GM Vaccines: From bench to bedside

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J.H.C.M. Kreijtz
B. Ramezanpour
K.D.S. Fernald
L.H.M. van de Burgwal

GM VACCINES: FROM BENCH TO BEDSIDE

An investigation performed for The Netherlands Commission on Genetic Modification (COGEM)

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Disclaimer Authors

The authors do not claim completeness of the data that was analysed since they were dependent on the data in the public domain and the information provided in the databases which was not always complete In this report are included: included information from databases (registered vaccines, clinical trials, and patents), websites, literature, interviews and conferences. The information regards registered vaccines, clinical trials and preclinical work. The authors of this report are not liable for any actions taken on the basis of the data in this report.

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Authors

Project leader: Dr. JHCM Kreijtz
Team members: B. Ramezanpour, MSc
K.D. S. Fernald, MSc
L.H.M. van de Burgwal, MSc

Preface

Since its introduction more than 30 years ago, gene technology has successfully contributed to the development of vaccines saving the life of millions of people and animals. Genetic modification is often used in the production of vaccine constituents but also to engineer harmless viruses and bacteria that can provide protection against disease. Proper risk assessment is always done before introduction of this type of vaccines into the population. To ensure optimal environmental risk analysis and to anticipate possible societal implications of emerging new vaccine gene technology, it is highly desirable to have an overview of the currently used recombinant vaccines and the expected developments in this field. This overview is not available. To accommodate the existing need, COGEM has commissioned the writing of an independent report on the market penetration of recombinant vaccines, the types of vaccines that are used, and the expectations of producers regarding future introduction of novel types of genetically engineered vaccines.

The research project was assigned to Dr. J.H.C.M. Kreijtz and his team of the Viroscience Lab, Erasmus MC, Rotterdam. Based on their expertise and analysis of the literature, an overview has been compiled about the genetically modified (GM) vaccines from bench to bedside. The report describes the current market of GM vaccines and indicates vaccines in clinical and preclinical stages of development. Deep interrogation of a patent database and expert interviews yielded valuable insight into the latest trends in the field of vaccine development. Together, the report signals the genetic modification is a firmly established and safe technology both in the production and design of vaccines worldwide. Currently about 10% of registered vaccines are gene technology-based products, but many more are under development. The panel of experts corroborate the technical potential of genetic modification for smart vaccine design and in the manufacturing process to make safer and more efficacious vaccines and signal the importance of timely discussion with registration authorities about the development and opportunities of new technologies.

Prof. dr. J.P.M. van Putten
Chairman advisory committee
Faculty of Veterinary Medicine, Utrecht University
COGEM subcommittee 'Medisch Veterinair'

Delineation of Genetically Modified Vaccine definitions

We have drafted the following delineation of genetically modified vaccine definitions to enable proper selection and interpretation of relevant data

Vaccine: A biological preparation that induces immunity to one or more antigens, typically derived from a disease-causing agent

Genetically modified organism (GMO): An organism of which the genome has been altered genetically by non-natural means in order to modify its phenotype

Genetically modified (GM) vaccine: A vaccine that is produced using genetic modification. Examples are recombinant antigen(s), (self-amplifying) DNA/RNA and vaccines that consist of genetically modified organisms

Non-genetically modified vaccine: A vaccine based on the wildtype pathogen or a part thereof, be it live-attenuated, inactivated or a single purified antigen

Genetically modified (GM) production platform: A platform that 'applies' a GMO for the production of pathogens or one or more antigens that are to be used as a vaccine

The term ***'genetically modified'*** is often also referred to as ***'recombinant'*** by others

Co-injected: The vaccine itself contains the GMO and because of this the use of co-injected vaccines requires a biological containment strategy to limit spreading of the GMO

Non-retrievable: The vaccine is an end product of a process in which a GMO was used, however the GMO is not included in the vaccine itself

