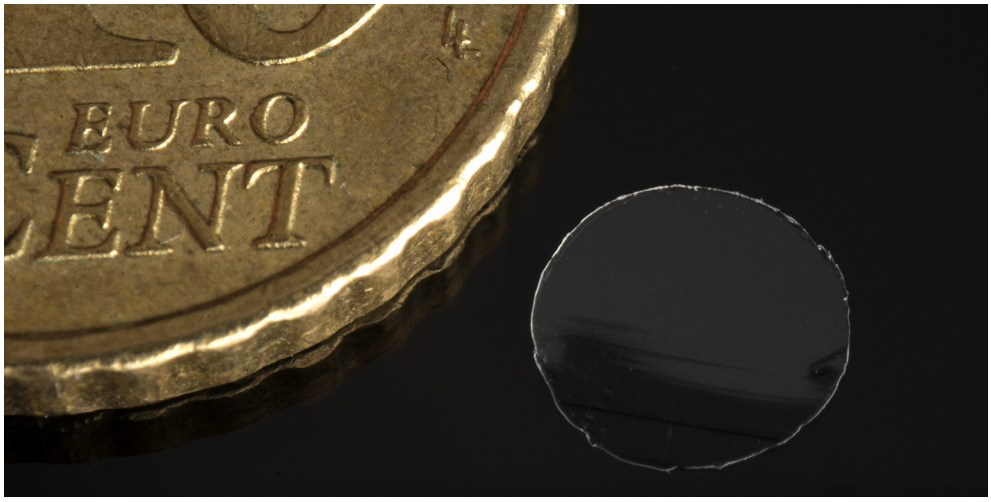


Figure 3 Multi-layered films or tablets. The layers of multi-layered dosage forms have different functions.

Extended release films and tablets

To effectively vaccinate via the sublingual and buccal mucosae, the contact time of the antigen with the mucosa is expected to be critical, although this remains to be demonstrated. Sustained release dosage forms adhere to the mucosa and direct transport of the antigen to the mucosa may improve the efficacy of sublingual and buccal vaccination. In this regard, extended release films or tablets that consist of multiple layers with different functions (Figure 3) may be applied. The sublingual mucosa is exposed to a high saliva flow, which might be advantageous for a better swelling and dissolution of oral tablets. On the other hand, it might

also cause loss of the antigen and adjuvant due to salivary washout. Therefore, the much lower saliva flow of buccal mucosa makes this route probably a better target for a sustained release system.



Oral mucosal films - Polymer-based oral dissolving films for buccal or sublingual vaccine delivery. The size of the film depicted here is suitable for *in vivo* testing in small animals.

Extended release tablets are typically based on gelling hydrophilic polymers, so-called hydrophilic matrix tablets. The polymers form a gel layer around the tablet when they make contact with water. The release profile is controlled by the overall swelling and erosion [72] [72]. Most polymers that are used as mucoadhesives are hydrophilic polymers that gelate upon contact with saliva, allowing adherence to the buccal mucosa by interfacial forces, such as hydrogen bonding, electrostatic interaction as well as van der Waal's bonds.

Borde *et al*. [73] prepared extended release tablets for the model antigen ovalbumin, based on two-layer tablets consisting of a mucoadhesive layer, which is composed of carbopol, and a controlled release layer. Since the tablets were not able to adhere to the floor of the mouth in mice, the tablets were applied upside down to the ventral side of the tongue, resulting in the release of the antigen towards the sublingual region. Immunization studies were performed with tablets containing 250 µg ovalbumin directly followed by sublingual administration of a 1 µg/mL CT solution (7.5 µL) as adjuvant. Immunization with a fast-releasing tablet was favorable over extended release formulations and comparable to the liquid reference (ovalbumin with CT) for the immune response. However, the production of saliva was induced by administering pilocarpine subcutaneously before administering the tablet to provide better swelling and adhesion of the tablets [73]. The authors concluded

that extended release formulations for sublingual vaccination have to be investigated more in detail. Extended release formulations for mice have to be optimized to direct the antigen toward the ventral sublingual mucosa. This will improve the antigen diffusion and prevent the salivary washout and the impact of enzymes by shielding.

Extended release films based on multiple layers are generally made by the solvent casting method [74] for which the antigen is added to a coating mass (e.g., cellulose, polysaccharides) and subsequently cast onto a film application apparatus. After drying and solvent evaporation, the films can be cut into single doses.

The viscosity and density of the coating mass are critical for successful film casting (layer-by-layer coating). Moreover, the vaccine antigen needs to be formulated in the coating mass with proper excipients to guarantee resistance to the applied drying stresses [75]. Formulations that have been proven to protect vaccines during the fabrication of coated solid dosage forms have been described in literature but are not yet used to produce vaccine-containing films. For example, the group of Prausnitz applied formulations based on trehalose, surfactant (poloxamer) and a polymer (PVP) to coat microneedles with vaccines such as the whole inactivated influenza virus [76] and measles [77] for dermal vaccination. An additional advantage of dried formulations is the improved thermal stability during storage.

PERSPECTIVES

The oral mucosa, in particular the sublingual and buccal regions are attractive sites for the delivery of antigens, since their accessibility, non-invasive and (immunological) advantages over other (mucosal) routes (section 2.1). Therefore, sublingual vaccine delivery has gained significant attention during the past few years, as shown by the numerous preclinical studies published in the last decade (Table 2).

The preclinical proof of concept of the sublingual route for vaccine delivery has been proven for several antigens. In general, high doses of antigen (10-500 µg) are administered in mice using a multiple dosing regimen (Table 2). However, well-designed studies including proper dose response and tracking (PK/PD) studies are still lacking. Nevertheless, the use of strong mucosal adjuvants seems to be necessary to induce protective immune responses upon sublingual vaccination using inactivated vaccines. Reported studies on screening and comparison of adjuvants for sublingual vaccination, unfortunately, are still limited. Of the adjuvants investigated thus far (CT, CTB, LT, LTB, mLT, dmLT, CpG, c-di-GMP, L18-MDP, FSL-

1, Poly(I:C), MPLA, Pam3CSK4, R848, murabutide, peptidoglycans, vitamin D3, chitosan and poly-gamma-glutamic acid), enterotoxins (and their mutants) and TLR-9 agonists resulted in the highest antibody responses whereas the other adjuvants did not improve immune responses upon sublingual delivery. The most used adjuvants for sublingual vaccine delivery are the enterotoxins CT and LT, which are considered too toxic for human use. However, genetically defined mutants of these toxins, which have reduced or minimal toxicity, seemed to be promising adjuvants to augment both systemic and mucosal immunity in response to sublingual administration.

Recombinant live carriers expressing the vaccine antigen seemed to elicit protective immunity by inducing both antigen-specific systemic as well as mucosal antibodies at distinct mucosal sites [28, 32]. The exact advantage of such live vaccines over inactivated vaccines is still not clear, but it could be related to factors such as receptor-mediated uptake, intracellular replication of antigens and co-delivery of immune potentiators and antigens to APCs.

An approach that resembles the characteristics of the live pathogens, e.g., by co-delivery of immune potentiator and antigen, is needed to develop an effective inactivated (subunit) vaccine for the sublingual route. Unfortunately, to date, limited studies have focused on such approaches that use tailored formulations, such as nanoparticles or conjugated antigen-adjuvant formulations. In the design of these formulations, mucoadhesive or receptor-binding properties may be built in to increase interaction with the oral mucosa and thereby facilitate antigen uptake.

In addition to delivery systems, dosage forms may improve the efficacy of sublingual and buccal vaccines. Today, research on dosage form optimization for sublingual or buccal vaccination appears marginal. The few reported studies use mouse models to evaluate sublingual vaccination *in vivo*. Nevertheless, the small space under the tongue of animals used for preclinical studies makes testing of sublingual solid dosage forms challenging. For example, Muragappan *et al*. faced the problem that sublingual tablets were not able to dissolve under the tongue of mice [40]. Moreover, two-layered extended release tablets failed to adhere to the floor of the mouth in mice, resulting in a suboptimal administration that applied the tablet upside down on the ventral side of the tongue [73]. Potential problems related to the sublingual application of slow release formulations are due to the lack of an expanse of smooth or (relatively) immobile mucosa, which make it difficult to keep the dosage form in contact with the sublingual mucosa. Other dosage forms, such as wafers, films and thermo-

responsive gels, should be explored as these may likely increase the potential of sublingual vaccine delivery. Sustained dosage forms (e.g., multi-layered oral films or tablets) are probably better suited for application on the buccal mucosa, which is considerably less permeable than the sublingual mucosa. The few current studies on buccal vaccine delivery use buccal injections instead of a topical application. However, the development of the extended release formulations would make topical buccal cheek vaccination easier. Compared to the sublingual route, buccal administration in small animals, such as mice, is more complicated since the buccal compartment has a minimal anatomical barrier with the sublingual compartment. Besides the animal size, the anatomical features of the oral cavity should be taken into account, because of the specialized histological characteristics and immunological competences of the oral mucosae in humans. However, most rodents, such as mice, rats and hamsters, have buccal mucosa that contains keratinized epithelium, in contrast to non-keratinized epithelium of sublingual and cheek mucosae in human. For buccal cheek administration, other animal models such as rabbits, dogs and pigs are more appropriate since they contain non-keratinized buccal mucosae. Moreover, the thickness of the buccal mucosa in these animal models (rabbit, 600 µm; dog and pig, 770 µm) is comparable to that of humans (500-800 µm) [78]. Current literature does not address to which extent mucoadhesive formulations increase the delivery of the antigen to oral mucosal APCs after sublingual or buccal administration, requiring proper tracking (pharmacokinetics, PK) studies in relation with immunological outcome (pharmacodynamics, PD) in adequate animal models (Table 3).

To select suitable formulations and dosage forms, an *in vitro* model for the mucosa that can predict the transport of an antigen might be useful. Porcine buccal mucosa is often chosen for *in vitro* studies on buccal delivery of medicines because of its close resemblance to human buccal mucosa with respect to structure, enzyme activity as well as permeability characteristics [79]. However, mechanistic studies evaluating the conditions for sublingual or buccal antigen delivery, such as optimal contact time of the dosage form and the differences between the oral mucosal routes, are lacking in literature. Moreover, the importance of specific molecular features of the antigen and/or antigen formulation, such as size (e.g., 1-10 nm proteins versus 10-250 nm viruses versus 250 nm-2 µm bacteria), surface charge or specific receptor ligands, is still not investigated systematically.

Most preclinical studies (*in vivo* animal models) are used to evaluate the vaccine candidate's efficiency to induce protective mucosal (and systemic) immunity. Secretory IgA (sIgA) provides antigen-specific immune protection in mucosal tissues. As a result, most preclinical studies on sublingual vaccination include the detection of sIgA in mucosal secretions. Unfortunately,

Table 3 Current challenges in the development of sublingual and buccal vaccines.

Challenges	Research should focus on:
Get more insight into pharmacokinetics and pharmacodynamics (PK/PD)	- Dose response studies Dose-sparing possibilities Multiple dose regime needed? Potent and safe adjuvants - *In vivo* imaging (tracking) studies
Get more insight into immunological mechanisms	- Role of oral DCs upon buccal/sublingual immunization - Antigen uptake and transport - Use of proper animal models - Readout for mucosal immunity
Development optimized vaccine formulations and proper dosage forms	- Tailored vaccine delivery: oral DCs as vaccine target - Stable vaccine formulations - Optimal contact time (mucoadhesive) - Optimal antigen release time - Use of proper animal models
Development of predictive assays for mucosal immunity	- Optimal sampling for mucosal readout - Validated assays for (pre-)clinical studies

most studies are designed for an optimal readout of systemic immune responses, making assumptions on mucosal immunity not fully conclusive. Moreover, in general, the presence of sIgA is determined in saliva, which is not the best readout for mucosal immunity, especially for rodents [80]. A more predictive method to measure mucosal immunity in mucosal tissues of rodents is the 'Perfext method', which is a direct method for quantitative assessment of *in vivo* antibody or cytokine production at the local level [81]. The method uses the collection of heparinized mucosal tissue of the animal followed by detergent treatment and the detection of antigen-specific antibodies by ELISA [53, 55].

Based on several preclinical studies, it would be highly interesting to follow the expected upcoming clinical studies on sublingual vaccine delivery (using live attenuated vaccines). These studies should build on the experience of previous preclinical studies that were designed to evaluate mucosal immunity as a correlate of protection, which would require standardized and validated assays predictive of mucosal immune protection [82]. For example, a method described by Saletti *et al*. allows measurements of both systemic and mucosal antibody responses to vaccines by detecting antigen-specific plasmablasts with a specific mucosal pedigree (e.g., α4β7, CCR10) on small amounts of whole blood [83]. Although it was developed for clinical samples, a certain type of mucosal readout would also be useful for preclinical studies, amid limited blood volumes that can be sequentially withdrawn from small animals. The full benefits of sublingual vaccination can only be revealed by the determination both local and systemic responses.

Current literature on sublingual and buccal vaccination with a broad range of antigens provides a strong base for further testing of these non-invasive vaccine delivery routes. This testing should include mechanistic studies on the superiority of live vaccines over inactivated antigens, including dose-response studies (Table 3). Based on the findings, tailored vaccine formulations for inactivated antigens may be designed in the near future. Development of these sophisticated formulations and optimized dosage forms that facilitate antigen uptake by the oral mucosa will be an important step forward towards successful sublingual and/or buccal vaccination. Finally, upcoming clinical studies that confirm the suggested safety and efficiency (by proper readout of both mucosal and systemic immunity) may result in the first approved sublingual vaccination strategy.

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Intranasal and sublingual delivery of inactivated polio vaccine

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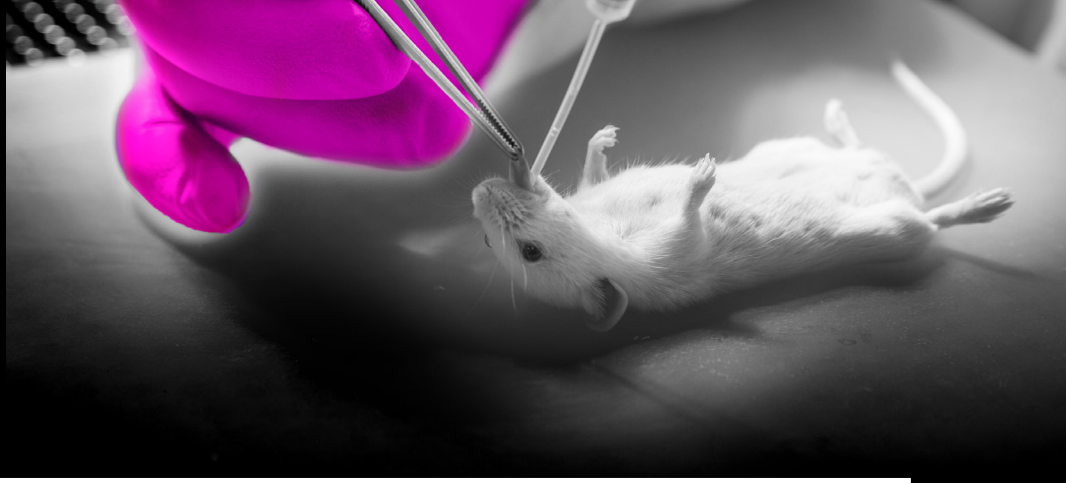

ABSTRACT

Polio is on the brink of eradication. Improved inactivated polio vaccines (IPV) are needed towards complete eradication and for the use in the period thereafter. Vaccination via mucosal surfaces has important potential advantages over intramuscular injection using conventional needle and syringe, the currently used delivery method for IPV. One of them is the ability to induce both serum and mucosal immune responses: the latter may provide protection at the port of virus entry.

The current study evaluated the possibilities of polio vaccination via mucosal surfaces using IPV based on attenuated Sabin strains (sIPV). Mice received three immunizations with trivalent sIPV via intramuscular injection, or via the intranasal or sublingual route. The need of an adjuvant for the mucosal routes was investigated as well, by testing sIPV in combination with the mucosal adjuvant cholera toxin.

Both intranasal and sublingual sIPV immunization induced systemic polio-specific serum IgG in mice that were functional as measured by poliovirus neutralization. Intranasal administration of sIPV plus adjuvant induced significant higher systemic poliovirus type 3 neutralizing antibody titers than sIPV delivered via the intramuscular route. Moreover, mucosal sIPV delivery elicited polio-specific IgA titers at different mucosal sites (IgA in saliva, fecal extracts and intestinal tissue) and IgA-producing B-cells in the spleen, where conventional intramuscular vaccination was unable to do so. However, it is likely that a mucosal adjuvant is required for sublingual vaccination. Further research on polio vaccination via sublingual mucosal route should include the search for safe and effective adjuvants, and the development of novel oral dosage forms that improve antigen uptake by oral mucosa, thereby increasing vaccine immunogenicity. This study indicates that both the intranasal and sublingual routes might be valuable approaches for use in routine vaccination or outbreak control in the period after complete OPV cessation and post-polio eradication.

INTRODUCTION

During the past decades, the global incidence of paralytic poliomyelitis has decreased by more than 99% since 1988. Type 2 wild poliovirus was eradicated in 1999 and the last reported case of type 3 wild poliovirus was from 2012. Since 2015, cases of type 1 wild poliovirus were only detected in the remaining endemic countries (i.e., Pakistan, Afghanistan and Nigeria) [1]. It is expected that wild poliovirus will be eradicated within a few years. However, to accomplish a polio-free world, eradication efforts should focus on both wild polioviruses as well as vaccine-derived viruses. Therefore, the endgame strategy of the Global Polio Eradication Initiative (GPEI) includes a phased withdrawal of the live-attenuated oral polio vaccine (OPV), the source of vaccine-derived viruses, and the worldwide inclusion of the inactivated polio vaccine (IPV) into all routine immunization programs [2]. The GPEI is pursuing several priority approaches for the development of a new generation of IPV [3]. To this extent, Intravacc has developed a new polio vaccine based on Sabin polio viruses, Sabin IPV (sIPV), that is being transferred to local vaccine manufacturers to support post-eradication goals in terms of biosafety and IPV availability [4-7].

A new generation of sIPV should not only be affordable and safe to produce, but preferably should also induce mucosal immunity, remain stable, and be easy to administer. This is important with regard to stockpiling and outbreak management in the period after cessation of OPV and after eradication. Several alternative polio vaccine delivery strategies are in development, with a focus on dermal delivery of polio vaccines [8]. Vaccination via mucosal sites has the benefits of needle free vaccine delivery . Moreover, mucosal immunization is able to elicit strong mucosal immunity, even at distant effector sites. As we know from OPV, polio-specific mucosal immunity in the gut is a powerful mechanism for protection and interruption of polio transmission [9].

The current study evaluated the potential of different mucosal routes, i.e., intranasal and sublingual, in mice. It was investigated whether intranasal or sublingual vaccination with sIPV is able to elicit functional systemic immunity (serum) as well as local immune responses at different mucosal sites.

MATERIALS AND METHODS

Vaccine

Monovalent Sabin IPV bulk material used in this study was produced as described previously [10]. For the preparation of trivalent sIPV, monovalent type 1, type 2 and type 3 were mixed and diluted in M199 medium (Bilthoven Biologicals, The Netherlands) to a nominal concentration of 1000-1600-3200 D-antigen units (DU) per mL for type 1, type 2 and type 3, respectively. Cholera toxin from Vibrio Cholerae was purchased from Sigma-Aldrich (St. Louis, MO).

Immunization study

The animal experiment was performed according to the guidelines provided by the Dutch Animal Protection Act, and was approved by the Committee of Animal Experimentation (DEC) of the National Institute of Public Health and Environment (RIVM). Balb/cOlaHsd mice (8-10 weeks old from Envigo, The Netherlands) were anesthetized with ketamine-xylazine, and received a single human dose (based on previous clinical studies [6, 11]) trivalent sIPV (10-16-32 DU/dose) via the intramuscular (IM, injection of 50 µL in hind limb), intranasal (IN, pipetting 10 µL in the nose) or sublingual (SL, pipetting 10 µL under the tongue) route at day 0, 7 and 28. Adjuvanted groups received 5 µg/dose cholera toxin. Upon SL immunization, mice were maintained in upright position to minimize the risk of swallowing. Blood samples were taken at day 0 (prior to immunization) and day 14 (after second immunization). At day 35, anesthetized animals received an intraperitoneal injection of 0.1 mL of 0.05 M pilocarpine (Sigma-Aldrich, St. Louis, MO) in PBS to induce saliva production. Saliva was collected and, subsequently, animals were sacrificed by bleeding. Post-mortem, fecal samples were isolated from the large intestine, weighted and stored at -80ºC until analysis. Spleens were placed in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 5% fetal bovine serum and placed on ice for the B-cell ELISPOT. Small intestines were harvested and placed in 3 mL PBS containing 50 mM EDTA (Gibco, Invitrogen) and protease inhibitors (Complete, Mini, EDTA free, Roche Applied Sciences). Small intestines were extensively vortexed and centrifuged for 15 min at 300 g (4ºC). Supernatants, mentioned further as intestinal wash, were collected and stored at -80ºC until analysis (IgA ELISA). Subsequently, small intestines were cut into small pieces, transferred to cryotubes, and 2 µL PBS containing 2% saponin (Sigma Aldrich, St. Louis, MO) and protease inhibitors was added per mg intestinal sample. After a fast freezing step, samples were centrifuged for 20 min at 4600 rpm and supernatants

were filtered through 0.22 µm filters (Merck Millipore, Darmstadt, Germany). Intestinal tissue samples were stored at -80ºC until further analysis. The presence of both excreted (intestinal washes) and intracellular (intestinal tissue samples) polio-specific IgA in small intestine was assessed by ELISA.

IgG and IgA ELISA

Enzyme linked immunosorbent assays (ELISA) were performed to determine polio-specific antibody titers in sera, saliva, feces, intestinal washes and intestinal tissue samples. Fecal extracts were prepared by adding fecal extract buffer, PBS containing 10% normal goat serum (Sigma Aldrich, St. Louis, MO) and protease inhibitors, to the fecal pellets (0.2 g/mL). Fecal extracts were extensively vortexed and, subsequently, centrifuged for 15 min at 13000 g. Supernatants were filtered through 0.22 µm filters and immediately tested. For the ELISA, polystyrene 96 wells microtiter plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated overnight at 4°C with bovine anti-poliovirus serum (Bilthoven Biologicals, Bilthoven, The Netherlands) in PBS (Gibco from Invitrogen, Paisley, UK). After washing coated plates with 0.05% (v/v) Tween 80 (Merck, Darmstadt, Germany) in tap water, trivalent inactivated polio vaccine diluted in assay buffer, PBS containing 0.5% (w/v) Protifar (Nutricia, Zoetermeer, The Netherlands) and 0.05% (v/v) Tween 80 (Merck, Darmstadt, Germany), was added and incubated for 2h at 37°C. Subsequently, plates were washed and threefold sample dilutions in assay buffer were added and incubated for another 2h at 37°C. After washing, plates were incubated with horse-radish peroxidase (HRP)-conjugated goat-anti-mouse IgG or HRP-conjugated goat-anti-mouse IgA (Southern Biotech, Birmingham, AL). After 1h incubation at 37°C, plates were washed and TMB substrate solution, containing 1.1 M sodium acetate (Bilthoven Biologicals, Bilthoven, The Netherlands), 100 mg/mL 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO), and 0.006% (v/v) hydrogen peroxide (Merck, Darmstadt, Germany), was added to each well. After 10-15 minutes, the reaction was stopped with 2 M sulfuric acid (Bilthoven Biologicals, Bilthoven, The Netherlands) and absorbance was measured at 450 nm by using a Biotek L808 plate reader. For the CT-specific ELISA, plates were coated with 1 µg/mL CT and blocked with 1% Protifar in PBS. ELISA was further performed as described above. Endpoint titers were determined by 4-parameter analysis using the Gen5™ 2.0 Data Analysis software (BioTek Instruments, Inc., Winooski, VT) and defined as the reciprocal of the serum dilution producing a signal identical to that of negative control samples at the same dilution plus three times the standard deviation.

Virus neutralization (VN) assay

Neutralizing antibodies against all three poliovirus types were measured separately by inoculating Vero cells with 100 TCID50 of the wild-type strains (Mahoney, MEF-1 and Saukett) as described previously [12, 13]. Twofold serial serum dilutions were made and serum/virus mixtures were incubated for three hours at 36°C and 5% CO2 followed by overnight incubation at 5°C. Subsequently, Vero cells were added and after 7 days of incubation at 36°C and 5% CO2, the plates were stained and fixed with crystal violet and results were read macroscopically. Virus neutralizing (VN) titers were expressed as the last serum dilution that has an intact monolayer (no signs of cytopathogenic effect).

B-cell ELISPOT

MultiScreen-HTS IP 96 wells filter plates (Merck Millipore, Darmstadt, Germany) were wet by adding 35% ethanol, immediately washed twice with PBS and, subsequently, coated overnight with 5 µg/mL monovalent IPV type 1, 2 or 3. As a positive control, wells were coated with a mixture of 7 µg/mL purified goat-anti-mouse kappa and 7 µg/mL purified goat-anti-mouse lambda (Southern Biotech). As a negative control, wells were left uncoated (PBS). After washing with PBS, plates were blocked with RPMI-1640 medium (Gibco, Invitrogen) with 2% Protifar (Nutricia, Zoetermeer, The Netherlands) for 1 hour at room temperature. Spleens were homogenized using a 70-µm cell strainer (BD Falcon, BD Biosciences) and cells were collected in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics (Penicillin-Streptomycin-L-Glutamine, 100x (Gibco, Invitrogen)). Erythrocytes were removed by ACK lysis buffer (Gibco, Invitrogen). After washing, cells were counted and 5x105 cells/well were added to coated plates. After overnight incubation at 37°C and 5% CO2 plates were washed extensively and wells were developed by stepwise incubations with AP-conjugated goat-anti-mouse IgA (Southern Biotech) and washing with PBS followed by the addition of BCIP-NBT liquid substrate (Sigma Aldrich, St. Louis, MO). Plates were kept in dark during spot development and thereafter, the reaction was stopped by discarding the substrate and extensively washing of both sides of the filter with tap water. Plates were dried overnight at 37°C and spots were counted using EliSpot reader (AID iSpot FluoroSpot Reader System, Autoimmun Diagnostika GmbH, Strassberg, Germany) and AID EliSpot software.

Statistical analysis

Data was statistically analyzed by comparing all groups by a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Probability (p) values of $p < 0.05$ were considered

statistically significant. Statistics were performed using GraphPad Prism version 6.07 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Systemic immunity elicited after mucosal sIPV administration

To assess whether systemic immunity was induced after vaccination of mice with sIPV via conventional intramuscular (IM) injection, via the nose (intranasal, IN), or under the tongue (sublingual, SL), polio-specific IgG antibodies were measured in serum. For both mucosal routes (SL and IN), the vaccine was also given in the presence of cholera toxin (CT), which is known as a strong mucosal adjuvant [14-16]. Prior to immunization, no polio-specific immune responses were detected (data not shown). After two immunizations (day 14), evident polio-specific IgG antibody titers were already induced in the group of mice vaccinated with sIPV via the conventional intramuscular route using needle and syringe (**Figure 1A**). Intranasal and sublingual administered sIPV without adjuvant induced significantly lower anti-polio type 1 IgG titers than the intramuscular control group (respectively, $p<0.05$ and $p<0.001$) (**Figure 1A and 1B**). However, the inclusion of CT as adjuvant significantly improved systemic IgG responses after intranasal sIPV vaccination. Animals that received sIPV plus CT via the intranasal route induced polio-specific IgG antibody titers similar to those obtained after intramuscular vaccination, both at day 14 (2 immunizations) (**Figure 1A**) and day 35 (3 immunizations) (**Figure 1B**). For the sublingual route, higher numbers of responders were observed after immunization with sIPV plus CT with a significant enhanced IgG antibody titers against polio type 3 induced after 2 immunizations (day 14) (**Figure 1A**). For the induction of detectable systemic IgG after sublingual sIPV delivery, an adjuvant (**Figure 1A**) and/or at least 3 vaccinations were needed (**Figure 1B**).

To investigate the functionality, the virus-neutralizing capacity of the sera was determined after three immunizations. All mice from the intramuscular control group showed distinct neutralizing antibody titers against all three poliovirus types (**Figure 2**). The presence of CT adjuvant was beneficial for the induction of virus-neutralizing antibodies after sIPV vaccination via the sublingual or intranasal route. For all serotypes higher numbers of animals responded after mucosal sIPV immunization plus CT (**Figure 2**). Besides, significantly higher virus-neutralizing (VN) titers were induced after intranasal vaccination of sIPV plus CT compared to intranasal vaccination in the absence of an adjuvant, both for type 1 ($p<0.05$) and type 3

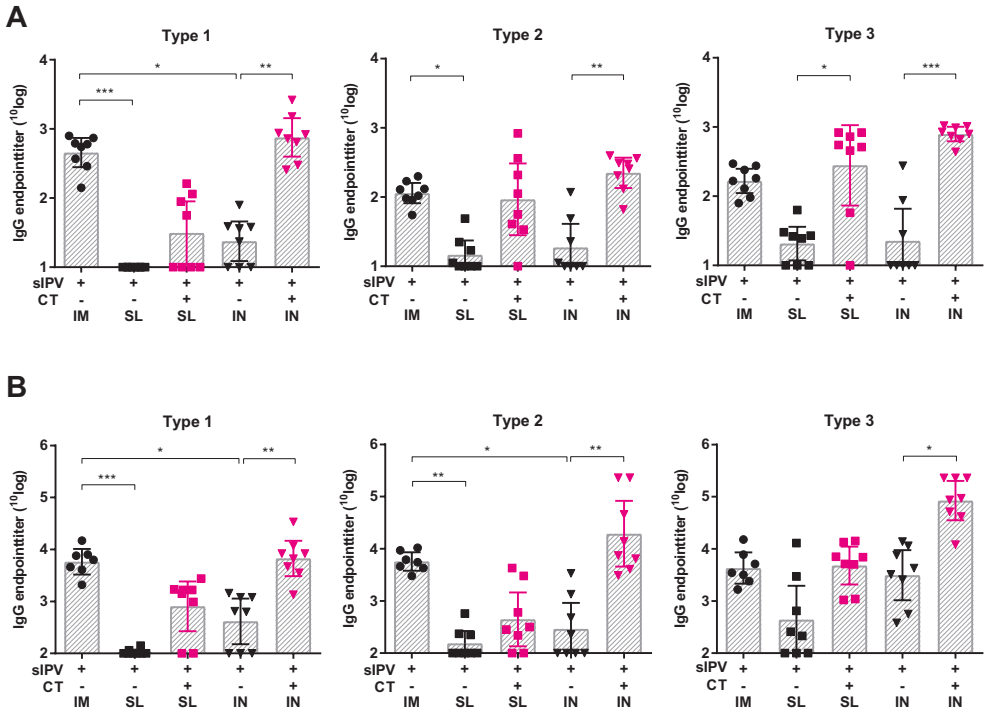

Figure 1 **Systemic immunity induced after vaccination with sIPV via parenteral or mucosal routes.** Polio-specific IgG antibody endpoint titers in serum from mice (n = 8) immunized with sIPV via conventional intramuscular (IM, circles) injection or via sublingual (SL, squares) or intranasal (IN, triangles) route. For both mucosal routes, vaccinations were given in the absence (black symbols) or presence (pink symbols) of the mucosal adjuvant cholera toxin (CT). Sera were collected one week after the second (day 14, panel A) and third immunization (day 35, panel B). Bars represent mean values and error bars depict 95% confidence interval values. Asterisks indicate significant differences between groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

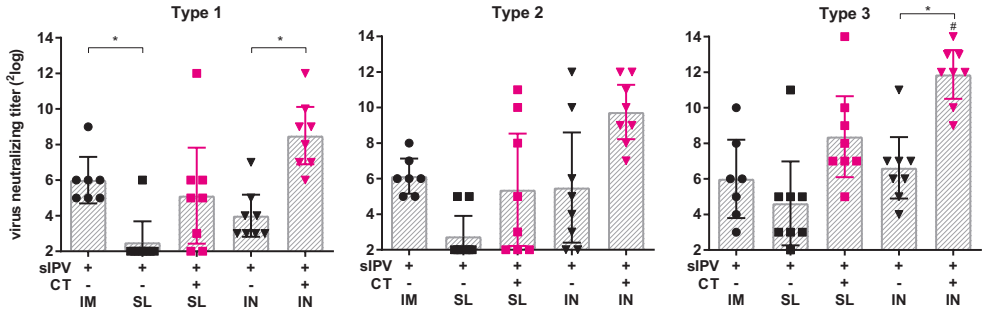

Figure 2 **Virus-neutralizing capacity of serum from mice immunized with sIPV via parenteral or mucosal routes.** Virus-neutralizing (VN) serum antibody titers were measured from mice (n = 8) immunized three times with sIPV via conventional intramuscular (IM) injection or via sublingual (SL) or intranasal (IN) route. For both mucosal routes, vaccinations were given in the absence (black symbols) or presence (pink symbols) of the mucosal adjuvant cholera toxin (CT). Sera were collected one week after the third immunization (day 35). Bars represent mean VN titers and error bars depict 95% confidence interval values. Asterisks indicate significant differences between groups (* $p<0.05$, ** $p<0.01$). Hashtags indicate a significant difference with the conventional IM group (# $p<0.05$).

($p<0.05$). Similar VN titers were obtained after sublingual vaccination in the presence of an adjuvant compared to conventional intramuscular immunization (without adjuvant), although some non-responders were observed after sublingual vaccination (Figure 2). Interestingly, superior type 3-specific VN titers were observed after sIPV (plus adjuvant) delivery via the nose, even when compared to intramuscular injected sIPV ($p<0.05$) (Figure 2).

To evaluate whether differences in systemic immune responses could be assigned to a difference in antigen penetration through and uptake by mucosae, antibody titers against the adjuvant, the highly immunogenic cholera toxin (CT), were measured in sera from mice that received sIPV plus CT. After three immunizations, all animals induced evident CT-specific IgG antibody titers (Figure 3). Even after vaccination via the sublingual route, no non-responders were observed, whereas in some animals no detectable polio-specific IgG titers were found (Figure 1). Significantly improved IgG antibody titers against CT were observed after intranasal vaccination compared with those obtained after sublingual immunization ($p<0.01$) (Figure 3).

Mucosal immune responses induced after SL and IN immunization with sIPV

Polio-specific IgA antibody endpoint titers were determined in different mucosal samples to evaluate mucosal immunity after sIPV immunization of previously mentioned administration routes. Both sublingual and intranasal administration of sIPV induced polio-specific IgA antibody responses in saliva (Figure 4A) and feces (Figure 4B), whereas the intramuscular route was unable to do so. Non-adjuvated sIPV delivery via the sublingual

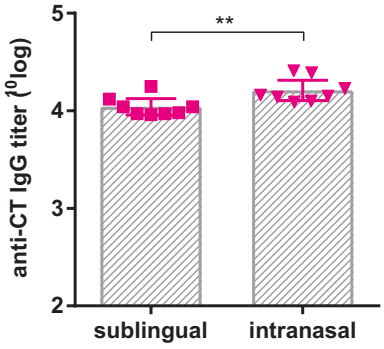

Figure 3 **Systemic antibody titers induced against cholera toxin (CT) delivered via sublingual or intranasal routes**. CT-specific IgG antibody titers were measured in serum from mice (n = 8) immunized with sIPV plus CT as adjuvant via sublingual or intranasal route. Sera were collected one week after the third immunization (day 35). Individual (pink symbols) and mean (bars) are depicted. Error bars represent 95% confidence interval values. Mean values differ significantly ($p=0.0093$) as analyzed by Mann-Whitney test.

route induced no detectable salivary IgA against polio type 1 and 2, and no polio-specific fecal IgA (Figure 4A and 4B). Sublingual vaccination of sIPV in combination with CT resulted in higher numbers of mice having detectable IgA titers in mucosal samples. For type 3, significant salivary IgA titers were elicited after sublingual administration of sIPV plus CT (Figure 4A). Also significantly enhanced polio-specific IgA titers (all serotypes) were observed in mice immunized intranasally with sIPV plus CT, both in saliva ($p<0.001$) and feces ($p<0.001$) (Figure 4A and 4B).

To evaluate the intestinal immune responses further, local IgA antibody production was determined by ELISA on detergent extractions of small intestinal samples. No intestinal immunity was induced after intramuscular vaccination with sIPV. Only for type 3, 25% of the animals induced polio-specific IgA in the intestine after sublingual delivery of sIPV, with or without adjuvant (Figure 4C). Again, animals immunized via the intranasal route with sIPV plus CT showed significantly improved IgA antibodies against polio type 1 ($p<0.001$), type 2 ($p<0.05$) and type 3 ($p<0.001$) (Figure 4C).

Polio-specific B cell responses elicited after SL and IN immunization with sIPV

The effect of the different immunization routes on the numbers of polio-specific plasma cells was evaluated in single cell suspensions from spleens. Whereas no IgA-secreting plasma cell responses were found in splenocytes from mice immunized via intramuscular injection, significantly enhanced numbers of IgA-producing B-cells were found in spleens of mice immunized via the intranasal route with either unadjuvanted sIPV (type 2 ($p<0.05$); type 3 ($p<0.01$)) or CT-adjuvanted sIPV (type 1 ($p<0.001$); type 2 ($p<0.001$); type 3 ($p<0.05$)) (Figure 5). The presence of CT enhanced the B cell responses after sublingual delivery of sIPV, since a higher number of animals showed IgA-producing B cell responses compared to the unadjuvanted sublingual group. Moreover, mice vaccinated sublingually with sIPV plus CT showed significantly enhanced numbers of polio type 2-specific IgA-secreting B cells ($p<0.01$) (Figure 5).