Definitions and Abbreviations

ALVAC	Acronym for vaccine technology based on administration of live Canarypox virus
Antigen-by-design	The process of designing an antigen that is subsequently generated through synthetic biology and can be presented in different vaccine formats, e.g. recombinant protein, DNA or vector vaccine
BARDA	Biomedical Advanced Research and Development Authority (United States)
BRICS	Acronym for the association of five major emerging national economies: Brazil, Russia, India, China, and South Africa
BSL-2	Biosafety level 2: the level of containment for laboratory facilities that is required to work with biological agents of moderate hazard to the laboratory workers and the environment (e.g. seasonal influenza A virus)
Chimeric vaccine	Vaccine that is based on two pathogens from one genus (e.g. Flaviviruses)
CHO cells	Chinese Hamster Ovary cell line
CPC code	Code used by the EPO (European Patent Office) and USPTO (United States Patent and Trademark Office) to allow for targeted searches for prior art.
DIVA vaccines	Differentiating Infected from Vaccinated Animals
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency (works closely with the 28 European Union Member States as well as the European Economic Area countries (Norway, Iceland and Liechtenstein)
FDA	Food and Drug Administration (United States)
HIV	Human Immunodeficiency virus
HPV	Human Papillomavirus
MS	Multiple Sclerosis
MVA	Modified Vaccinia virus Ankara
PCV vaccine	Vaccine against Porcine CircoVirus type 2
R&D	Research and development
RNA	Ribonucleic Acid
RVFV	Rift Valley Fever Virus
Vaccinee	A person or an animal that is being vaccinated
WHO	World Health Organization

Vaccine types

Inactivated vaccine

A vaccine in which the pathogen is killed by chemical or physical treatments. The pathogen can then be used as a whole (whole-inactivated vaccines (WIV)) or can be broken down into smaller pieces consisting of fragments or proteins (e.g. subunit or split virion vaccines).

Live-attenuated vaccine

A vaccine in which the targeted pathogen is used in an attenuated or 'weakened' form. This attenuation can be achieved in various manners: with chemicals, by temperature adaptation or by introducing mutations or deleting certain genes or parts thereof applying recombinant DNA techniques.

Recombinant protein vaccine

The vaccine contains one or more antigens in the form of proteins that have been produced by organisms that are genetically modified to express the recombinant protein(s) and are in that way used as vaccine production platforms (e.g. bacteria, yeasts, plants).

Toxoid

A toxoid is based on the toxin produced by certain bacteria. Toxins can be inactivated through thermal or chemical treatment in order to destroy toxic property but retain antigenicity thus generating a toxoid. Vaccination with the toxoid can induce an immune response to the original toxin.

VLP: Virus-like particle

A vaccine formulation that consists of structures that resemble a virion (virus particle), but does not contain viral genes (lacks RNA/DNA). The VLP is constructed by expressing multiple structural proteins of a virus in the same platform based on a genetically modified organism (e.g. insect cells in combination with a baculovirus expression system, as used for the production of Cervarix®[1], an HPV vaccine) that then produces the VLP's.

Vector vaccine

A vaccine that is based on the use of a certain microorganism as a vehicle for the delivery of a foreign gene derived from the pathogen that is targeted by the vaccine. E.g. a Modified Vaccinia virus Ankara can be used as a vector to deliver the HA gene from influenza A virus to function as an Influenza HA vaccine. Vectors can either be replication competent (e.g. adenovirus serotype 5) or replication deficient (e.g. MVA). In the description of the clinical trials obtained from the ICTRP database (see Chapter 3), dendritic cell vaccines were indicated as vaccines but it can be debated whether this should be corrected to immunotherapy based on the European guidelines. In the analysis of the clinical trial data we have included the dendritic cell vaccines. The dendritic cells in these vaccines are modified through e.g. recombinant DNA technology or infection with a viral vector. Dendritic cell vaccines are meant for smaller target populations and in that manner differ from the typical vaccines that are more universally applied.

Virosome

A vaccine based on a vesicle with a phospholipid membrane in which viral proteins are embedded. These vesicles are capable of fusion with target cells, hereby delivering the viral proteins in the cell and thus mounting a stronger immune response.