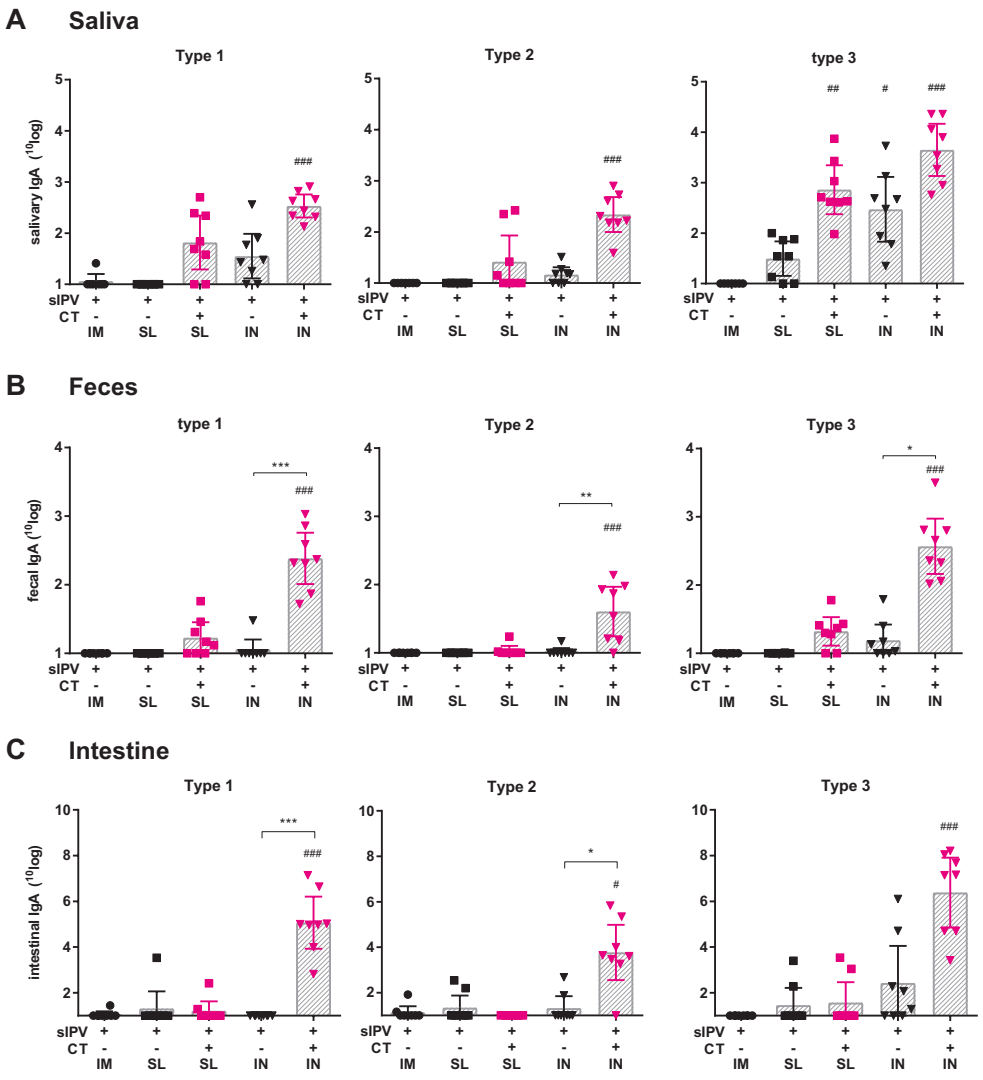

Figure 4 **Mucosal immunity induced after sIPV delivery via mucosal routes.** Polio-specific IgA endpoint titers in saliva, fecal extracts and intestinal tissue samples from mice (n = 8) immunized with sIPV in the absence (black symbols) or presence (pink symbols) of the mucosal adjuvant cholera toxin (CT) were measured. Immunizations were given via conventional intramuscular (IM) injection or via sublingual (SL) or intranasal (IN) routes. One week after the third immunization polio-specific IgA antibody titers were measured in saliva (panel A), fecal extracts (panel B) and intestinal tissue samples (panel C) were measured. Bars represent mean values and error bars depict 95% confidence interval values. Asterisks indicate significant differences between groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) and hashtags indicate significant differences with the conventional IM control group (# $p<0.05$, ## $p<0.01$, ### $p<0.001$).

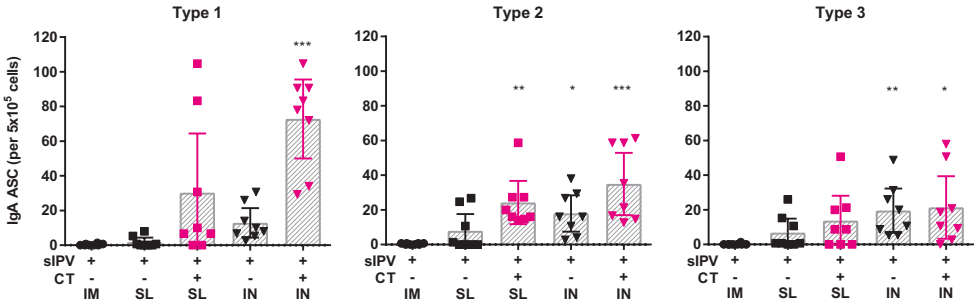

Figure 5 **IgA-secreting B cell responses elicited after mucosal sIPV immunization.** An ELISpot assay was performed to detect polio-specific IgA-secreting B cells from spleens from mice (n = 8) immunized with sIPV in the absence (black symbols) or presence (pink symbols) of the mucosal adjuvant cholera toxin (CT). Immunizations were given via conventional intramuscular (IM) injection or via sublingual (SL) or intranasal (IN) route. Bars represent mean antibody-secreting cell (ASC) numbers and error bars depict 95% confidence interval values. Asterisks indicate significant differences with the conventional IM control group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

DISCUSSION

The goal of the current study was to evaluate whether mucosal administration of sIPV could elicit both systemic immunity and polio-specific mucosal IgA at distinct mucosal sites. sIPV delivered via the sublingual or intranasal route was able to induce systemic polio-specific IgG responses with poliovirus-neutralizing capacity. Besides, mucosal vaccination of sIPV elicited polio-specific IgA antibody titers at distinct mucosal sites including strong intestinal responses after IN immunization and IgA-producing B cell responses in the spleen both after IN and SL immunization.

Mucosal compartmentalization restricts the induction of intestinal immunity upon intranasal immunization [17, 18]. The existing paradigm that only gut-resident dendritic cells (DCs) can recruit T cells to the gastrointestinal tract is difficult to reconcile with our findings and other reports of gastrointestinal T cell responses and protective gut immunity after intranasal immunization of influenza [19, 20] or Salmonella antigen [21]. Ruane *et al*. showed that lung DCs, which were targeted by intranasal immunization, stimulated gut-homing integrin α4β7 expression on CD4+ T cells in the lungs, and induced cell migration to the gastrointestinal tract in mice [22]. Therefore, it might be that intranasal vaccine delivery is

able to recruit antigen-specific immune cells to the gut and thereby inducing local intestinal immunity, which is in agreement with our findings.

Mucosal vaccine delivery has several practical advantages over vaccination via parenteral routes using needle and syringes, as previously mentioned. However, only relatively few mucosal vaccines for human use are licensed [23]. With the exception of some cholera vaccines, which have a very strong intrinsic immune potentiating capacity [24], all these mucosal vaccines are live attenuated vaccines, like OPV or intranasal influenza vaccines (Flumist/Fluenz) [8, 25]. In contrast to OPV, mucosal polio vaccination based on IPV is expected to require the inclusion of an adjuvant to evoke appropriate immunity against polio [13], which was confirmed in the current preclinical study. Both intranasal and sublingual vaccination of sIPV plus cholera toxin (CT) as adjuvant were able to significantly enhance functional systemic immunity and polio-specific IgA titers in mucosal samples compared to immune responses obtained after mucosal sIPV vaccination without adjuvant. However, CT and the Escherichia coli-derived heat-labile toxin (LT) are well known as potent mucosal adjuvants, but are also associated with adverse effects in humans. Concerns has been raised after an undesired association between facial nerve paralysis (Bell's palsy) and the intranasally delivered inactivated influenza vaccine (Nasalflu) containing an enzymatically active LT adjuvant [26]. Probably the neuronal-binding capacity of the LT-derived adjuvant was the cause of this adverse effect suggesting that nasal administration of LT or CT molecules is inadvisable [27]. Migration to or accumulation in the central nerve system might be avoided by vaccine administration under the tongue [28-30]. Moreover, in a recent Phase 1 study (NCT00820144) conducted in France, the sublingual administration of recombinant CT B subunit in healthy subjects was found to be safe. Therefore, a new safe mucosal adjuvant with strong immune potentiating capacity should be included in the further development of a mucosal (Sabin) IPV although adverse effects after sublingual immunization are not documented as far as we know. Since the mucosal route is minimally addressed for IPV yet, current experience is limited to the use of a double mutant of LT (dmLT) in combination with the sublingual route as described by White *et al*. [31]. Adjuvants (e.g., LPS derivative PagL, oil-in-water emulsions, CpG ODN) that have shown their potential for (Sabin) IPV via the parenteral route could also be evaluated for mucosal vaccination [12, 32].

In the last decade, sublingual vaccine delivery has gained significant attention as shown by the numerous published preclinical studies that provide a strong base for further testing of this non-invasive route [33]. Our findings and earlier research indicates that the inclusion of an adjuvant might be needed to avoid tolerance or low-to-undetectable immune responses after

sublingual delivery of IPV [31]. Besides the inclusion of an adjuvant as immune potentiator, novel oral dosage forms to improve contact time or to facilitate transport through the mucosal barrier, may be required to achieve successful vaccination. White *et al*. studied the sublingual route for IPV (based on Salk strains) using a thermoresponsive gel (TRG) delivery system [31]. Whereas sublingual administration of IPV as a liquid or as TRG in the absence of an adjuvant was not able to induce any immune response in mice, sublingual administered IPV as TRG in combination with the mucosal adjuvant dmLT led to systemic poliovirus-neutralizing antibody titers, and salivary and fecal IgA production [31]. A comparison between the liquid and TRG formulation (both with or without adjuvant) is missing, but it is expected that mucoadhesive dosage forms that prolong the residence time of the vaccine at the oral mucosa and thereby facilitate antigen uptake by local antigen-presenting cells, are needed [13, 33]. Research on sublingual polio vaccination may, besides the use of novel adjuvants, comprise development of extended release formulations, including solid dosage forms that at the same time improve the thermostability of the vaccine as well. Earlier studies revealed that dried IPV can be more resistant to higher temperatures compared to liquid IPV [34-36].

The phased withdrawal of OPV and inclusion of IPV into all global routine immunization programs will create a market for non-invasive delivery of polio vaccines, even a considerable time after eradication either for routine immunization or stock piling. Improved polio vaccine delivery strategies should be suitable for the final target population: infants. The current study demonstrated the potential of both the intranasal and sublingual routes for polio vaccination with IPV based on Sabin strains. Intranasal vaccination showed to be more efficient in eliciting both systemic and mucosal immune responses compared with the sublingual route. However, besides possible redirection to olfactory bulbs (Bell's palsy), the risk of wheezing in young children exists [8]. The sublingual route could be an easy and safe polio immunization approach. Nevertheless, for the induction of evident immunity upon sIPV vaccination under the tongue, strong mucosal adjuvants might be required. Therefore, further research on polio vaccination via the sublingual route should include the search for a safe and effective adjuvant and the development of novel oral dosage forms that improve antigen uptake by the oral mucosa.

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Manuscript in preparation

Polymer-based oral dissolving films for polio vaccination

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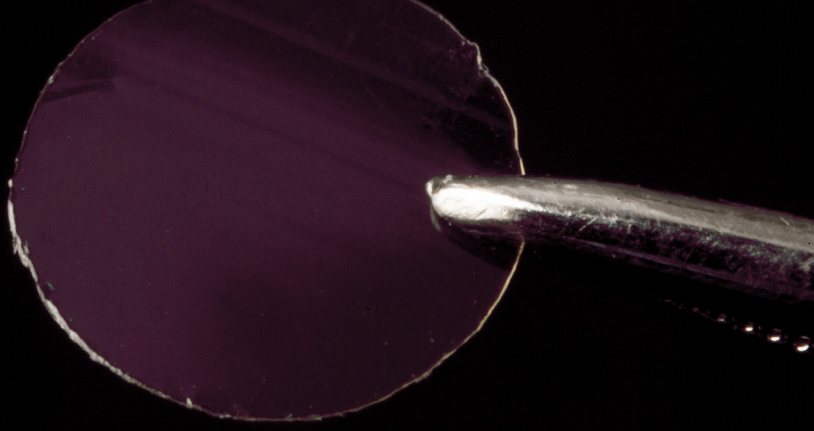

ABSTRACT

Alternative delivery technologies for polio vaccination might be of importance in the development of improved polio vaccines that are needed towards complete polio eradication and their use in the period thereafter. Vaccination via oral mucosa, like the sublingual or buccal route, could be an easy applicable and safe polio immunization approach for routine immunization or as a tool for outbreak intervention after cessation of the live oral polio vaccine.

Aim of the current study was to evaluate the possibility to make polymer-based films containing trivalent sIPV and suitable for oromucosal vaccination. A combination of a Design of Experiments approach and the evaluation of excipients with already proven stabilizing capacity of polio antigens was used to develop sIPV-containing oral film formulations while preserving its D-antigenicity for 85-100% for type 1, 60-85% for type 2, and 50-75% for type 3. This study revealed that a combination of excipients based on sorbitol, magnesium chloride and monosodium glutamate, has strong stabilizing potential for sIPV-films, even when combined with different film formers, i.e., hydroxypropyl cellulose, sodium glutamate or sodium carboxymethyl cellulose. This pivotal study showed the promise of dried polymer-based sIPV-films that might be suitable for sublingual or buccal polio vaccination. Further optimization is required during future product development studies, especially with respect to the mechanical properties of the film formulations.

INTRODUCTION

Since eradication of polio is one of the top global health priorities, the need for inactivated polio vaccines (IPV) that are more affordable, more effective, and safer than existing polio vaccines is higher than ever. This new generation of IPV, for instance based on attenuated Sabin strains, should preferably also induce mucosal immunity, remain stable outside the cold-chain, and be easy to administer. Such a vaccine is valuable with regard to stockpiling and outbreak control in the period after complete cessation of the oral polio vaccine (OPV) and after polio eradication [1]. Vaccination via mucosal sites has the benefits of needle free vaccine delivery and may induce strong mucosal immunity, even at distant effector sites. The induction of polio-specific immunity in the gut protects against polio infection and may interrupt the person-to-person transmission of poliovirus [2].

A previous study in mice revealed that sublingual immunization with fluid Sabin IPV (sIPV) induced systemic poliovirus-neutralizing immune responses as well as polio-specific IgA-producing B cells in the spleen. Moreover, sublingual sIPV delivery elicited polio-specific IgA antibodies at different mucosal sites, where conventional intramuscular vaccination was unable to do so [3]. In the same study, intranasal sIPV vaccination showed to be more efficient in eliciting polio-specific immune responses as compared with the sublingual route [3]. However, besides the concern of uptake by nervous tissue via the olfactory bulbs, which may cause adverse effects (like Bell's palsy), the risk of wheezing in young children exists, making the intranasal route potentially less suitable for infants [1]. Sublingual vaccine delivery has gained significant attention as shown by the numerous studies on this innovative and non-invasive route [4]. For sublingual IPV delivery, the inclusion of an adjuvant may be required to circumvent tolerance or low-to-undetectable immune responses [3, 5]. Besides, novel oral dosage forms that facilitate transport through the oral mucosa, for example by extending the contact time of the antigen, might be desirable for successful vaccination.

Several dosage forms exist for sublingual and buccal delivery of marketed drugs. However, not all of them will be suitable for oral mucosal vaccine delivery and only few have been used to explore sublingual delivery of vaccines. Novel dosage forms that might have the ability to retain the antigen at the sublingual or buccal delivery site are (mucoadhesive) oromucosal films or -tablets, and thermoresponsive gels. Currently, only thermoresponsive gels, which are aqueous solutions at room temperature but transform into gels when at body temperature (e.g., upon contact with the mucosa), have been non- and preclinical evaluated as new dosage form for sublingual delivery of IPV. Sublingual administration of these gels,

containing Salk IPV plus a double mutant heat-labile toxin (dmLT) as adjuvant, induced polio-specific functional antibodies in serum as well as polio-specific IgA antibodies in mucosal samples [5]. Among the solid oral dosage forms are the (mucoadhesive) extended release films and tablets. Converting the vaccine into the dry state might improve the thermostability and could therefore reduce vaccine system costs tremendously. When vaccines no longer require cold storage, or could be kept out of the cold-chain long enough for their transport to remote areas in developing countries, logistic costs will decrease and vaccine availability will be improved [6, 7].

Aim of the current study was to evaluate the possibility to make polymer-based oromucosal films containing trivalent sIPV. First, a target product profile (TPP) describing the desired properties and characteristics of a final product was defined. Among those characteristics are the critical quality attributes (CQAs), which should be within an appropriate limit, range or distribution to ensure the desired product quality, efficacy and safety of the product when used [8, 9]. Further on, different film forming polymers (i.e., hydroxypropyl cellulose (HPC), sodium alginate and sodium carboxymethyl cellulose (CMC)) were selected from literature and tested in combination with excipients that were able to stabilize the antigen during the drying process. D-antigenicity of prepared oral films was assessed in an ELISA directly upon drying. To gain insight into the effects of the main components, i.e. film forming polymer and plasticizers, on both D-antigen recovery and physical/mechanical film characteristics, a Design of Experiments (DoE) approach was used. Subsequently, oral film formulations were further optimized based on D-antigen recovery of each serotype. It was investigated whether the addition of sugars or the combination sorbitol, magnesium chloride and monosodium glutamate could further improve the D-antigen recovery of each serotype in order to yield an oral film formulation with minimal loss of antigenicity during the drying process.

MATERIALS AND METHODS

Materials

Monovalent Sabin IPV bulk material used in this study was produced as described previously [10]. For the preparation of trivalent sIPV, monovalent type 1, type 2 and type 3 were mixed and, subsequently, concentrated using 10 kDa Amicon® Ultra Centrifugal Filters (Merck Millipore, Billerica, MA).

The excipients hydroxypropyl cellulose (HPC), glycerol, D-sorbitol, D-trehalose dehydrate, sucrose, maltodextrin, L-glutamic acid monosodium salt monohydrate (MSG), magnesiumchloride hexahydrate and TRIS (Trizma Base) were purchased from Sigma Aldrich (St. Louis, MO). To prepare 10 mM phosphate buffer, 10 mM disodium hydrogen phosphate heptahydrate (Na2HPO4 * 7H20 from Merck, Darmstadt Germany) was added to 10 mM potassium dihydrogen phosphate (Na2HPO4 from Merck, Darmstadt, Germany) until pH 7.0 was reached. All excipients used were of reagent quality or higher grade.

Methods

Preparation of casting solutions

For the preparation of stock solutions, excipients were dissolved in 10 mM TRIS (for HPC-containing films) or phosphate buffer (SA- or CMC-containing films) (under constant stirring). After complete dissolution, the pH was adjusted to 7.0 ± 0.1 and, subsequently, formulations were mixed with concentrated sIPV bulk using an Intelli-Mixer. Air bubbles were removed by a short spin in an Eppendorf centrifuge. Placebo film formulations were prepared for viscosity measurements and determination of film characteristics as described previously [11]. The viscosity of the casting solutions was measured directly after preparation at ambient temperature using a viscometer (Brookfield, Middleboro, USA). Depending on the viscosity of the casting solution spindle T-B or S04 was used.

Standard film casting method

The solutions were cast onto a release liner (Primeliner® 410/36, Loparex, Apeldoorn, The Netherlands) with a quadruple film applicator using a casting height of 1000 µm. The release liner was fixed to a COATMASTER film casting apparatus (Erichsen, Hemer, Germany) by vacuum suction. The casting speed was 10 mm/s and, subsequently, the film layer was dried at 30°C and at ambient relative humidity. Placebo films were punched using an Artemio perforator (Artemio, Wavre, Belgium) in squares of 1.8 x 1.8 cm, yielding stamp-shaped oral films used in tests for mechanical and physical properties of film formulations as described below.

Ring-based film casting method

Formulations containing sIPV were cast into metal rings (Ø 1.6 cm) in a volume of 201 µL/ring (corresponding with casting height of 1000 µm) onto polyethylene terephthalate foil (Silphan S75 M371, Siliconature, Treviso, Italy) as release liner (see Figure 1B). CMC-containing films were cast in a volume of 301 µL/ring (casting height 1500 µm)), since films with holes were obtained when casting at 1000 µm casting height. Subsequently, the film layer was dried at 30°C and ambient relative humidity for up to 20h. This novel ring-based method was developed to avoid spreading or shrinkage of the liquid formulations with low viscosity and allow film casting using small volumes to minimize the amount of antigen needed.

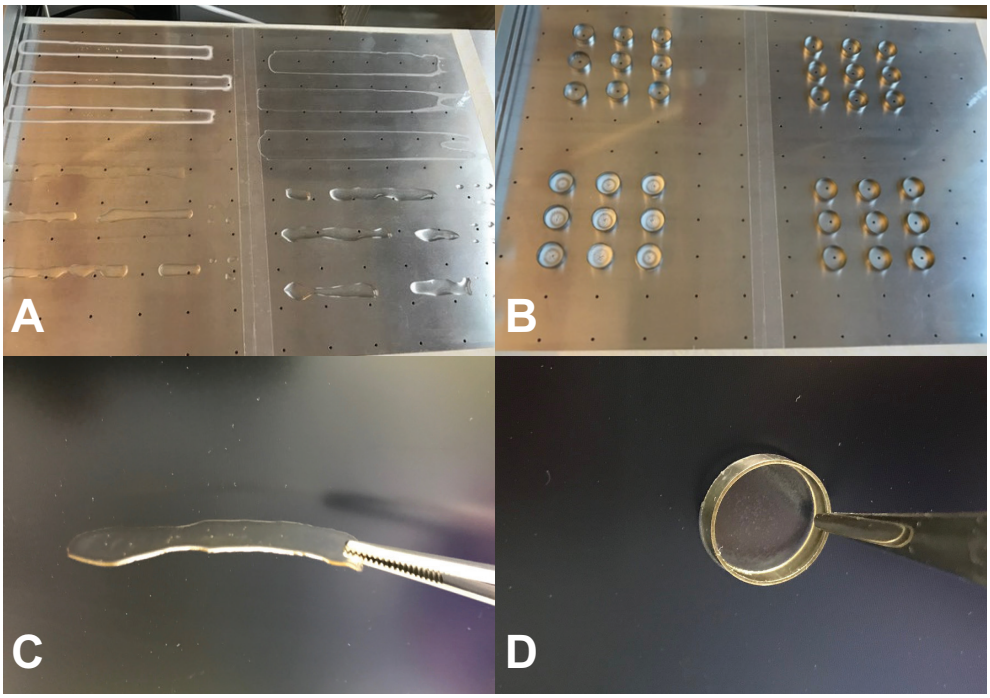

Figure 1 Preparation of films was performed using different film production methods. Since standard film casting method using a film casting apparatus (A) resulted sometimes in spreading (A: upper right formulation) or shrinkage (A: lower formulations) of the liquid formulations with low viscosity, a ring-based film casting method (B) was developed. sIPV-containing oral film formulations consisting of sodium carboxymethyl cellulose, sorbitol, magnesium chloride and monosodium glutamate resulted in transparent films that were easily removable from the release liner, either when cast with the standard (C) or ring-based film casting method (D). Due to shrinkage of the casting solution on the release liner directly upon standard casting, an irregular shaped film is formed (C), whereas the ring-based method yield a more uniform film surface.

Physical and mechanical characterization of oral films

Placebo film formulations were prepared to evaluate physical and mechanical properties of oral films. The thickness was measured using a microscrew meter (Mitutoyo, Neuss, Germany) at five different points of the prepared film. Uniformity of mass was determined according to the Ph. Eur. 9th edition: uniformity of mass for single-dose preparations. Twenty randomly chosen oral films were weighed individually on an analytical balance and average mass was calculated. Residual water content of placebo film formulations was measured using an infrared moisture analyzer (Sartorius MA40, Sartorius Göttingen, Germany). Approximately 1.5 gram of oral films were weighed and heated at 105°C for at least 1.5h until equilibrium in weight was reached. Loss on drying was calculated as the difference (in %) in mass between the initial weight and the final weight at equilibrium:

$$\text{Loss on drying} = \frac{\text{Initial film weight (t=0) - Film weight (t=1.5h)}}{\text{Initial film weight (t=0)}} \times 100 \qquad (1)$$

The mechanical properties of oral films were analyzed as described previously [11]. A minimum of six punched films were tested using an Instron series 5500 load frame with a load cell of 100N (Instron, Norwood, USA). Films were fixed between two clamps that moved away from each other with a crosshead speed of 50 mm/min until film tearing or breakage. Tensile strength (N/mm2), Young's modulus (N/mm2) and elongation at break (%) were calculated using the following equations:

$$\text{Tensile strength} = \frac{\text{load at auto break}}{\text{cross-sectional area of film}} \times 100 \qquad (2)$$

$$\text{Young's modulus} = \frac{\text{slope of stress-strain curve}}{\text{film thickness x cross-head speed}} \qquad (3)$$

$$\text{Elongation at break} = \frac{\text{increase in length at break}}{\text{initial film length}} \times 100 \qquad (4)$$

D-antigen ELISA

Immediately after drying, sIPV-containing films were dissolved and analyzed for antigenically active D-antigen by ELISA as described earlier with some small adaptations [12]. Microtiter plates were coated overnight with serotype-specific bovine anti-polio serum

and, subsequently, blocked with 1% bovine serum albumin (Sigma Aldrich, St. Louis, MO) in PBS for 30 min at 37°C. After washing with 0.05% Tween 80 (Merck, Darmstadt, Germany) in PBS, serial dilutions of sIPV samples (reconstituted films or liquid controls) were added and incubated for 2h at 37°C. Subsequently, plates were washed and serotype-specific monoclonal antibodies (HYB295-17-02 (type 1), HYB294-06-02 (type 2), HYB300-06 (type 3) from Thermo Fisher Scientific, or 4-8-7 (Bilthoven Biologicals, Bilthoven, The Netherlands) were added and incubated for 2h at 37°C. After another washing step, HRP-labeled anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) was added, plates were incubated for 1h at 37°C, washed, and SureBlue tetramethylbenzidine (TMB) Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. After 10 min, the reaction was stopped with 0.2 M sulfuric acid and absorbance at 450 nm was measured. Assay data were analyzed by four-parameter logistic curve fitting and D-antigen content was calculated relative to the reference standard.

Table 1 Design of Experiments worksheet. Composition of all formulations tested in a full factorial design were displayed in the design matrix.

Experiment No.	HPC (% w/v)	Glycerol (% w/v)	Sorbitol (% w/v)
N1	10	0	0
N2	15	0	0
N3	10	2.5	0
N4	15	2.5	0
N5	10	0	5
N6	15	0	5
N7	10	2.5	5
N8	15	2.5	5
N9	12.5	1.25	2.5
N10	12.5	1.25	2.5
N11	12.5	1.25	2.5

Design of Experiments (DoE)

A Design of Experiments (DoE) approach was used to evaluate the effects of HPC (10-15% w/v), glycerol (0-2.5% w/v) and sorbitol (0-5% w/v) on both D-antigen recovery upon drying and mechanical film properties. A full factorial screening design (Table 1) was performed and analyzed using Modde software (version 12, Umetrics AB, Umea, Sweden).

Models were fitted using multilinear regression (MLR) and subsequent optimized by deleting non-significant terms leading to a model with the best model performance parameters, i.e., goodness of fit (R^2), goodness of prediction (Q^2), model validity and reproducibility.

RESULTS AND DISCUSSION

Target product profile

The establishment of a target product profile (TPP) is a helpful tool to focus research on a certain technology and product development efforts in order to support efficient and directed product development [9]. A TPP describes the desired properties and characteristics of a final product. The current study was started with the establishment of a TPP for sIPV-containing orally dissolving film formulations appearing as (semi)-transparent films (Table 2). Moreover, some of the characteristics were defined as critical quality attribute (CQA), because these properties should be within an appropriate limit to ensure the desired product quality and thereby adequate performance and safety of the product when used [8]. The TPP is a dynamic summary that changes as knowledge during product development increases. Therefore, the anticipated quality target product profile (QTPP) might also be subjected to changes (Table 2).

Since the proposed administration route differs from the conventional route for sIPV (intramuscular or subcutaneous injection), dose-finding studies should define the single human dose for oral dosage forms, like oromucosal films. Moreover, the size of the film depends on what is acceptable for the intended target population and should thus be defined later in development (Table 2). Variability will affect efficacy, so content uniformity should be within certain limits, which will be defined upon selection of validated assays for the assessment of content and conform relevant guidelines. Moreover, a high or low pH will disrupt the polio particle thereby affecting the vaccine's efficacy. The pH limits were set on pH 6.5 - 7.0.

Unfortunately, mechanistic studies designed to evaluate and define the optimal conditions for sublingual (or buccal) vaccine delivery are lacking in literature. It remains speculative what, for example, the optimal release profile of an oral dosage form for sublingual vaccination is. Moreover, to what extend residual water content of oral films might affect product performance or stability is still unknown. Hence, CQA targets, but also critical process parameters having an impact on CQAs, should be determined during future product development and based

on risk assessments. Although they are relevant for the final product, it was decided not to focus extensively on all critical quality characteristics, like water content, dissolution and disintegration time, during the current (preliminary) study on sIPV-oral films.

Table 2 Target product profile of an IPV-containing oromucosal film. The current table summarizes the desired target product profile of an orally dissolvable film containing Sabin IPV.

Attributes	Target product profile (TPP)	Anticipated QTPP[1] - CQA[2]	Justification
Dosage form	Oromucosal film	No	n.a.
Appearance	(semi-)transparent film Size: t.b.d. Thickness: 5-200 µm	No	Target set to ensure recipient acceptability
Target population	Infants	No	
Administration route	Buccal or sublingual	No	
Dose	t.b.d.	Yes	Dose finding studies should define dose needed for buccal or sublingual route. *NB: Currently used (parenteral) sIPV dose: 10-16-32 DU.*
Content uniformity	t.b.d.	Yes	Variability will affect efficacy
Pharmaco-kinetics	Dissolution rate: t.b.d. Antigen release within 24h	Yes	Release profile is important for bioavailability and antigen uptake. Also recipient acceptability plays a role.
Storage	2-8°C	No	Defined based on stability results.
Stability	At least 36 months shelf-life at 2-8°C	Yes	Ideally, oral sIPV films should be at least as stable as the liquid sIPV vaccine. Depending on acceptable dose range (efficacy and safety).
Container closure system	t.b.d.	Yes	Needed to achieve target shelf-life and ensure the product integrity during storage. *NB: If defined properly without having impact on product quality and integrity, this attribute is not critical.*
pH	6.5 < pH < 7.0	Yes	High or low pH will disrupt antigen and affect efficacy.
Residual water content (RMC)	t.b.d.	Yes	Depending on design space, acceptable RMC range and subsequent stability and D-antigen recovery as result of formulation and drying process. *NB: Limited amounts of water in oral dosage forms will not impact patient safety or efficacy.*
Disintegration	t.b.d.	Yes	Film strength will affect release profile and may impact efficacy.

[1] Quality target product profile (QTPP) – prospective summary of the quality characteristics; critical quality attributes.
[2] Critical Quality Attribute (CQA) - should be within defined targets to ensure the desired product quality.
Abbreviations used: n.a. - not applicable; t.b.d. - to be determined

Table 3 Oral film formulations tested in full factorial screening experiment using a Design of Experiments (DoE) approach including hydroxypropyl cellulose (HPC), glycerol and sorbitol as excipients. Viscosity of the casting solutions was measured and dried oral films that were removable from the release liner were further characterized.

Exp. No.	Viscosity (mPa.s, 20 rpm)	Removable from release liner	Mass (mg)	Thickness (µm)	Residual moisture (%)	Elongation at break (%)	Young's Modulus (N/mm2)	Tensile strength (N/mm2)	Disintegration time (s)
N1	296	+	25.2 ± 2.3	61.9 ± 5.1	2.93	20.2 ± 7.9	315.5 ± 49.3	2.8 ± 0.6	16.2 ± 2.1
N2	1490	+	35.9 ± 2.0	86.4 ± 5.1	3.14	28.1 ± 6.9	319.4 ± 22.9	3.2 ± 0.3	29.0 ± 3.2
N3	270	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N4	1700	+/-	39.9 ± 3.4	91.4 ± 10.1	7.69	93.1 ± 35.6	71.2 ± 11.2	0.5 ± 0.2	19.7 ± 3.0
N5	458	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N6	2900	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N7	634	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N8	3170	+/-	48.7 ± 2.9	137.1 ± 27.4	8.73	17.1 ± 6.0	61.3 ± 25.4	0.6 ± 0.2	14.8 ± 2.7
N9	890	+/-	38.5 ± 5.1	101.5 ± 12.1	6.39	14.6 ± 3.7	93.9 ± 30.8	0.8 ± 0.3	14.4 ± 4.7

n.d. – not detectable

Table 4 Oral film formulations tested in full factorial screening design including hydroxypropyl cellulose (HPC), glycerol and sorbitol as excipients. D-antigen recoveries were determined per serotype directly upon drying.

Exp. No.	HPC (% w/v)	Glycerol (% w/v)	Sorbitol (% w/v)	D-antigen recovery (%)		
				Type 1	Type 2	Type 3
N1	10	0	0	9.1	n.d	10.8
N2	15	0	0	13.6	n.d	20.0
N3	10	2.5	0	n.d.	n.d	6.0
N4	15	2.5	0	n.d.	n.d	5.5
N5	10	0	5	48.2	32.8	34.7
N6	15	0	5	40.8	19.7	34.6
N7	10	2.5	5	30.3	42.9	36.8
N8	15	2.5	5	27.0	39.5	39.0
N9	12.5	1.25	2.5	7.8	22.2	27.0
N10	12.5	1.25	2.5	7.1	17.8	24.4
N11	12.5	1.25	2.5	7.5	16.2	24.5

n.d. – not detectable

Full factorial screening design

To gain insight into the effects of the main components of oral film formulations, i.e. film forming polymer hydroxypropyl cellulose (HPC), and plasticizers glycerol and sorbitol, on both physical/mechanical film characteristics and D-antigen recovery, a full factorial screening design was performed. Viscosity of the (placebo) casting solutions was measured and ranged from 270 to 634 mPa.s and from 1490 to 3170 mPa.s for formulations containing respectively 10% (w/v) (N1, N3, N5 and N7) and 15% (w/v) HPC (N2, N4, N6 and N8) (Table 3). All solutions were homogenous and no air bubbles occurred during the casting procedure, so all were suitable for casting. Formulation N1, consisting of 10% (w/v) HPC only, spreads too much directly after casting due to the relatively low viscosity, whereas appropriate spreading occurred upon casting of formulation N2. Formulations containing the lowest concentration HPC (10% (w/v)) displayed holes and were defined as unsuitable for further testing due to their handling difficulty, since these formulations yielded very thin and fragile films that, in the presence of sorbitol and/or glycerol, cannot be removed from the release liner (Table 3). The inclusion of sorbitol resulted in sticky films that were at least difficult or unable to subtract from the release liner, so these formulations (N5, N6 and N7 of table 3) were not further analyzed for mechanical film properties. The sorbitol-containing formulations that could be taken from the release liner upon drying consisting of 15% w/v HPC, 2.5% w/v glycerol and 5% w/v sorbitol (N8) or 12.5% HPC, 1.25% glycerol and 2.5% w/v/ sorbitol (N9).

Upon removal from the release liner, oral film formulations were further characterized for their physical (i.e., mass, thickness, residual water content) and mechanical properties (elongation at break, Young's Modulus, tensile strength and disintegration time). Increasing the concentration of excipients resulted in oral films with increased mass and thickness (Table 3). Moreover, addition of excipients resulted in higher percentages of residual water (defined as weight loss on drying) and negatively influenced the tensile strength (Table 3).

The oral films disintegrated all within 30 seconds (Table 3). This is compliant with guidelines for orally disintegrating tablets in European Pharmacopoeia (EP) and United States Pharmacopoeia (USP) (disintegration within 180 s) [11, 13, 14]. However, these recommendations might be less useful for our vaccine-containing films as antigen-transport through oral mucosa and subsequent uptake by immune cells might be time-consuming steps, which might need a prolonged residence time of the vaccine-formulation into the mouth. As described in section 3.1, it remains speculative what the optimal release profile of vaccine-containing oral films will be. As such, clearly defined requirements for disintegration time of oromucosal (fast or slow release) films have to be defined, subsequently by pharmaceutical guidelines.

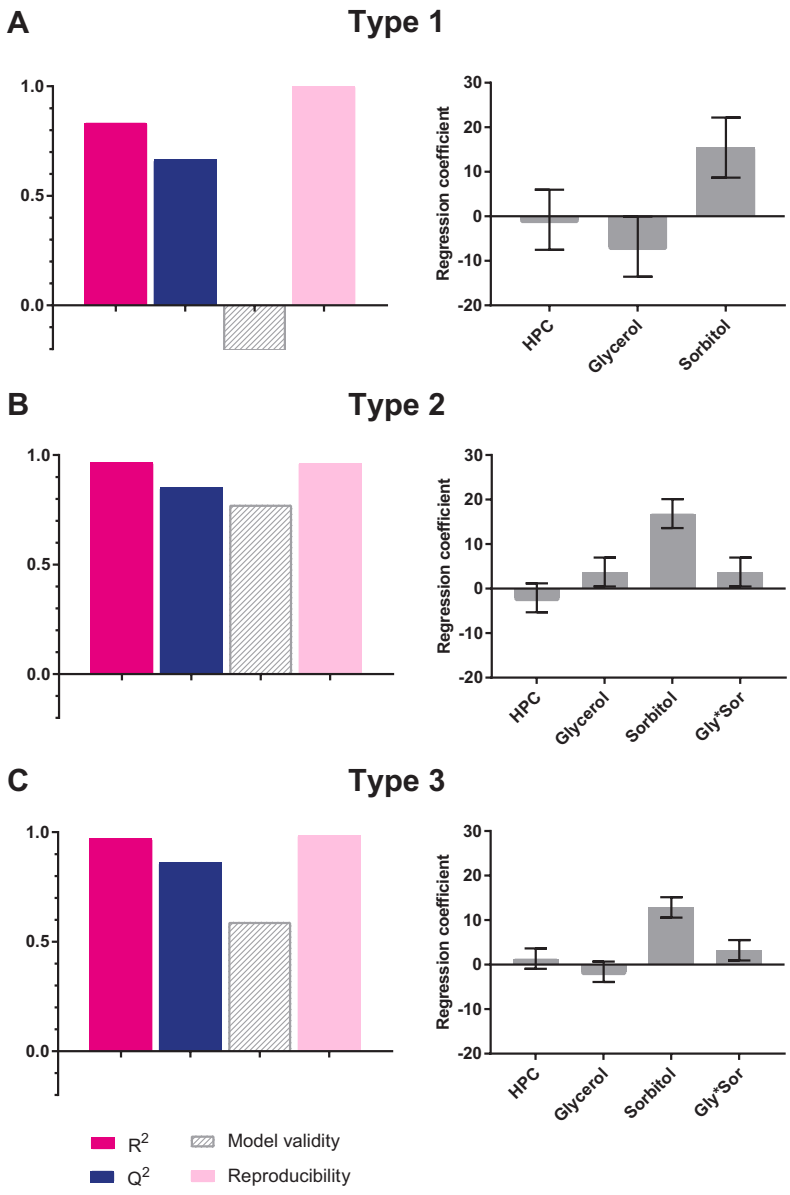

Figure 2 Regression models for the D-antigen recoveries type 1 (A), type 2 (B) and type 3 (C) of oral film formulations directly after drying. On the left, summaries of fit for all models are depicted: goodness of fit (R^2, values >0.5 indicate a good model), goodness of prediction (Q^2, values >0.5 indicate a good prediction power), model validity (values >0.25 indicate that the model is smaller than the experimental error) and reproducibility (values >0.5 indicates a small experimental error). On the right, main and interaction effects of the components (i.e., hydroxypropyl cellulose (HPC), sorbitol (Sor) and glycerol (Gly)) that contribute (per serotype) to the best fitted model, according to their model performance parameters are shown in model coefficient plots. Note: The negative model validity value for type 1 (A) here is a direct result of the extremely good replicates and a reproducibility value close to 1.