[1] http://www.rivm.nl/dsresource?objectid=rivmp:116768&type=org&disposition=inline&ns_nc=1

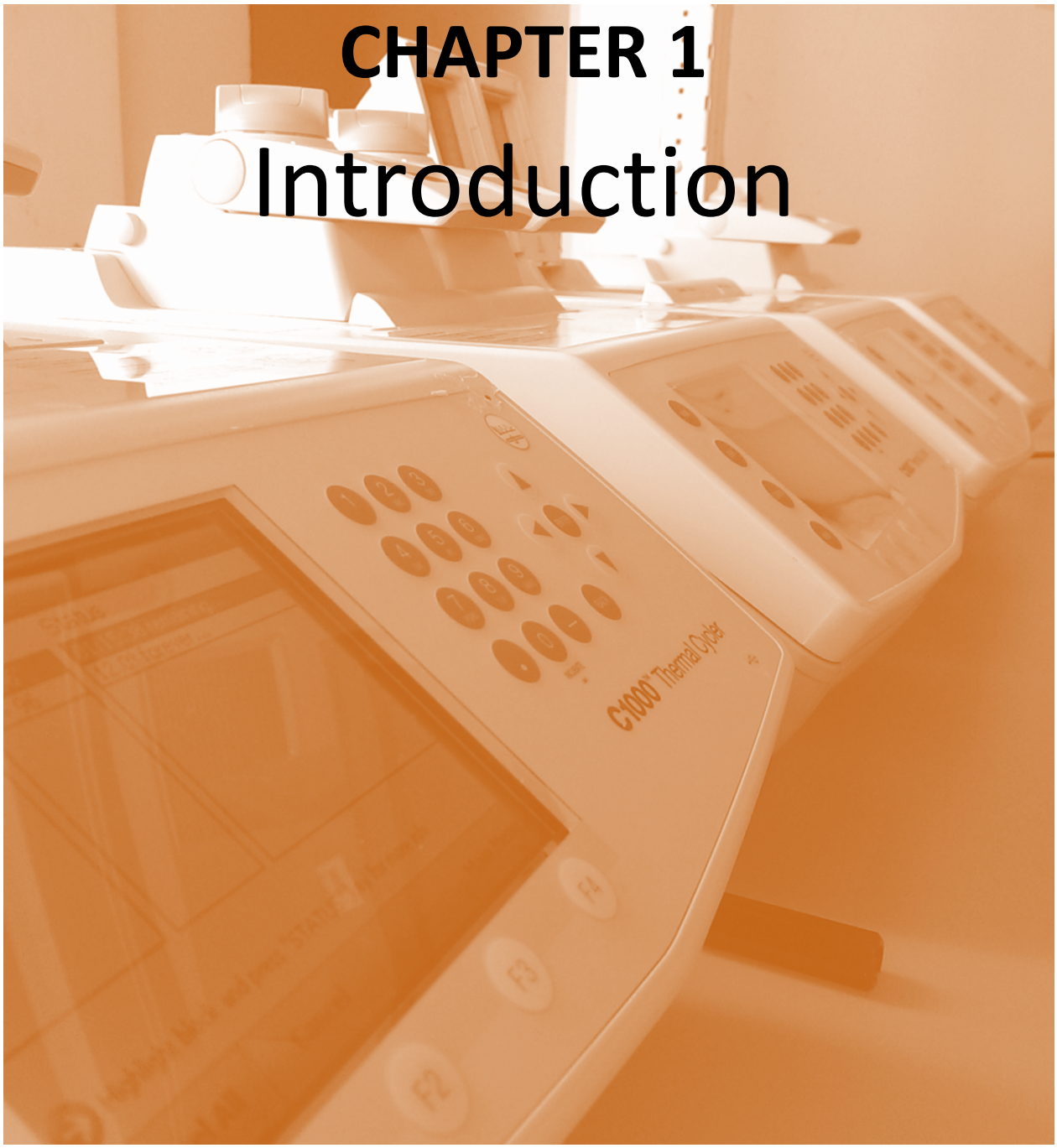
Limitations of this study

We do not claim completeness of the data we have analysed since we were dependent on the data in the public domain and the information provided in the databases which is not always complete (e.g. patents are only filed 18 months after their submission) or indexed in a manner that allowed for a systematic search for genetically modified (GM) vaccines. In order to be able to process the data we used the definitions as defined on page 6-8 of this report and made assumptions based on what our research team found to be relevant for the state-of-art and technological advancements in GM vaccine development. Where possible we indicate limitations of the searches and available information so that the spectrum of the data set is clear to the reader.