To evaluate the effect of the excipients on D-antigen recovery upon drying, all formulations from the screening design were prepared freshly wherein sIPV was mixed (Table 1). To avoid spreading of the liquid formulations with low viscosity and allow film casting using small volumes to minimize the amount of antigen needed, vaccine-containing casting solutions were converted in films using the ring-based casting method. Directly after drying, films were dissolved and D-antigenicity was determined by ELISA (Table 4). Multilinear regression (MLR) models were fitted using the ELISA results and optimized per serotype, which resulted in valid models for the prediction of D-antigen recoveries of dried sIPV-films according to the model performance parameters for type 1 (Figure 2A), type 2 (Figure 2B) and type 3 (Figure 2C). Model validity for the type 1 D-antigenicity was negative, which is likely a model artifact and a direct result of the very low variation in the replicates (N9-N11) resulting in a reproducibility value close to 1 [15]. In general, most of the D-antigen content was lost during the drying process as indicated by D-antigen recoveries of <50% for all serotypes with the highest D-antigen recoveries obtained in the presence of sorbitol in the formulation (N5-N9) (Table 4). The stabilizing effect of sorbitol was also confirmed by the significant positive regression coefficients of the prediction models for D-antigen recovery type 1, 2 and 3 directly after drying (Figure 2). The film forming polymer HPC did not influence D-antigen recovery, whereas the addition of glycerol seemed to affect type 1 D-antigenicity negatively (Figure 2A), but have a slight stabilizing effect on type 2 D-antigenicity during the drying process (Figure 2B). Both for type 2 and 3, a significant interaction effect was observed when glycerol and sorbitol were combined in the formulation (Figure 1B-C). Response contour

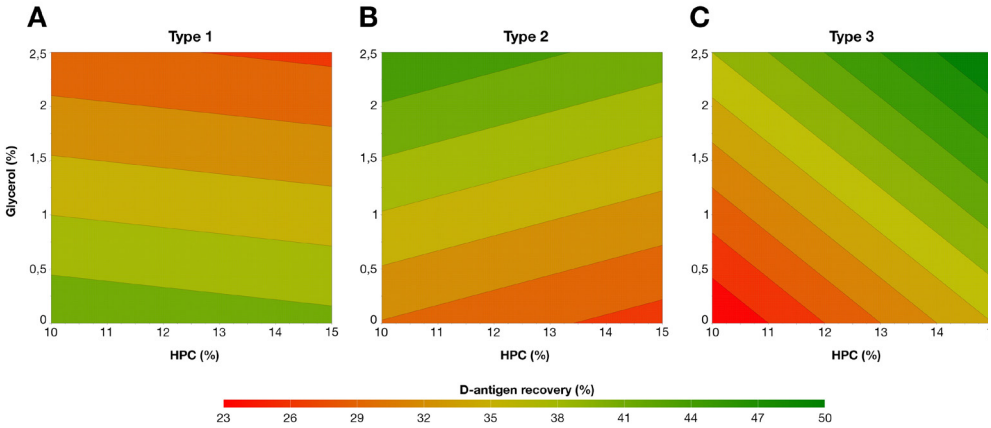

Figure 3 Response contour plots showed predicted D-antigen recoveries type 1 (A), type 2 (B) and type 3 (C) of formulations containing HPC (10-15% w/v, on x-axis) and glycerol (0-2.5% w/v, on y-axis) in combination with 5% (w/v) sorbitol.

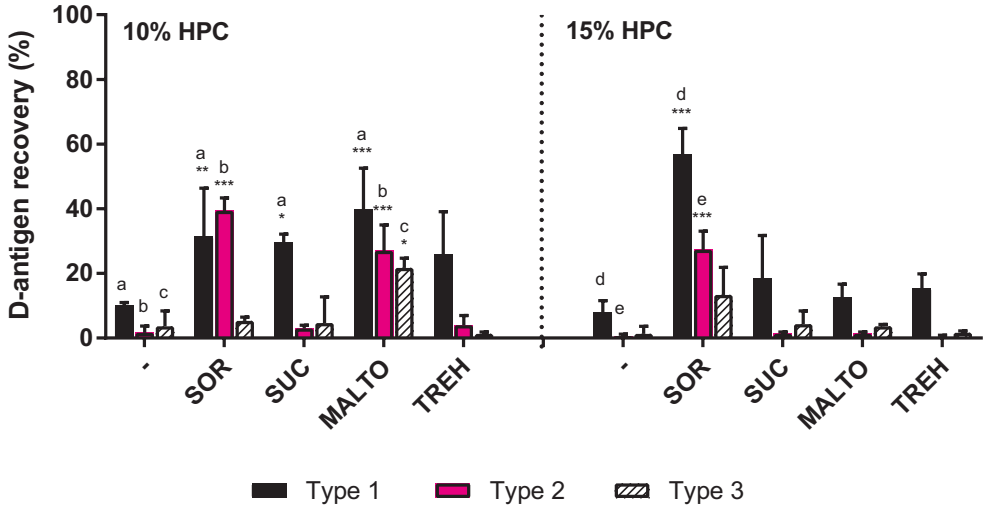

Figure 4 D-antigenicity of oral film formulations containing 10% (w/v) or 15% (w/v) hydroxypropyl cellulose (HPC) as film former and 5% (w/v) of sorbitol (SOR), sucrose (SUC), maltodextrin (MALTO) or trehalose (TREH). Directly upon drying, oral films were dissolved and D-antigen recoveries were determined for type 1 (black bars), type 2 (pink bars) and type 3 (striped bars) by ELISA. Mean values (n=3) and standard deviations are depicted. Asterisks indicate significant differences of formulations when compared with control formulation without additional sugars (-) marked with the same letter above bars (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

plots showed the challenge of developing a trivalent dried Sabin IPV since each serotype showed a different optimal combination of excipients (Figure 3). Earlier studies revealed the difficulty of drying IPV, which was based on Salk strains instead of the Sabin strains based IPV formulated here, using different drying methods [16-18]. Therefore, it was decided to focus on D-antigenicity, instead of film characteristics, in the upcoming experiments in order to improve vaccine's stability during the drying process.

Sugar screening

To increase the D-antigen recovery of the film formulations after drying, additional excipients may be included. Sugars have the ability to protect biologicals, like proteins or vaccines, against dehydration stresses [19]. The carbohydrates sucrose, maltodextrin and trehalose were screened in combination with 10% (w/v) or 15% (w/v) HPC. Since sorbitol already showed to confer protection during drying, sorbitol was also included. Glycerol was excluded from this experiment, since no clear positive effect on the D-antigen recovery of HPC-containing film formulations directly upon drying was found (data not shown). For formulations containing 10% HPC (w/v), both sorbitol, sucrose and maltodextrin conferred protection of sIPV serotype 1. However, only the addition of sorbitol or maltodextrin

significantly improved D-antigen recoveries of sIPV type 2, whereas only adding maltodextrin resulted in higher sIPV type 3 recoveries (Figure 4). Oral films consisting of 15% (w/v) HPC profited only from the inclusion of sorbitol as revealed by significant improved D-antigen recoveries for type 1 and 2 (Figure 4). These results indicated that a more complex formulation is needed to obtain an oral dissolvable film formulation while maintaining D-antigenicity. This is in contrast with literature showing D-antigen recoveries of more than 50% for all serotypes with vacuum-dried Salk IPV in combination with only one of the carbohydrates, which were also tested in the current study [16, 18]. However, the combination of sorbitol, magnesium chloride ($MgCl_2$) and monosodium glutamate (MSG) stabilized Salk IPV both during freeze-drying and vacuum-drying to a higher extent than a formulation with sorbitol only [16, 18].

Effect of $MgCl_2$ and MSG during drying

Certain excipients or combinations of excipients, like $MgCl_2$ and MSG, have the ability to drastically improve IPV stability during drying [16, 18]. In order to evaluate the effect of $MgCl_2$ and MSG in an oral film formulation, these excipients were tested in combination with sorbitol in HPC-films. Until now, highest type 3 D-antigen recoveries were obtained for the oral film consisting of 10% (w/v) HPC and 5% (w/v) maltodextrin, so this formulation was also tested in combination with $MgCl_2$ and MSG. Unfortunately, formulations consisting of 15% (w/v) HPC and 5% (w/v) of a sugar/polyol in combination with MSG did not result in homogeneous casting solutions, so only 10% (w/v) HPC-films were evaluated in this experiment. In agreement with previous work, the combination of sorbitol, $MgCl_2$ and MSG was able to protect the polio vaccine during drying as revealed by significant higher type 2 and type 3 D-antigen recoveries directly upon drying when compared with the control formulation consisting of only 10% (w/v) HPC and 5% (w/v) sorbitol (Figure 5). D-antigen recoveries of type 1 were significantly higher than those obtained with only $MgCl_2$ or MSG added to the control formulation. For type 3, the most vulnerable serotype, the combination of sorbitol, $MgCl_2$ and MSG resulted in a substantial increase in recovery after drying. Using the carbohydrate maltodextrin, as a substitute for sorbitol, did not result in improved stabilization, but showed instead a significant drop in the D-antigen recovery of type 3. This experiment showed the potential of a sIPV-containing oral film formulation with less than 25% loss in D-antigen content during drying. The formulation consisting of 10% (w/v) HPC, 5% (w/v) sorbitol, 3% (w/v) $MgCl_2$ and 3% (w/v) MSG resulted in an oral film with D-antigen recoveries of 110 ± 10% for type 1, 83 ± 7% for type 2, and 76 ± 3% for type 3.

However, although D-antigen recoveries were ideal or at least high, all sorbitol-containing formulations resulted in sticky films that were difficult to remove from the release liner and felt very fragile. It would be hard to apply such a formulation into the oral cavity. This may be solved by further matrix optimization with focus on better physical properties with remaining high antigen recovery. Alternatively, preparation of bilayered oral films might result in formulations with suitable film mechanical properties allowing easy handling and application [20, 21].

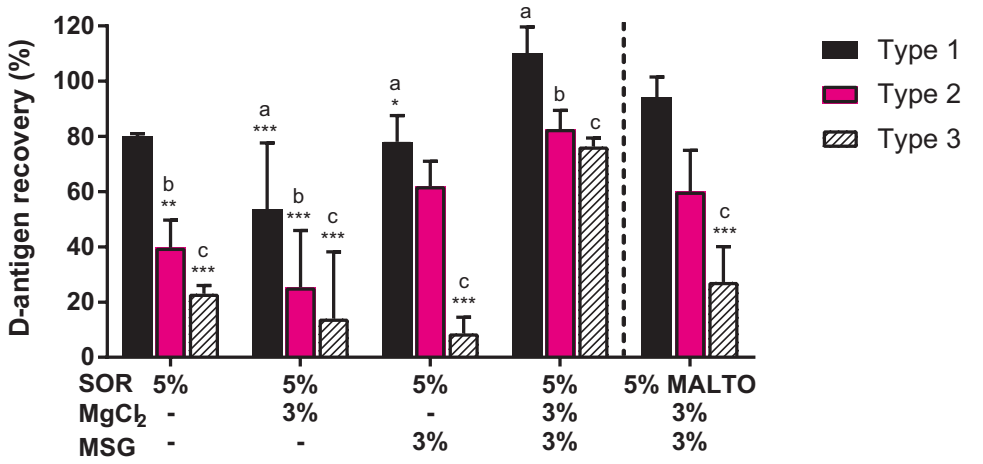

Figure 5 D-antigenicity of oral film formulations containing 10% (w/v) hydroxypropyl cellulose (HPC) as filmformer and 5% (w/v) of sorbitol (SOR) or maltodextrin (MALTO). The stabilizing effect of magnesium chloride ($MgCl_2$) and monosodium glutamate (MSG) was investigated. Directly upon drying, oral films were dissolved and D-antigen recoveries were determined for type 1 (black bars), type 2 (pink bars) and type 3 (striped bars) by ELISA. Mean values (n=3) and standard deviations are depicted. Asterisks indicate significant differences of formulation when compared with the formulation marked with the same letter above bars (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Sodium alginate and sodium carboxymethyl cellulose as film formers

Two polymers, i.e., sodium alginate (SA) and sodium carboxymethyl cellulose (CMC), were selected from literature and evaluated as film forming component in sIPV-containing oral films in order to try to improve film characteristics and maintaining high D-antigen recoveries. Preliminary experiments revealed that the addition of glycerol to SA-containing films resulted in more flexible films that can be easily removed from the release liner (data not shown). Therefore, oral film formulations comprising of 5% (w/v) SA, 5% (w/v) sorbitol and 1.25% glycerol were prepared and it was investigated whether the addition of both $MgCl_2$ and MSG was able to further improved D-antigen recoveries directly upon drying. In agreement with previous experiments with HPC as film former, the combination of sorbitol, $MgCl_2$ and MSG

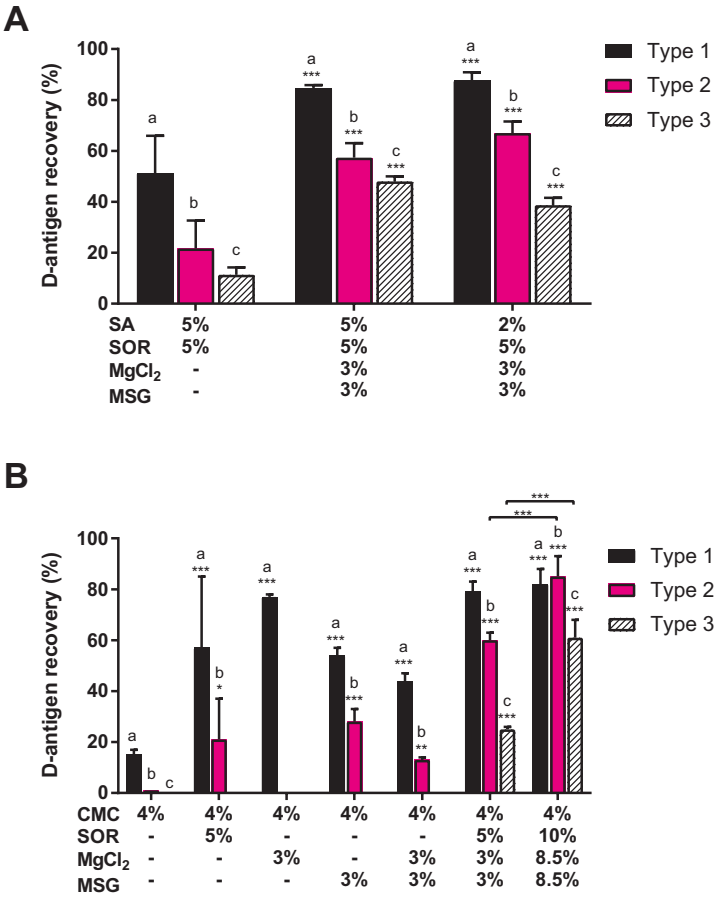

Figure 6 D-antigenicity of oral film formulations comprising of film formers sodium alginate (SA) (A) or sodium carboxymethyl cellulose (CMC) (B) as film former combined with a basic combination of sorbitol, magnesium chloride ($MgCl_2$) and monosodium glutamate (MSG). Directly upon drying, oral films were dissolved and D-antigen recoveries were determined for type 1 (black bars), type 2 (pink bars) and type 3 (striped bars) by ELISA. All SA-containing films containing also 1.25% (v/v) glycerol (A). Mean values (n=3) and standard deviations are depicted. Asterisks indicate significant differences of formulation when compared with the formulation marked with the same letter above bars (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

was able to protect the polio antigen during drying in an oral film formulation using SA as film former as exposed by significant higher D-antigen recoveries for all serotypes when compared to those obtained after drying the same formulation in the absence of $MgCl_2$ and MSG (Figure 6A). Using a lower concentration of film former, 2% (w/v) instead of 5% (w/v) SA, resulted in comparable D-antigen recoveries of 85-90% for type 1, 55-65% for type 2, and 40-50% for type 3.

Subsequently, the combination of sorbitol, $MgCl_2$ and MSG was evaluated in a formulation consisting 4% (w/v) CMC as film former. Besides, it was examined what the stabilizing effect was of each of these excipients was on the polio particle during drying. Directly upon drying, D-antigenicity of oral film formulations were assessed. Addition of each of the excipients (i.e., sorbitol, $MgCl_2$ and MSG) resulted in significant higher type 1 D-antigen recoveries, whereas the combination thereof resulted in significantly improved antigenicity of sIPV for all serotypes (**Figure 6B**). Type 3 showed again to be the most vulnerable serotype to stabilize during drying with detectable, but low recoveries (25 ± 1%) when using 5-3-3% (w/v) of respectively sorbitol, $MgCl_2$ and MSG. Addition of increasing amounts of the stabilizing components resulted in enhanced type 2 and type 3 D-antigen recoveries. The higher excipient concentrations (i.e., 10-8.5-8.5% (w/v) of respectively sorbitol, $MgCl_2$ and MSG) were based on the optimal formulation in our previous study on lyophilized IPV [16].

CONCLUDING REMARKS

The oral mucosa, especially the sublingual region, might be an attractive vaccine delivery site since it is densely populated with specialized dendritic cells while the adjacent submucosal tissue is drained by lymphatic vessels, through which free antigen as well as antigen-loaded dendritic cells can migrate to regional lymph nodes [4, 22]. However, although mucosal surfaces are the main route for pathogen entry, yet the induction of effective (mucosal) immunity elicited by vaccine antigens is a major challenge. Mechanistic studies designed to evaluate and define optimal conditions for sublingual or buccal vaccine delivery of macromolecules or even particles are still lacking in literature. Ideally, a minimal contact time of the dosage form for optimal antigen transport through oral mucosa should be defined. Also the question whether solid oral dosage forms would be preferred over liquid formulations are not answered. The risk of swallowing and/or salivary wash-out when administering liquid formulation to the oral mucosa exists, but liquid administration may also improve antigen uptake due to a larger contact area between vaccine and sublingual mucosa. Ideally, a head-to-head comparison is made between different oral films and (thermoresponsive) gel formulations with distinctive retention times to optimize mucosal contact area and contact time for sublingual delivery. Moreover, antigen transport through oral mucosa and uptake by immune cells could also be affected by muco-adhesive and penetration-enhancing components. The inclusion of such components in oral films might improve antigen delivery

via sublingual (or buccal) film application as well. Furthermore, for the induction of evident immunity (i.e., systemic and local mucosal immunity) upon vaccination under the tongue, inclusion of adjuvants may be required.