Although we had the ambition to make a complete database spanning all GM vaccines (available and in development), we found out early on that this was a mission impossible within the time span and resources of the project. Other complications were: lack of clear GMO indications for vaccines in the different databases and incompleteness of certain databases (searching all university and company websites by hand was not possible within the current project setting). In order to compensate for these limitations we aimed at a quantitative and categorized analysis of the patents, clinical trials and registered vaccines in order to provide an overview of the direction in which the field is going. The interviews and conferences proved to be of great value to provide depth and insights on the role of genetic modification in the vaccine field.

The veterinary field is not as well documented as the human field, especially for zoo and wildlife animals not much is known on available vaccines and those that are still under development. Therefore these were discussed in more general terms in the indicated sections. Furthermore veterinary vaccines are registered based on the data obtained from registration studies, however data on these studies is not available in the public databases. Therefore we were dependent on literature and public databases to get an indication of the 'clinical' development of veterinary vaccines, which proved to be insufficient and thus we were not able to map the clinical stage of veterinary vaccines within the scope of this project.

In the scientific literature the discrimination between non-recombinant and recombinant technology and vaccine construction through genetic modification is ill defined. Through targeted searches we aimed to compensate for this. We chose to search for reviews instead of individual research articles since the number of hits during exploratory searches ran in the tens of thousands and analysis of such a dataset would not fit within the time schedule and financial resources of this project.



CHAPTER 1
Introduction

1. Introduction

1.1 Background

Since the introduction of the concept of vaccination by Edward Jenner in his publication in 1798, the field of vaccinology has come a long way. Traditionally, vaccines are derivatives of the targeted pathogen, be it the inactivated pathogen itself, a part of it or a live-attenuated version of it. Vaccines based on these conventional technologies have been and are still successfully used to fight infectious diseases, limit the impact of epidemics and pandemics and eradicate human and veterinary infectious diseases. There are numerous pathogens, however, for which no vaccines are available. Furthermore, some vaccine-matched pathogens adapt and drift into escape variants, forcing the necessity of frequent vaccine antigen updates. Additionally, conventional vaccine technology does not always match the current standards and requirements for vaccine production anymore.

The issues sketched ask for 'smarter' vaccine design, construction and production. The introduction of genetic modification (recombinant technology) and other molecular biological techniques allows for isolation, modification and optimization of vaccine antigens and can facilitate smart vaccine design. With such technology the generation of vaccines against virtually all pathogens becomes possible. And these technologies even open opportunities for vaccine development beyond the realm of infectious diseases. New indications and application fields of vaccines are: tumor immunology, autoimmune diseases, allergies and addiction. In the veterinary field also more practical indications are targeted, e.g. DIVA (Differentiating Infected from Vaccinated Animals) vaccines.

A striking illustration of these developments is the use of genetic modification for the generation of high-growth reassortant influenza vaccine strains. These processes use reverse genetics or defined antigen production in genetically modified cell lines, bacteria and other, more novel production platforms such as plants and even algae. The technology also makes it possible to construct more complex vaccines based on vectors such as adenoviruses and various poxviruses that can be used to encode one or more foreign genes.

These novel vaccine platform technologies are in different stages of development and in the veterinary field multiple genetically modified (GM) vaccines are already available. The first recombinant human vaccine, Recombivax HB®, was approved in 1986, seven years after the characterization of the hepatitis B antigen. After this, no new GM vaccines were licensed for human years for decades. In recent years, however, human vaccine development is catching up. Some examples: the live-attenuated seasonal influenza vaccine (Flumist®/Fluenz®, Medimmune, USA) is produced with recombinant DNA technology and is available in Europe and the US. The first recombinant protein influenza vaccine (Flublok®, Protein Sciences, USA) is available in the US since last year and the first viral-vectored vaccine (IMOJEV®, Sanofi Pasteur, Australia), for Japanese Encephalitis, is available in Australia. These are a few of the successful GM vaccines made it from the concept phase towards market authorization.