Aim of the current study was to evaluate the possibility to make polymer-based oral dissolvable films containing trivalent sIPV. In order to facilitate the formulation screening and optimization an antigen sparing ring-based film casting method was developed. Maintaining the antigenicity of the polio particle during film casting and drying showed to be challenging, especially for sIPV type 3. Addition of the excipients sorbitol, $MgCl_2$ and MSG protected the antigen during the drying process, irrespective of the film forming component that was used. This study yielded oral film formulations containing sIPV while preserving its D-antigenicity for 85-100% for type 1, 60-85% for type 2, and 50-75% for type 3. Amongst these sIPV film formulations, the sIPV film based on CMC-containing formulations showed highest D-antigen recoveries and is most appropriate for further product development in which several aspects need to be considered. The product profile of sIPV-containing oral films consisting of 4% (w/v) CMC, 10% (w/v) sorbitol, 8.5% (w/v) $MgCl_2$ and 8.5% (w/v) MSG gives a clear indication of the attributes, like stability, dissolution, disintegration and water content, that need to be evaluated during further development (**Table 5**). Due to shrinkage of the casting solution when using the standard casting method, it was not possible to obtain reproducible oral film formulations upon casting on a film casting apparatus. Since scalability of the production process is an important issue, further optimization of both formulation (e.g., inclusion of surfactant) and production process (e.g., release liner, casting speed) might be desirable. Moreover, based on future insights in the contribution of oral films to immunogenicity of sublingually administered sIPV, attention should be paid to further optimization of film characteristics, including mechanical properties for ease of handling and release kinetics.

Table 5 Product profile of sIPV-containing orodissolvable film consisting of 4% (w/v) sodium carboxymethyl cellulose, 10% sorbitol, 8.5% magnesium chloride and 8.5% (w/v) monosodium glutamate cast using the ring-based casting method. Mean values and standard deviation (n=3) are shown.

Attributes	Product profile (PP)	Target[1] achieved?
Dosage form	Orally dissolving film	Yes
Appearance	Transparent film Removable from release-liner	Yes Yes
Size Thickness	Ø 16 mm circle Not measured	Unknown Unknown
D-antigen recovery	82 ± 6% (type 1) 85 ± 8% (type 2) 61 ± 7% (type 3)	Unknown
Content uniformity Mass	Not measured 100 ± 2 mg	Unknown
Stability	Not measured	Unknown
Dissolution	Not measured	Unknown
Disintegration	Not measured	Unknown
pH	7.0 ± 0.1	Yes
Water content [2]	7.1 ± 1.8%	Unknown

[1] *For expected targets see target product profile (TPP) in table 2.*

[2] *Theoretical water content determined as percentage weight loss upon drying.*

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9

Summary, concluding remarks and perspectives

SUMMARY

Global polio eradication is closer than ever. The endgame strategy of the Global Polio Eradication Initiative (GPEI) includes a phased withdrawal of the live attenuated oral poliovirus vaccine (OPV) and the worldwide inclusion of the inactivated poliovirus vaccine (IPV) into all routine immunization programs [1]. Furthermore, the GPEI is pursuing several priority approaches for the development of a new generation of polio vaccines. Ideally, new polio vaccines should be administered through alternative (needle-free) delivery routes, provide mucosal immunity, be safe to manufacture, have a long shelf-life, be stable outside the cold-chain, and be affordable for low-income countries. Live polio vaccines attenuated with modern molecular techniques partially fulfill these requirements, but effective inactivated polio vaccines may be faster to develop and be more acceptable to the general public.

The aim of this thesis was to develop improved formulations and novel vaccine delivery strategies for polio vaccination using IPV. To improve storage stability of IPV, thermostable solid IPV formulations were developed for possible use in developing countries without the need of a cold-chain. Moreover, an alternative delivery system (i.e., Bioneedle) and mucosal delivery routes (i.e., intranasal and sublingual) were evaluated for use in IPV vaccination.

Chapter 2 provides the current status of alternative polio vaccine delivery strategies. The feasibility of these strategies is given by highlighting challenges, hurdles to overcome, and formulation issues relevant for optimal vaccine delivery. Important variables for the development of improved IPV are the route of administration, the selection of adjuvants, the vaccine formulation, and the use of (non-invasive) delivery methods.

Chapter 3 focuses on the development of a lyophilized IPV formulation with minimal loss during the drying process and improved stability when compared with the conventional liquid IPV. Extensive excipient screening was combined with a Design of Experiment (DoE) approach. Although earlier research revealed that lyophilization of a trivalent IPV while conserving its antigenicity is challenging, we developed a formulation that showed minimal loss of D-antigen during drying and subsequent storage at higher temperature. This study yielded a highly stable lyophilized polio vaccine formulation, which may be distributed without the need of a cold-chain.

Further research on lyophilized IPV was conducted and chapter 4 describes a clear difference in rat potency between lyophilized IPV and liquid IPV serotype 3 (upon drying 2 to 3-fold lower than in liquid form), whereas type 1 and 2 had unaffected antigenicity/

immunogenicity ratios. In addition, an approach to obtain a hexavalent vaccine by reconstituting lyophilized IPV with liquid pentavalent vaccine, containing diphtheria toxoid, tetanus toxoid, whole cell pertussis, Haemophilius influenza type B and hepatitis B (DTwP-Hib-HBV), was evaluated. Reconstituting dried IPV in the presence of thimerosal, a compound used in production of or added as antimicrobial in certain pentavalent vaccines, resulted in a fast temperature dependent loss in IPV antigenicity. Therefore, the use of lyophilized IPV as a component in a hexavalent vaccine by mixing licensed pentavalent vaccine, requires neutralization of thimerosal, to overcome the detrimental effect on the polio D-antigen. Use of a scavenger, like L-cysteine, to bind thimerosal (or mercury containing degradation products thereof), resulted in a hexavalent vaccine mixture in which polio D-antigen was more stable allowing an on-site mix-and-shoot approach.

Chapter 5 describes the development of an alternative delivery of a thermostable IPV in the solid state. Bioneedles, which are biodegradable mini-implants, are administered without the use of needles and syringes. Also, they have the potential to be stored and transported outside the cold-chain. Trivalent IPV was formulated in Bioneedles while remaining most of the polio D-antigenicity during the lyophilization process (D-antigen recoveries of >90% for all serotypes). Accelerated stability testing revealed that IPV in Bioneedles was more resistant to elevated temperatures than liquid IPV. *in vivo* imaging indicated that IPV administered via Bioneedles remained at the site of administration as long as subcutaneously injected liquid IPV, i.e. 3 days. This demonstrated that Bioneedles are not a controlled release vehicle, but dissolve quickly without forming a local depot (at least for IPV and the formulation used). Finally, an immunogenicity study showed that IPV-filled Bioneedles were able to induce virus-neutralizing antibody titers similar to those obtained by liquid intramuscular injection when administered in a booster regime, demonstrating the potential of Bioneedles as a syringe-free alternative delivery technology for polio vaccination.

Because of their large surface area and immunological competence, mucosal tissues are attractive administration and target sites for vaccination. An important characteristic of mucosal vaccination is its ability to elicit local immune responses, which act against infection at the site of pathogen entry. However, mucosal surfaces are endowed with tolerance mechanisms to prevent the immune system from overreacting to the many environmental antigens they encounter. In chapter 6, the characteristics of and approaches for sublingual and buccal vaccine delivery are described and compared with other mucosal vaccine delivery routes. Moreover, this review chapter highlights promising developments in the search for vaccine formulations, including adjuvants and suitable dosage forms, which are

likely critical for designing a successful sublingual or buccal vaccine. Finally, the challenges, hurdles to overcome and formulation issues relevant for sublingual or buccal vaccine delivery are outlined.

In chapter 7 the possibilities of polio vaccination via mucosal surfaces using IPV based on attenuated Sabin strains was evaluated. Both intranasal and sublingual Sabin IPV immunization induced systemic polio-specific serum IgG in mice that were functional as measured by poliovirus neutralization. Moreover, mucosal Sabin IPV delivery elicited polio-specific IgA titers at different mucosal sites (IgA in saliva, fecal extracts and intestinal tissue) and IgA-producing B cells in the spleen, where conventional intramuscular vaccination was unable to do so. However, it is likely that a mucosal adjuvant is required for sublingual immunization. This study indicates that both the intranasal and sublingual routes might be valuable approaches for use in routine vaccination or outbreak control in the period after complete oral polio vaccine cessation and post-polio eradication.

Chapter 8 describes the development of Sabin IPV-containing polymer-based oromucosal films suitable for sublingual or buccal vaccination. The combination of a Design of Experiment (DoE) approach and the evaluation of excipients with already proven stabilizing capacity for polio antigens was used to develop oral film formulations while preserving most of its D-antigenicity. This study revealed that the combination of sorbitol, magnesium chloride and monosodium glutamate has strong stabilizing potential for sIPV-films, even based on different film formers, i.e., hydroxypropyl cellulose, sodium alginate or sodium carboxymethyl cellulose. Although further optimization is required during future product development studies, especially with respect to the mechanical properties of the film formulations, this study showed the promise of dried polymer-based sIPV-films that might be suitable for sublingual or buccal polio vaccination.

GENERAL DISCUSSION

The phased withdrawal of OPV is mandatory to achieve global polio eradication, since OPV causes poliomyelitis in rare cases. The inclusion of IPV into all routine immunization programs will spur the need for better and more affordable IPV, because current manufacturing capacity and relatively high manufacturing costs prevent this. Ideally, a new generation of IPV should be easy and safe to administer, provide mucosal immunity, be safe to manufacture, have a long shelf-life, be stable outside the cold-chain, and be affordable for low-income countries. Alternative delivery strategies using improved formulations may fulfill at least some of these preferred vaccine characteristics. The relevance of this ideal target product profile also depends on the polio status worldwide (i.e., current situation with both OPV and IPV in use, after complete OPV cessation, post-eradication, or eventually, without routine polio vaccination), but also on the aim, i.e., for use in routine immunization programs or as outbreak control to interrupt transmission (**chapter 2**).

Thermostable IPV

Most vaccines, polio vaccines included, currently require storage and transport in refrigerators or freezers as exposure to higher temperatures may result in loss of the vaccines potency [2]. Unreliable access to electricity is a challenge that limits vaccine coverage in low and middle-income countries or may lead to administration of vaccines partially deteriorated due to storage and transport at too high temperatures. Replacement of existing vaccines with thermostable vaccines can relieve bottlenecks in vaccine supply chains and thus increase vaccine availability [3]. The economic impact of thermostable vaccines is immense. When vaccines no longer require cold storage, or could be kept out of the cold-chain long enough for their transport to remote areas, logistic costs will decrease. The cold-chain contributes about 20% of total system costs of vaccination, whereas vaccine wastage, at least partially caused by inappropriate storage and transport, add another ~20% to system costs [4]. Therefore, substantial reduction occurs in medical costs and diminished productivity losses as more vaccines reach the target population [5]. Many attempts have been made to develop thermostable formulations for antigens, including influenza [6-8], rotavirus [9], human papillomavirus [10, 11] and polio (**chapter 3, 4 and 5**). The question is which of these formulations may ultimately reach the market, since the development of thermostable vaccines brings several technological and regulatory challenges. Investments needed to tackle these hurdles may finally be worthwhile, since cost savings could compensate even for doubling or

even tripling the price charged for thermostable formulations of vaccines [5].

This thesis describes the development of IPV formulations with improved thermostability by converting the vaccine into the dry state. Lyophilization of Salk IPV in the presence of excipients sorbitol, magnesium chloride and monosodium glutamate resulted in a dried IPV formulation stable at temperatures up to 37°C for several weeks, whereas the conventional liquid IPV formulation showed significant loss in antigenicity when stored above ambient temperatures (chapter 3 and 4). However, the drying procedure might have a detrimental effect on the immunogenicity of the type 3 polio particle as revealed by a 2- to 3-fold lower rat potency as anticipated based on the measured antigenicity (**chapter 4**). This phenomenon (the loss of correlation between antigenicity and immunogenicity) was also seen in studies using solid IPV formulations administered via alternative delivery technologies, like Bioneedles (chapter 5) and dissolvable microneedles [12].

Stabilizing IPV, whether based on Salk or Sabin strains, by converting it into the solid state is very challenging with type 3 being the most vulnerable serotype for both Salk IPV and Sabin IPV. A formulation comprising sorbitol, monosodium glutamate and magnesium chloride protects the polio antigen from dehydration stress, even using different drying methods, like lyophilization (**chapter 3, 4 and 5**), vacuum drying [13] or air drying (**chapter 8**). A head-to-head comparison of the thermostability profiles of both (wildtype) Salk IPV and Sabin IPV is lacking in literature, so it remains speculative what the dissimilarities are between those polio particles. It would be interesting to investigate the impact of extensive thermostability testing, but also of different drying techniques on the integrity of Salk IPV versus Sabin IPV. A complicating matter in the assessment of their antigenic and immunogenic properties is the fact that the D-antigen is not well defined. The D-antigen ELISA can be used as *in vitro* alternative for the *in vivo* rat potency test for release of polio containing vaccines according to the European Pharmacopoeia monograph [14]. However, the set-up of the ELISA is crucial and should therefore be standardized among all polio vaccine manufacturers and research groups [15]. Moreover, a standardized *in vitro* measurement of the D-antigen, the calibration free concentration assay, which combines quantity and quality, may be suitable [16]. Biosensor analysis allows also a more extensive antigenic characterization by assessing different antigenic sites, a so-called antigenic fingerprint [16]. Furthermore, biophysical techniques might give a more clear view of the structural stability of IPV [17] and mechanisms involved in degradation or destabilization of (s)IPVs. With a combination of certain techniques, like field flow fractionation - multi-laser light scattering (FFF-MALS), circular dichroism (CD) and fluorescence spectroscopy, both particle size and (secondary and tertiary) structural integrity may be characterized.

Alternative delivery of polio vaccines

Different alternative administration methods and routes for polio vaccines have been developed and evaluated over the years (chapter 2). One of these delivery technologies are Bioneedles, which are hollow mini-implants from biodegradable polymers that can be filled with antigen followed by a lyophilization process. Bioneedles have the potential to avoid the cold-chain and they are administered without the use of needles and syringes. The feasibility of Bioneedles as vaccine delivery system has been shown preclinically with different antigens, including alum-adjuvanted tetanus toxoid [18], LpxL1-adjuvanted hepatitis B surface antigen [19], CAF01-adjuvated tuberculosis vaccine [20], and influenza vaccines [6]. In chapter 5 of this thesis, Bioneedles were filled with a trivalent IPV-formulation, containing excipients that were able to protect the polio antigen from degradation during lyophilization as evaluated earlier using glass vials (chapter 3 and 4). Lyophilized IPV, also when formulated in Bioneedles, was more resistant to elevated temperatures than liquid IPV. Moreover, IPV-filled Bioneedles were able to induce virus-neutralizing antibody titers similar to those obtained by liquid intramuscular injection when administered in a booster regime. Although this thesis demonstrated the preclinical proof of concept of Bioneedles for IPV, several steps should be taken in the further development of this alternative delivery system for polio vaccination, including toxicity and dose finding studies. Besides, safety concerns with respect to cross-contamination need to be addressed in the development of the applicator used for the administration. Thermostable vaccines formulated in Bioneedles might be very useful in lower- and middle-income countries, where the logistics for vaccine storage and transport under refrigerated conditions (cold-chain) are very limited or at least unreliable, interrupting the vaccine supply chain [3, 21-23]. Additionally, when an appropriate applicator for Bioneedle administration is developed, vaccine-filled Bioneedles may be quickly administered during mass vaccination campaigns. A phase 1 clinical study in healthy volunteers showed already that solid Bioneedles without any antigen were well tolerated [6]. Further (pre)clinical studies, using an approved administration device, should prove the practical use, safety and efficacy of Bioneedles for human vaccination.

The Global Polio Eradication Initiative (GPEI) is pursuing several priority approaches for the development of a new generation of IPV. To this extent, Intravacc has developed a new polio vaccine based on attenuated Sabin polio viruses, Sabin IPV, that is being transferred to local vaccine manufacturers to support post-eradication goals in terms of biosafety and IPV availability [24-27].

Important variables for the development of improved IPV are the route of administration,

the selection of adjuvants, vaccine formulations, and the use of (non-invasive) delivery methods [28]. Besides the use of Bioneedles for alternative IPV delivery, this thesis explored the possibilities of polio vaccination via mucosal surfaces using Sabin IPV (chapter 7). Mucosal vaccine delivery has the benefits of needle-free vaccination, like the relatively easy administration (which may avoid the need of trained personnel), eliminating the risk of needle-stick injuries or reuse of needles, and minimal generation of waste [29, 30]. Moreover, mucosal immunization has the potential to evoke strong mucosal immunity at the virus entry site. As we know from OPV, polio-specific mucosal immunity in the gut is a powerful mechanism for protection and interruption of polio transmission [31, 32]. In contrast to OPV delivered via the oral route, mucosal polio vaccination based on IPV might require the inclusion of an adjuvant to elicit appropriate immunity against polio, which was confirmed by the preclinical study in mice described in chapter 7. Both intranasal and sublingual (under the tongue) vaccination using Sabin IPV plus cholera toxin (CT) as strong mucosal adjuvant were able to significantly enhance functional systemic immunity as well as polio-specific IgA titers in mucosal samples compared to immune responses obtained after Sabin IPV vaccination without adjuvant (chapter 7). Although CT and the Escherichia coli-derived heat-labile toxin (LT) are well-known as potent mucosal adjuvants, these immune-potentiating components are not desired for further clinical testing as they are associated with adverse effects in humans. Concerns have been raised after an unwanted association between temporary facial nerve paralysis (Bell's palsy) and the intranasally administered inactivated influenza vaccine containing a detoxified mutant of LT (Nasalflu) [33]. Due to the possible neuronal binding capacity of CT or LT(-derived) molecules, resulting in migration to and accumulation in the central nerve system, nasal administration of certain toxin-based adjuvants is undesirable [34]. Therefore, the further development of a mucosal (Sabin) IPV should include the search for a safe mucosal adjuvant with strong immune potentiating capacity. Since in literature the mucosal route is minimally addressed for IPV yet, current experience is limited to the use of a double mutant of LT (dmLT) in combination with the sublingual route [35]. Other adjuvants that have shown their potential for (Sabin) IPV via the parenteral route could also be evaluated for mucosal vaccination, these include the LPS-derivate PagL [36], alphavirus-based GVI3000 [16], CAF01 [37], chitosan [38], CpG oligodeoxynucleotides [39] or vitamin D [40]. However, for a fast track to market, adjuvants with a proved safety record in humans might be preferred over components with immune potentiating capacity or delivery systems that are not licensed for other vaccines or even have been tested in clinical trials yet. With this point of view, CAF01 (phase I) [41], flagellin (phase II) [42], saponins (phase II) [43], CpG oligonucleotides (phase III) [44], or poly(I:C) (phase III) [45] might be worthwhile to test in combination with IPV delivered

via mucosal routes. Furthermore, the toxin-based dmLT (NCT02052934) and B subunit of CT (NCT00820144) have found to be safe in clinical phase I trials when administered via the sublingual route.