GM vaccines and GM-based production platforms require proper regulation, depending on the level of complexity of the genetic modification. Incorporating the national and international guidance and regulations (e.g. from CBG, EMA and FDA) in the vaccine development process is a complex exercise.

Here we present insights and trends on GM vaccine development reaching from patents to registered vaccines and perspectives from experts in the field. This report can be used as a reference framework for the evaluation of current rules and regulations and the formulation of new guidance documents for the future.

1.2 Project Outline

1.2.1 Goal of the project

The goal of this project, as defined by the COGEM, is to provide insight in the 'market penetration' of GM vaccines, which types are there and what are the expectations of manufacturers and other experts in the field?

In order to provide a structured overview of the current market and the vaccine development pipeline[2], the project is divided in two parts, each with their own research questions, applying to both the human and veterinary field.

1.2.2 Part A: Generate an overview of available GM vaccines and GMO production platforms

Research questions

1) What GM vaccines are currently in preclinical development?

2) What GM vaccines are in clinical phase 1/2/3?

3) What GM vaccines are in the phase of market authorization?

4) What GM vaccines are already on the market?

To address these questions two search strategies were used. The first search strategy focused on databases for registered vaccines, clinical trials and patents. The second search was aimed at literature describing preclinical and clinical vaccine studies. Detailed information on the search terms and data analysis of the search results can be found in the respective chapters in this report.

Table 1 Overview of Chapters

Chapter	Search	Development phase	Goal
2	Registered vaccines	Market	Inventory of registered GM vaccines
3	Clinical trial registers	Bedside (phase 1/2/3/4)	Inventory of clinical trials with GM vaccines
4	Review articles	Bench (preclinical)	Overview of preclinical and to a certain extent clinical evaluation of

[2] Based on discussions with the steering committee it was decided to focus on the GM vaccines and not so much the GMO production platforms since these not necessarily result in a GM vaccine per se. Therefore the latter are addressed in this report but to a minimal extent.

			GM vaccines
5	Conferences	Full pipeline	Register the latest trends and innovations
6	Patents	Concept	Overview of novel technologies for GM vaccines and GM vaccine production

The latest developments and key vaccine innovations were inventoried at three different conferences focussing on vaccine development and GM based vaccines as discussed in chapter 5. The observed trends are integrated with the results and information distilled from the interviews (Part B as described below).

Combined, the database and literature searches and the conference data form an overview of the current state of practice and what vaccines are on the verge of entering (pre)clinical development. Herewith we fulfil Part A of the project.

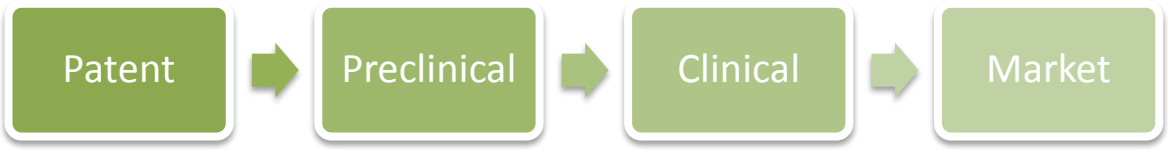


Figure 1 The route of a vaccine from the drawing table to the market

1.2.3 Part B: Mapping the potential of GM vaccines and GMO-based vaccine production

Research questions

1) What is the technical potential of genetic modification in vaccine development?

2) What is the market potential of genetically modified vaccines?

3) What is the application potential of genetically modified vaccines?

4) What are the hurdles in the development and registration pipeline of genetically modified vaccines?

To address these questions, interviews were held with experts-in-the-field as described in Chapter 7. These experts were selected from the different sectors of the vaccine field in order to cover the views from the different phases of the vaccine development pipeline: concept → preclinical → clinical → market. We not only asked them what the potential of GM vaccines and GMO-based vaccine production is but also what they consider to be necessary to use the full potential of these technologies to make sure that they do not strand in the (pre)clinical phase as unredeemed promises for the future. Details on the interview strategy are described in Chapter 7.

As an extension of Part B, a GM vaccine forum is organised as the closing session of a renowned vaccine conference with representatives from two vaccine disciplines (Academia and Regulatory Authority).

After an introductory lecture to set the scene on the role of genetic modification in vaccine development, two lectures will be held followed by a discussion on how the innovations in GM vaccine development can be further boosted to bring more GM vaccines from bench to bedside.[3]
Although the traditional vaccine development stages, as depicted in Figure 1, often start with patent applications and end in market implementation, in this report we follow the opposite trajectory. This will provide the reader a clear overview of current vaccines and future trends in order of market proximity.

1.3 Report Outline

In Chapter 2 – The Market, the registered human and veterinary vaccines are discussed, followed by Chapter 3 – Clinical development in which we discuss the clinical trials that are described for GM vaccines. Next is Chapter 4 – Preclinical development in which the trends in vaccine technology are addressed based on literature on *in vitro* and *in vivo* evaluation of new vaccines. The latest trends from three GM- and vaccine-oriented conferences held in 2014 are discussed in Chapter 5 – Latest trends, followed by the more conceptual and early-stage vaccine technologies that are described in the patent literature in Chapter 6 – Patents. In Chapter 7 – Expert opinions, the results from the interviews are presented. In principle in every chapter both human and veterinary vaccines are discussed, unless information for the latter was not publicly available.

Finally in Chapter 8, a summarizing discussion is provided in combination with highlights, which can also be found at the end of each chapter, and in the last chapter recommendations are provided, based on the data presented in this report.

[3] http://www.terrapinn.com/conference/world-vaccine-congress-europe/programme.stm

CHAPTER 2

The Market

2. Market

The aim of this chapter is to provide an overview of registered GM vaccines, both for human and veterinary applications, regarding vaccine pathogens (Figure 5), application fields (Figure 6 and Figure 7), vaccine development / production technologies (Figure 8).

2.1 Methodology

Various governmental databases have been explored in order to gather all registered vaccines for human use in the following regions; USA, EU, four of the five BRICS countries (Brazil, India, China and South-Africa; Russia was not included due to the language barrier), Australia and Japan, see Figure 2. The BRIC countries have been selected due to their fast growing economies and industrialization potential. Governmental databases from all these countries sufficed to provide us with the licensed vaccines.