The sublingual route, vaccination under the tongue, has gained increased attention in recent years. In this thesis, the characteristics of and approaches for sublingual (and buccal) vaccine delivery are described and compared with other mucosal vaccine delivery sites (chapter 6). Besides the attractive features of mucosal vaccine delivery in general, which are mentioned above, sublingual vaccination may circumvent the safety issues that are associated with nasal vaccines. Possible deposition of vaccine components to the olfactory bulbs and nerves, which can cause Bell's palsy, might be avoided by sublingual administration [46-48]. For successful vaccination via the sublingual route, the main challenges are to overcome the mucosal barrier, improve tissue penetration, and increase vaccine potency. To reach immune competent cells, vaccine antigens have to overcome enzymatic and pH induced degradation, entrapment in the oral mucosa, and wash-out by salivary flow. The development of innovative oral dosage forms might be beneficial to improve antigen delivery into the sublingual tissue, and to facilitate access to resident antigen presenting cells. Chapter 8 of this thesis describes the development of polymer-based oral films containing Sabin IPV. The development of alternative delivery technologies using (trivalent) IPV in a dried form is challenging, due to the vulnerability of the polio antigen during the drying procedure and a possibly altered polio type 3 particle with reduced potency [12, 13, 49-51]. However, optimization of an IPV-containing oral film formulation should not only focus on maintaining the vaccine's potency, but also achieve optimal (mechanical) film properties. The design of films and the subsequent properties may influence the ease of handling and enhance antigen transport through the oral mucosa and uptake by immune cells. Unfortunately, mechanistic studies designed to evaluate and define optimal conditions for sublingual (or buccal) vaccine delivery of macromolecules or even particles are lacking in literature. Ideally, these studies should define a minimal contact time of the dosage form for optimal antigen uptake. Moreover, even the question whether solid oral dosage forms would be preferred over liquid or gel-like formulations should be answered. The risk of swallowing and salivary wash-out exist, but liquid administration may also improve antigen uptake due to a larger contact area between vaccine formulation and the sublingual mucosa. Thermoresponsive gels, which are aqueous solutions at room temperature that transform into gels when at body temperature on mucosal surfaces, might therefore be a good alternative in between liquid and solid dosage forms. The proof of concept of sublingual delivery of thermoresponsive gels containing IPV plus dmLT

as adjuvant in mice was described by White *et al*. [35]. However, this and our preclinical work on sublingual administration of IPV in mice revealed the poor immunogenicity of IPV when delivered via this relatively novel vaccination route.

Besides the sublingual immunotherapy products on the market, which are aimed at immune regulation instead of activation of the adaptive immune system, no sublingual (preventive) vaccine has been licensed to date. This may indicate the difficulty of this theoretically attractive immunization route. Nevertheless, the preclinical proof of concept of the sublingual route for vaccination against infectious diseases has been proven for several antigens (**chapter 6**). Live attenuated viruses and vector-based vaccines showed promising results in several rodent models as well as in non-human primates. In contrast, vaccines based whole inactivated or subunit pathogens might require adjuvant, which was also confirmed by this thesis by testing the sublingual route for Sabin IPV (**chapter 7**). Repeated boost immunization schedules might be essential for the induction of protective immune responses upon sublingual vaccine delivery. Besides homologous prime-boost strategies, using the same formulation and immunization route both for prime and booster vaccination, an increasing amount of research focuses on heterologous prime-boost approaches. Certain vaccination strategies, using different routes, different vaccine antigens, different vaccine concepts (e.g., live-inactivated or DNA-protein), or a combination thereof, are being investigated and some of them are also tested in combination with the sublingual route [52-55].

The real potential of sublingual vaccination still has to be proven in clinical studies. Recent findings of a head-to-head comparison of sublingually with intranasally applied live attenuated influenza vaccine (FluMist) indicate the promise of sublingual vaccination, although this may require live vaccine antigens or strong adjuvants. Clinical trials with enterotoxins, e.g., dmLT and CT-B subunit, via the sublingual route are underway. These clinical studies should provide insight in the general applicability of the sublingual route for vaccine delivery as well as the requirement and role of adjuvants, the potential and need of formulation strategies, as well as immunological readout and optimal correlates of protection for each vaccine specific sublingual vaccination strategies.

CONCLUDING REMARKS AND PERSPECTIVES

The cessation of OPV and inclusion of IPV into all global routine immunization programs may create a market for non-invasive delivery of polio vaccines. However, it remains unclear how large this market will be, since IPV demand in the post-eradication era is uncertain. A new generation of IPV should not only be affordable and safe to produce, but preferably should also induce mucosal immunity, remain stable at unrefrigerated conditions, and be easy to administer. This is also important with regard to stockpiling and outbreak management in the period after complete OPV withdrawal and post-polio eradication. Since more research groups have access to (Sabin) IPV via support from nongovernmental organizations (e.g., BMGF, WHO) and/or (new) Sabin IPV producers, more efforts to develop alternative administration technologies for IPV are expected in the coming years.

Furthermore, it is expected that other novel approaches, like the heterologous prime-boost schedules, will get more attention for polio vaccination upcoming years. IPV boosts mucosal immunity in recipients who have received OPV earlier [56, 57]. Furthermore, heterologous prime-boost approaches using a combination of parenteral and mucosal administration can significantly increase mucosal responses [53, 55, 58, 59]. Further (pre)clinical studies, whether based on homologous or heterologous prime-boost regimes, should include proper readout of mucosal immunity using modern techniques. The readout of mucosal immunity should not only be restricted to the detection of secretory IgA at mucosal sites, but those secretions (e.g., saliva, feces) and lavages (e.g., bronchoalveolar, intestinal) need also be used to investigate whether the antibodies are functional with respect to neutralizing the poliovirus. Besides the assessment of secretory IgA at mucosal sites, more and more attention is given to the duration as well as memory of mucosal immune responses. Until now preclinical vaccination studies on alternative delivery strategies lack the mucosal readout based on profiles of homing receptors. Measurement of circulating antibody-producing B cells expressing the mucosal integrin α4β7 might facilitate detection of mucosal secretory IgA responses at an early stage and could act as surrogate of mucosal immunity upon polio vaccination [60, 61].

In-depth knowledge on immune activation after immunization via sublingual mucosa is still lacking. Reports on systematic mechanistic studies for sublingual vaccination are limited. Systems biology approaches and other innovative strategies can provide comprehensive insights into immunity elicited by vaccine candidates delivered via mucosal routes, as well as

Table 1 Alternative delivery methods and routes that are currently under development for IPV delivery.

Alternative delivery methods (route of administration)	Pros	Cons	Current phase	Ref
Jet injector (intradermal)	+ Easy administration + No reformulation needed + Fractional dosing (?)	- Dependence of cold-chain - Administration device needed	Licensed (Phase 4)[1]	[68]
Microneedle patches (intradermal)	+ Easy administration + Thermostability might be improved (dissolvable MNs) + Fractional dosing (?)	- Packaging might be complicated (sensitive to humidity) - Reformulation needed	Phase 3 *NB1: several concepts in preclinical phase* *NB2: Phase 1 for influenza vaccine*	[67]
Bioneedles (subcutaneous)	+ Fast administration + Thermostable	- Reformulation needed - Applicator needed	Preclinical *NB: Phase 1 with solid Bioneedles without antigen*	[51]
Nasal spray (intranasal)	+ Easy administration + Inducing mucosal immunity + No reformulation needed	- Risk of wheezing in young children might exist - Possible deposition of antigen or adjuvant to CNS (Bell's palsy) - Dependence of cold-chain	Preclinical *NB: licensed for influenza vaccine (Nasalflu)*	[70]
Thermoresponsive gels (sublingual)	+ Easy administration + Inducing mucosal immunity	- Reformulation needed	Preclinical	[35]
Oral films (sublingual or buccal)	+ Easy administration + Inducing mucosal immunity + Thermostability might be improved	- Reformulation needed - Packaging complicated (sensitive to humidity)	Preclinical	

[1] *Post-marketing surveillance*

the kinetics of provoked immune responses [62]. Unfortunately, to get mechanistic knowledge on the oral mucosa as vaccine delivery route, researchers are still dependent on animal models, which are often not predictive for humans. The development of *in vitro* models for sublingual tissue and resembling immune cells might facilitate the screening of new vaccine candidates and adjuvants suitable for sublingual delivery and development of oral dosage forms [63-65]. Inherent to the status of development still many challenges will have to be addressed before good models are present. Better knowledge of the human mucosal immune system, especially during early life, is needed to ascertain the usefulness of alternative immunization routes, like the sublingual route. The mucosal immune system is highly compartmentalized and the limited understanding of molecular and cellular mechanisms that induce antigen-specific IgA antibodies has hampered the development of safe mucosal vaccines capable to promote IgA production at distant mucosal sites [48]. Nevertheless, *in vitro* models combined

with systematic *in vivo* studies may generate new insights, such as the role and potential impact of mucosal surface characteristics, penetration of antigens, presence of receptors and immune cells and identification of the best inductive sublingual immune cells.

This thesis describes the development of improved formulations and alternative delivery strategies for polio vaccination. Solid dosage forms that have improved thermostability, like biodegradable Bioneedles comprising lyophilized IPV or polymer-based oral films, would be favorable to reach remote areas in developing countries for which proper logistics are not available. Replacing the currently used polio vaccines with a thermostable vaccine may yield significant cost savings, even when the premium price is up to three times the price of the current non-thermostable vaccine [5]. However, one of the main challenges for future introduction of IPV formulations administered via other routes than the subcutaneous route is the acceptance by the final stakeholders, which include governmental organizations shaping their immunization programs, global vaccine procurement organizations (e.g., UNICEF), but also key opinion leaders, vaccine producers and vaccine recipients. The feasibility of (multiple) fractional doses of IPV using needle-free injector devices have already been demonstrated in several clinical trials and, as endorsed by WHO that started stockpiling PharmaJet Tropis®, this concept remains an option for outbreak control or can extend coverage if vaccine supplies are limited [66-69]. Ongoing and newly initiated research on innovative delivery methods for polio vaccination will teach us what the viability is of these approaches.

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Appendix

Nederlandse samenvatting
List of publications
Curriculum vitae

NEDERLANDSE SAMENVATTING

Polio

Poliomyelitis, ook wel polio genoemd, is een ernstige en besmettelijke infectieziekte die veroorzaakt wordt door het poliovirus. Nadat het poliovirus het lichaam, meestal via de mond, is binnengedrongen, zal het zich in de darmen vermenigvuldigen. Het virus kan van mens op mens worden overgedragen via de ontlasting van besmette personen. In veel gevallen veroorzaakt infectie met het poliovirus helemaal geen of slechts milde (griepachtige) symptomen. Echter, in minder dan 1% van de gevallen zal het poliovirus doordringen tot het centraal zenuwstelsel (het ruggenmerg of de hersenstam) waardoor spierverlammingen op kunnen treden. De verlammingen treden meestal op aan de benen of armen, omdat het virus de zenuwen beschadigt die de bewegingen van deze lichaamsdelen aansturen. Omdat de zenuwen van slik- en ademhalingsspieren meestal worden aangetast, overlijdt ongeveer 5 tot 10% van de polio patiënten met verlammingsverschijnselenOverige patiënten houden blijvende verlammingen of herstellen, na intensieve fysiotherapie, maar gedeeltelijk. Bovendien krijgt ongeveer 40% van de patiënten die een poliovirus infectie hebben doorgemaakt zelfs na tientallen jaren opnieuw te maken met spierzwakte, pijn in spieren en gewrichten, hevige vermoeidheid en vermindering van spierweefsel. Dit wordt het ‘post-polio syndroom’ genoemd.

Besmette personen scheiden het poliovirus gedurende enkele weken uit waardoor het zich snel kan verspreiden in de populatie, met name in ontwikkelingsgebieden waar de hygiëne en sanitaire voorzieningen slecht zijn. Vooral jonge kinderen die nog onvoldoende ‘toilet-training’ hebben gehad zijn een directe bron van besmetting die kan leiden tot een polio uitbraak. Er bestaat geen medicijn om polio patiënten te genezen. Wel kan polio voorkomen worden met behulp van vaccinatie. Wanneer voldoende kinderen zijn gevaccineerd tegen polio, zal het virus zich niet verder kunnen verspreiden onder de bevolking en zal het verdwijnen omdat de mens de enige natuurlijke gastheer is voor poliovirus.

Polio vaccinatie

Er bestaan twee vaccins tegen polio. Het eerste poliovaccin werd in de jaren ’50 ontwikkeld door Jonas Salk. Dit is het ‘geïnactiveerde poliovirus vaccin’, vaak afgekort als IPV (vanuit het Engelse ‘inactivated poliovirus vaccine’), bestaat uit met formaldehyde geïnactiveerd (gedood) poliovirus. Er bestaan drie serotypes die zodanig van elkaar verschillen dat alle drie serotpyes ook in het vaccin moeten zitten. Het vaccin wordt toegediend via een injectie in de

spier en biedt bescherming door in het bloed antistoffen op te wekken die specifiek gericht zijn tegen de drie types van het poliovirus. Hoewel het vaccin goed werkt en veilig is, was het moeilijk om er voldoende van te produceren. In de jaren ’60 ontwikkelde Albert Sabin het orale poliovirus vaccin, afgekort als OPV, dat bestaat uit levend verzwakte poliovirus (Sabin) stammen. Omdat het verzwakte vaccin zich vermenigvuldigt na toediening kan met een lagere dosis dan het geïnactiveerde vaccin worden volstaan. Een ander belangrijk voordeel van dit oraal (via de mond) toegediende vaccin is dat het niet alleen in het bloed antistoffen opwekt, maar ook in de darmen waar het poliovirus het lichaam binnendringt bij een infectie. Daarbij kan in gebieden in de wereld waar de hygiëne en sanitaire voorzieningen slecht zijn, vaccinatie met OPV resulteren in passieve vaccinatie van personen die zelf niet gevaccineerd zijn. Bij een polio uitbraak kan door middel van OPV vaccinatie de overdracht van persoon op persoon in korte tijd gestopt worden waardoor verdere verspreiding van het (wildtype) poliovirus binnen de hele gemeenschap tegengegaan wordt.

Tabel 1 De voor- en nadelen van de verschillende polio vaccins: het levend verzwakte oraal toegediende polio vaccin (OPV) en het geïnactiveerde polio vaccin (IPV).

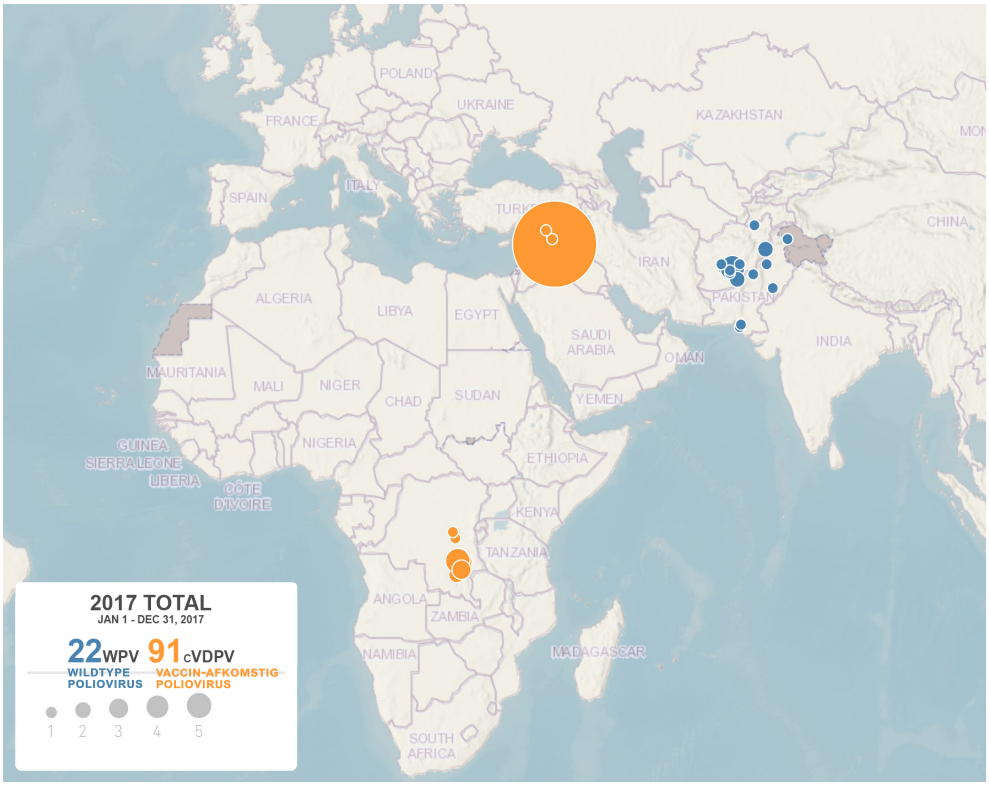
	Oraal polio vaccine (OPV)	Inactief polio vaccine (IPV)
Voordelen	+ Goedkoop *(betaalbaar voor ontwikkelingslanden)* + Makkelijke toediening *(druppelen op tong, soms op suikerklontje)* + Lokale bescherming (in de darm) *(na natuurlijke polio infectie zal het lichaam direct een beschermende reactie geven en het virus opruimen)* + Effectief *(biedt langdurige bescherming)*	+ Veilig *(vaccin kan geen polio veroorzaken)* + Redelijk stabiel *(vaccin wordt bij 2-8°C bewaard en verzonden)* + Zeer effectief *(slechts 1 of 2 vaccinaties nodig om langdurige bescherming te bieden)*
Nadelen	- Risico op poliomyelitis *(vaccinvirus kan soms muteren naar een vorm die verlammingsverschijnselen kan veroorzaken)* - Risico op nieuwe polio uitbraak *(vaccinatie kan leiden tot hercirculatie van terug gemuteerd poliovirus)* - Zeer gevoelig voor hogere temperaturen *(vaccin wordt in vriezer bewaard en getransporteerd)*	- Duur *(ruim vijf keer duurder dan OPV, tot voor kort voornamelijk gebruikt in Westerse landen)* - Geen lokale bescherming *(lichaam kan poliovirus niet direct in de darm opruimen. Geïnfecteerde persoon wordt niet ziek, maar kan het virus wel verspreiden)* - Getraind personeel nodig voor toediening - Risico op hergebruik van naalden

IPV is zeer veilig. Echter, OPV veroorzaakt in extreem zeldzame gevallen (2 tot 4 keer op een miljoen gevaccineerden) verlammingsverschijnselen. Daarnaast komt het binnen een gemeenschap waarin de vaccinatiegraad laag is (zeldzaam) voor dat het verzwakte vaccinvirus blijft circuleren en kan veranderen in een vorm die wel polio veroorzaakt. Deze vaccin-afkomstige poliovirussen kunnen dus leiden tot nieuwe polio-uitbraken. Een belangrijk voordeel van OPV is dat het vaccin goedkoper is te produceren en beter betaalbaar is voor landen met lage en middeninkomens. Omdat IPV geen levend vaccin is, heeft het de risico’s

op polio symptomen en ontstaan van nieuwe uitbraken niet. Echter, dit vaccin is ruim vijf keer duurder dan OPV. Daarbij brengt de toediening door middel van injectie in de spier waarbij gebruikt wordt gemaakt van spuit en naald, andere nadelen met zich mee. Vaccinaties zullen gegeven moeten worden door getraind personeel en er zijn steriele injectiematerialen en procedures nodig om hergebruik van naalden en ongelukken met naalden (prikincidenten) te voorkomen. In tabel 1 staan de voor- en nadelen van zowel OPV als IPV opgesomd.

Polio de wereld uit

In 1988 werd het Global Polio Eradication Initiative (GPEI), oftewel de wereldwijde publiek-private samenwerking van nationale overheden en partijen zoals o.a. de wereldgezondheidsorganisatie (WHO), UNICEF en de Bill & Melinda Gates Foundation, opgericht. Dit initiatief is in het leven geroepen met als doel om polio wereldwijd uit te roeien. Het aantal poliogevallen is sindsdien met meer dan 99.9% afgenomen. In de late jaren '80 veroorzaakte poliovirus verlammingen bij meer dan 1000 kinderen per dag. In 2017 was



Figuur 1 Geografsche weergave van alle poliogevallen die werden gemeld in het jaar 2017. De blauwe bolletjes geven de meldingen van wildtype poliovirus en de oranje bolletjes de gevallen van vaccin-gerelateerde polio gevallen. *(bron: www.polioeradication.org)*

dat afgenomen tot 22 poliogevallen van het wildtype poliovirus en 91 vaccin-gerelateerde poliogevallen (**zie figuur 1**). Er zijn nog drie landen waarin poliovirus voorkomt: Afghanistan, Nigeria en Pakistan.

Twee van de drie wildtype poliovirussen zijn inmiddels wereldwijd uitgeroeid. De laatste melding van polio type 2 dateert van 1999 en de meest recente melding van wildtype poliovirus type 3 werd gedaan in November 2012 (**zie tabel 2**). Echter, het blijkt een grote uitdaging te zijn om echt alle polio infecties te stoppen en het poliovirus volledig de wereld uit te helpen, onder andere door conflictgebieden, politieke instabiliteit, zeer moeilijk te bereiken populaties, en soms gebrekkige infrastructuur. Daarom is er in 2013 een uitgebreid strategisch eindplan gelanceerd waarin verschillende stappen staan beschreven om polio dan echt de wereld uit te helpen en hoe de wereld vervolgens polio-vrij kan blijven. Allereerst moet de verspreiding van het wildtype poliovirus zo snel mogelijk gestopt worden, maar moeten nieuwe polio uitbraken als gevolg van vaccin-afkomstige poliovirusssen ook worden tegengegaan. Belangrijke maatregelen die in gang gezet zijn, zijn het versterken van poliovaccinatieprogramma's wereldwijd én het gefaseerd terugtrekken van het gebruik van OPV voor routine vaccinaties. Er wordt nu alleen nog maar gevaccineerd met een bivalent OPV, met daarin alleen de verzwakte poliovirus stammen van type 1 en type 3. Immers, poliovirus type 2 is al sinds 1999 de wereld uit en alle meldingen van polio type 2 infecties worden sindsdien veroorzaakt door het vaccinvirus (OPV type 2). Daarom zijn alle landen die voorheen trivalent OPV (met type 1, 2 en 3) gebruikten in hun vaccinatie programma's overgestapt op bivalent OPV om zo het risico op hercirculatie van polio type 2 te verkleinen. Daarbij wordt in deze landen tenminste 1 dosis trivalent IPV gegeven om de kinderen wel bescherming te geven tegen het type 2 poliovirus. Zeker in gebieden waar het risico op polio infectie nog aanwezig is. Al deze maatregelen resulteren in de huidige situatie waarin de uitroeiing van het poliovirus zijn voltooiing nadert.

Een ander doel van het 'strategische eindplan' richt zich op hoe de verschillende regio's in de wereld polio-vrij kunnen blijven en hoe alle voorraden van het poliovirus en de poliovaccins vervolgens veilig bewaard moeten worden na uitroeiing van het poliovirus.

Hoewel er al vele successen zijn geboekt, blijken sommige doelen van het eindplan tot op heden niet haalbaar binnen de gestelde termijnen. Hieraan liggen zowel externe (bv. groeiende conflictgebieden) als interne factoren (bv. beperkte beschikbaarheid van IPV, onvoldoende toezicht). Nu het poliovirus alleen nog maar voorkomt in een zeer beperkt aantal gebieden in de wereld, zal de uitroeiing van polio afhankelijk zijn van de inzet van vaccinatieteams die

ervoor moeten zorgen dat ieder kind in hoog-risico gebieden gevaccineerd wordt. Zelfs de kinderen die in extreem afgelegen en moeilijk bereikbare gebieden wonen, of op de vlucht zijn.

Tabel 1 Overzicht van wereldwijd alle polio gevallen van de afgelopen jaren (2012-2018). Zowel meldingen van wildtype poliovirus als hercirculatie van poliovirus afkomstig van het vaccinvirus (OPV) zijn weergegeven.

		2012	2013	2014	2015	2016	2017	2018
WILDTYPE POLIOVIRUS								
Endemische landen[1]	Type 1	217	160	340	74	37	22	14
	Type 2	0	0	0	0	0	0	0
	Type 3	21	0	0	0	0	0	0
Niet-endemische landen	Type 1	6	256	19	0	0	0	0
	Type 2	0	0	0	0	0	0	0
	Type 3	0	0	0	0	0	0	0
VACCIN-AFKOMSTIG POLIOVIRUS								
Endemische landen[1]	Type 1	0	0	0	0	0	0	0
	Type 2	33	55	52	3	2	0	5
	Type 3	0	0	0	0	0	0	0
Niet-endemische landen	Type 1	0	0	1	20	3	0	4
	Type 2	35	10	3	9	0	96	14
	Type 3	3	1	0	0	0	0	3

[1] Landen waar circulatie van het poliovirus onder de bevolking onafgebroken plaats vindt: Afghanistan, Nigerië en Pakistan.

Nieuwe generatie poliovaccins

Zoals hierboven beschreven is de uitroeiing van polio aanstaande. Juist nu is er grote behoefte aan nieuwe en verbeterde poliovaccins. Idealiter kan een nieuwe generatie poliovaccins de voordelen van de huidige vaccins (IPV en OPV) combineren; is het eenvoudig (naaldvrij) toe te dienen, biedt het lokale bescherming (in de darm), is het veilig om te produceren, lang houdbaar en betaalbaar voor lage inkomenslanden? Daarbij zou een poliovaccin dat buiten de koelkast of vriezer bewaard en vervoerd kan worden van grote waarde zijn. Door verschillende onderzoeksgroepen in de wereld wordt er gewerkt aan de

ontwikkeling van alternatieve manieren om poliovaccins toe te dienen. Naaldvrije vaccinatie strategieën hebben als groot voordeel dat er geen risico bestaat op hergebruik van naalden en prikincidenten. In **hoofdstuk 2** wordt een overzicht gegeven van de verschillende alternatieve toedieningsroutes die momenteel voor polio in verschillende stadia van ontwikkeling zijn. Met name de toediening via de huid (dermaal) is voor poliovaccins uitgebreid onderzocht, omdat het idee is dat via dermale vaccinatie met een lagere dosis vergelijkbare bescherming verkregen wordt in vergelijking tot de conventionele toediening via naaldinjectie (in de spier). In dit hoofdstuk staat ook beschreven waar de moeilijkheden liggen om goede nieuwe vaccinformuleringen te ontwikkelingen die, idealiter, ook eenvoudig zijn toe te dienen.

Thermostabiele vaccins

De meeste vaccins, en poliovaccins zijn geen uitzondering, moeten gekoeld of soms zelfs ingevroren bewaard en vervoerd worden, omdat blootstelling aan hogere temperaturen de effectiviteit van het vaccin kan aantasten. Echter is in lage- en midden inkomens landen de beschikbaarheid van elektriciteit niet vanzelfsprekend, waardoor het niet eenvoudig is om de zogenoemde ‘cold-chain’ (koude keten), ofwel onafgebroken gekoelde condities tijdens opslag en vervoer van vaccins, in stand te houden. Het is niet zeldzaam dat partijen vaccins weggegooid moeten worden omdat ze niet onder de juiste condities bewaard zijn. Dit komt de beschikbaarheid van vaccins uiteraard niet ten goede. De vervanging van bestaande vaccins door verbeterde (thermostabiele) vaccinformuleringen die buiten de koeling bewaard kunnen worden zouden vaccin bevoorrading kunnen vereenvoudigen en de wereldwijde beschikbaarheid van vaccins sterk verbeteren.

Een veelgebruikte manier om de stabiliteit van vaccins te verbeteren is door het te drogen tot een vaste stof. In **hoofdstuk 3** staat de ontwikkeling van een gevriesdroogde IPV formulering beschreven. Het is een uitdaging gebleken om IPV te vriesdrogen met behoud van effectiviteit van het vaccin. Na een uitgebreide screening van allerlei suikers, zouten en aminozuren werden uiteindelijk een aantal stoffen geselecteerd die in staat waren het IPV te stabiliseren tijdens het droogproces. Door vervolgens gebruik te maken van statistische modellen (een ‘Design of Experiment’ benadering) is een optimale formulering ontwikkeld waarbij het IPV gevriesdroogd kan worden met minimaal verlies tijdens het droogproces en verbeterde thermostabiliteit in vergelijking tot het huidige vloeibare IPV. Deze verbeterde vaccin formulering kan mogelijk buiten de ‘koude keten’ vervoerd worden en dus bijdragen aan de beschikbaarheid van IPV in moeilijk bereikbare gebieden.

In **hoofdstuk 4** staat het vervolgonderzoek van het gevriesdroogde IPV beschreven. Hieruit blijkt dat er een duidelijk verschil bestaat tussen de effectiviteit van het gevriesdroogde type 3 vaccin (2 tot 3 keer minder immunogeen getest in een diermodel) en het vloeibare vaccin, waar voor type 1 en type 2 geen verschillen zijn waargenomen tussen het vloeibare en gevriesdroogde vaccin. Mogelijk is het type 3 poliovirusdeeltje tijdens het drogen toch iets aangetast. Dit effect is kennelijk alleen aantoonbaar in proefdieren en niet in de *in vitro* test. De oorzaak van dit verschil is momenteel niet bekend. Daarnaast is er gekeken naar de mogelijkheid om een hexavalent vaccin (6 vaccins in 1) te ontwikkelen door een al bestaand vloeibaar pentavalent vaccin tegen difterie, (a-cellulair) kinkhoest, tetanus, Heamophilius influenza type B en hepatitis B (DaKT-Hib-HepB, 5 vaccins in 1) samen te voegen met het gevriesdroogde IPV. De aanwezigheid van thiomersal, een antimicrobieel middel, in het bestaande pentavalent DaKT-Hib-HepB vaccin zorgt er echter voor dat het poliovaccin binnen een dag afneemt in werkzaamheid. Buiten de koelkast (bij kamertemperatuur (25°C) of lichaamstemperatuur (37°C)) is dit afbraakproces zelfs al na enkele uren zichtbaar. In **hoofdstuk 4** is aangetoond dat het schadelijke effect van thiomersal op het poliovaccin grotendeels ongedaan gemaakt kan worden door cysteïne toe te voegen. Cysteïne is een natuurlijk voorkomend aminozuur en is door een specifieke functionele groep (thiol, SH-groep) in het molecuul in staat om de thiomersal weg te vangen door hieraan te binden. Het is daardoor mogelijk om een 6-in-1 vaccin te verkrijgen door gevriesdroogd IPV op te lossen met een bestaand 5-in-1 combinatievaccin, een zogenoemd mix-and-shoot oplossing.

Bionaalden

Zoals benoemd wordt het vloeibare IPV normaliter toegediend door middel van intramusculaire injectie (in de spier) met spuit en naald. Deze conventionele manier van toediening heeft enkele nadelen, zoals het risico op prikongelukken waardoor kans bestaat op besmettingen met bijvoorbeeld hepatitis B. Daarbij is het afvoeren van naaldafval een ingewikkeld en prijzig proces waarbij hergebruik van naalden niet uitgesloten wordt, met name in ontwikkelingslanden. De vraag naar alternatieven voor naalden wordt echter ook steeds groter in westerse landen door de angst en stress die kan ontstaan bij kinderen die gevaccineerd worden (en hun ouders). Dit is nadelig voor de acceptatie van vaccins. In **hoofdstuk 5** staat de ontwikkeling van een alternatieve toediening voor IPV beschreven waarbij gebruik werd gemaakt van Bionaalden. Dit zijn holle mini-implantaten gemaakt van een biologisch afbreekbaar zetmeel, die gevuld kunnen worden met een vaccin waarna een vriesdroogproces volgt. Wanneer een met vaccin gevulde Bionaald onderhuids wordt

toegediend (idealiter onder hoge druk met een applicator), zal de Bionaald oplossen en het vaccin vrijkomen in het lichaam. De vriesdroogformulering die werd ontwikkeld in hoofdstuk 2 is hier toegepast in het Bionaald-concept, een alternatieve toedieningsvorm waarbij geen spuiten met naalden nodig zijn. Daarnaast hebben Bionaalden als belangrijk voordeel dat ze mogelijk buiten de koude keten bewaard en vervoerd kunnen worden. Het IPV in de Bionaalden bleek beter bestand te zijn tegen hogere temperaturen dan het conventionele vloeibare IPV. Een studie in ratten liet zien dat na twee vaccinaties, de IPV-Bionaalden vergelijkbare bescherming opwekten in vergelijking tot het vloeibare vaccin. We hebben hierbij de potentie van Bionaalden als alternatieve poliovaccinatie methode laten zien waarbij met name de thermostabiliteit van IPV-Bionaalden van grote waarde kan zijn in lage- en middeninkomens landen.

Mucosale vaccinatie: via de neus en onder de tong

Door de grote oppervlaktes en immunologische eigenschappen zijn de slijmvliezen, ook wel mucosa genoemd, interessante routes voor vaccintoediening. Een van de belangrijkste eigenschappen is dat mucosale vaccinatie niet alleen bescherming geeft in het bloed, maar daarnaast ook lokale immuunreacties kan opwekken op slijmvliezen elders in het lichaam, daar waar ziekteverwekkers, zoals virussen en bacteriën, het lichaam binnen kunnen dringen. In **hoofdstuk 6** staan de mogelijkheden van vaccinatie via slijmvliezen in de mond beschreven: buccale (binnenkant wang) en sublinguale (onder de tong) vaccinatie. De laatste jaren is er in toenemende mate onderzoek gedaan naar sublinguale toediening van vaccins. Hoofdstuk 6 geeft een overzicht van de resultaten die hierover gepubliceerd zijn en beschrijft wat de moeilijkheden zijn die moeten worden onderzocht voordat er succesvolle sublinguale vaccins op de markt zullen verschijnen. De toevoeging van een adjuvant, een stof die het immuunsysteem stimuleert, aan het vaccin lijkt voor vaccinatie onder de tong onvermijdelijk. Het immuunsysteem in de mondholte wordt namelijk voortdurend uitgedaagd om wel of niet te reageren op van alles wat er in de mond terecht komt. Er heeft zich daarom een tolerantie mechanisme ontwikkeld waardoor het immuunsysteem niet alles als indringer detecteert en dus niet overal meer op reageert. Bovendien zijn de slijmvliezen juist bedoeld om indringers niet tot het lichaam door te laten dringen. Bij vaccinatie willen we juist wel dat er een immuunreactie plaatsvindt en vandaar dat er manieren moeten worden gezocht om het tolerantiemechanisme te omzeilen en het vaccin op de juiste plek zijn werk te laten doen. Speciale toedieningsvormen en formuleringen, zoals orale films of gels die na toediening blijven plakken aan de orale mucosa, kunnen mogelijk bijdragen om het gewenste effect te bereiken na vaccinatie via de mondholte.

In **hoofdstuk 7** is onderzocht of IPV-toediening via mucosale routes potentie heeft om als nieuwe generatie poliovaccins te dienen. Zowel poliovaccinatie via de neus (intranasaal) als onder de tong (sublinguaal) zijn onderzocht door muizen via deze routes te vaccineren met IPV en de opgewekte immuunreacties te meten in zowel het bloed als ook in mucosale afscheidingen. Zowel intranasale als sublinguale IPV vaccinatie bleken in staat om in het bloed poliovirus-neutraliserende antistoffen op te wekken. Daarnaast werden er ook mucosale antistoffen tegen polio gemeten in het speeksel, de ontlasting (feces) en de darmen, terwijl conventionele toediening via injectie in de spier dat niet doet. Uit deze studie blijkt ook dat voor sublinguale IPV vaccinatie de toevoeging van een adjuvant nodig is om het immuunsysteem voldoende te stimuleren. Zowel de intranasale als sublinguale route lijken waardevolle poliovaccinatie strategieën om te gebruiken in vaccinatieprogramma's of om een onverwachte polio-uitbraak snel te kunnen stoppen in de periode waarin OPV niet meer gebruikt wordt.

Zoals hierboven beschreven, kunnen nieuwe vaccinformuleringen mogelijk zorgen voor een optimale opname van het vaccin door de orale mucosa en de gewenste immuunreactie daaropvolgend. In **hoofdstuk 8** staat de ontwikkeling van gedroogde orale films voor sublinguale of buccale poliovaccinatie beschreven. Net als in **hoofdstuk 2** bleek het wederom een uitdaging om het IPV te drogen, nu in films, met behoud van de werkzaamheid van het vaccin. De combinatie van sorbitol, magnesium chloride en glutamaat, die tijdens vriesdrogen in staat was het vaccin te stabiliseren, was ook nu succesvol bij de ontwikkeling van een filmformulering. Een andere uitdaging was om de gewenste eigenschappen van de film te behouden tijdens het drogen; een film die makkelijk toe te dienen is moet bijvoorbeeld flexibel zijn, makkelijk vast te houden zijn en niet te plakkerig (mogen niet blijven plakken aan je hand, wel aan de mucosa). De studie die in **hoofdstuk 8** beschreven staat laat zien dat het mogelijk is om een filmformulering te ontwikkeling met behoud van de antigene structuur (de structuur die door antistoffen wordt herkend) van het poliovaccin. Hoewel optimalisatie nodig is tijdens verdere productontwikkeling, waarbij met name gekeken moet worden naar de gewenste eigenschappen van de filmformulering, lijken orale films potentie te hebben voor sublinguale of buccale poliovaccinatie.

Conclusies en vooruitblik

Het gefaseerd terugdringen van OPV en includeren van IPV in alle routine vaccinatieprogramma's over de gehele wereld, creëert een markt voor poliovaccins die worden toegediend via alternatieve routes. Een nieuwe generatie IPV moet niet alleen betaalbaar en veilig zijn, maar idealiter ook lokale mucosale immuunreacties opwekken, stabiel blijven buiten de koude keten, en makkelijk toe te dienen zijn. Dit is ook belangrijk met het oog op de periode waarin poliovaccins langere tijd opgeslagen moeten worden om plotseling massaal ingezet te worden bij een uitbraak (eventueel na mogelijke bioterroristische aanslag) nadat polio de wereld uit is.

Dit proefschrift beschrijft de ontwikkeling van verschillende verbeterde formuleringen en alternatieve toedieningsstrategieën voor poliovaccinatie. Gedroogde vaccinformuleringen zijn beter bestand tegen hogere temperaturen en kunnen mogelijk buiten de koelkast bewaard en vervoerd worden. Bionaalden met daarin gevriesdroogd IPV of gedroogde filmformuleringen zouden gebruikt kunnen worden om afgelegen gebieden in ontwikkelingslanden te bereiken waar dat met de reguliere logistiek onmogelijk is. Het vervangen van de huidige poliovaccins door thermostabiele IPV formuleringen zouden enorme kostenbesparingen opleveren, zelfs wanneer de initiële vaccinprijs tot drie keer hoger is dan de huidige vaccins.

Toekomstige (klinische) studies waarbij innovatieve toedieningsvormen voor poliovaccinatie verder onderzocht moeten worden zullen ons leren wat de haalbaarheid is van deze nieuwe vaccinatie strategieën.

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CURRICULUM VITAE

Heleen Kraan was born on the 15th of January in 1984 in Gouda. After graduating from St.-Antoniuscollege in Gouda in 2002, she started her study Biology at Utrecht University and obtained her Bachelor's Degree. In 2008, she started working at the Netherlands Vaccine Institute (NVI). Next to her job, she did a pre-Master program in 2009 after which she continued her study with the Master's program Drug Innovation at Utrecht University. In 2012, she obtained her Master's degree in Pharmaceutical Sciences. In the same year, she started her PhD study on alternative routes and new delivery strategies for polio vaccines within the Delivery Technology project at the Institute for Translational Vaccinology (Intravacc). This work was performed under the supervision of Prof. dr. Gideon Kersten (Intravacc, University of Leiden), Prof. dr. Erik Frijlink (University of Groningen) and dr. Jean-Pierre Amorij. In recent years, she worked on numerous projects on vaccine formulation and delivery of different antigens. Currently, she is working as project leader and scientist within the Analysis, Delivery and Formulation department at Intravacc.