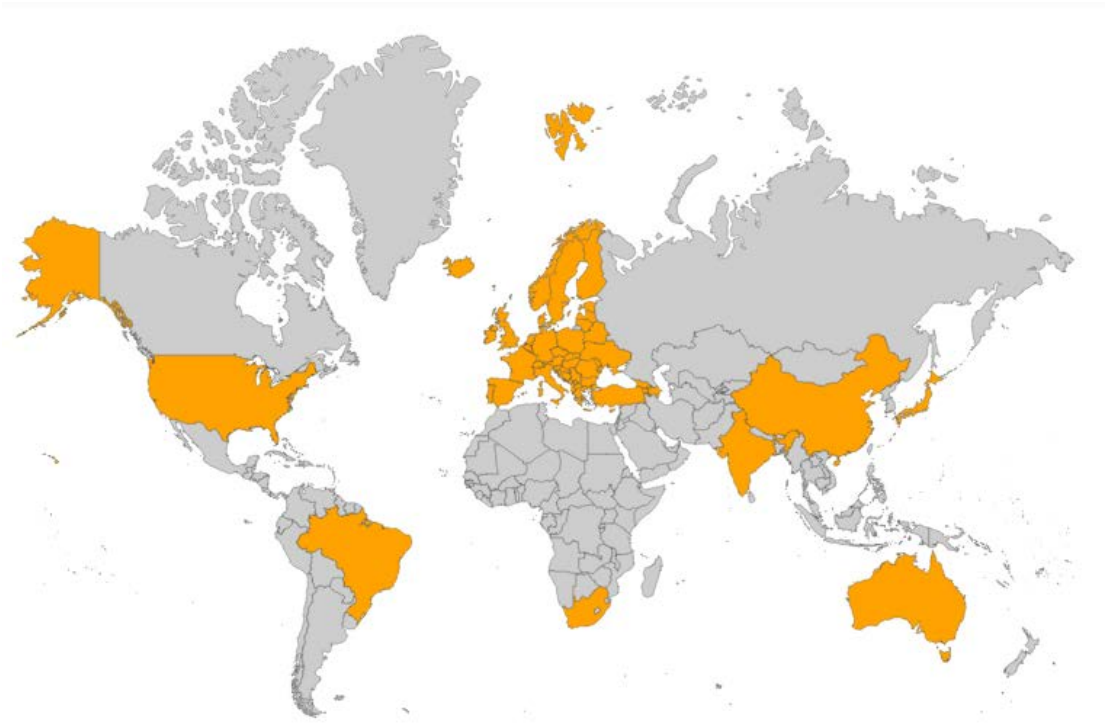


Figure 2 World map with the countries for which the registered human vaccines were inventoried

Furthermore, veterinary vaccines have been selected from the vetvac database. This database contains commercially available livestock vaccines worldwide[4]. The database reflects the information that is provided by manufacturers and hence if a vaccine is described as genetically modified by the manufacturer, this information has been integrated into the database.

Table 2 illustrates the governmental databases per country from which the registered vaccines have been collected. Most vaccines are registered in China and India with a total of 317 and 218 vaccines respectively. According to our data, Brazil has the least licensed vaccines. Fiocruz foundation guarantees

[4] http://www.vetvac.org

the Brazilian self-sufficiency in essential vaccines to respond to public health demands of the Brazilian vaccination schedule of the ministry of health. Their intention of being self-sufficient regarding product and service developments that meet the needs of the Brazilian public health could explain the low number of internationally registered vaccines from Brazil. In addition, some licensed vaccines may be missed in our research due to the language barrier.

Table 2 Databases used for collection of vaccines registered in specified countries

Country	Database	Results[5]
	HUMAN	
US	U.S. Food and Drug Administration (FDA)	100
EU	European Medicines Agency (EMA)	41
Brazil	Oswaldo Cruz Foundation, known as Fiocruz[6]	9
India	Central drugs standard control organization (CDSCO), Medguide India	218
China	China Food and Drug Administration (CFDA)	317
South Africa	South African vaccination and immunization center (SAVIC)	37
Australia	Government Department of Health, Register of Therapeutic Goods	75
Japan	Pharmaceuticals and Medical Devices Agency	24
Total		**821**
	VETERINARY	
Global	Vetvac	**2697**

[5] The following search terms have been applied in order to identify/classify the selected vaccines (the asterisk (*) being used as a boolean character): Genet*, Modif*, Engin*, DNA / RNA, Recombin*, Vector, Chimeric, VLP/ Virus-like, Virosome. The final dataset was checked manually to delete false-positive results that were obtained with these search terms.

[6] Institute guarantees the Brazilian self-sufficiency in essential vaccines.

2.2 Registered GM vaccines

According to the governmental databases from the selected countries, there are 821 registered human vaccines available on the market. 10% (83 out of 821) of these vaccines are GM vaccines. This classification is defined as: vaccines that are produced using genetic modification (e.g. recombinant antigen(s), (self-amplifying) DNA/RNA and vaccines that consist of genetically modified organisms). Current research illustrates that China has the most licensed human GM vaccines on the market, followed by EU and India, respectively. Figure 3 illustrates results obtained from various databases (Table 2) selected for registered human vaccines and human GM vaccines per region. In the following paragraphs these results will be further analyzed on the basis indications that these vaccines target (paragraph 2.3; 2.4) and the vaccine technology platforms (paragraph 2.5).

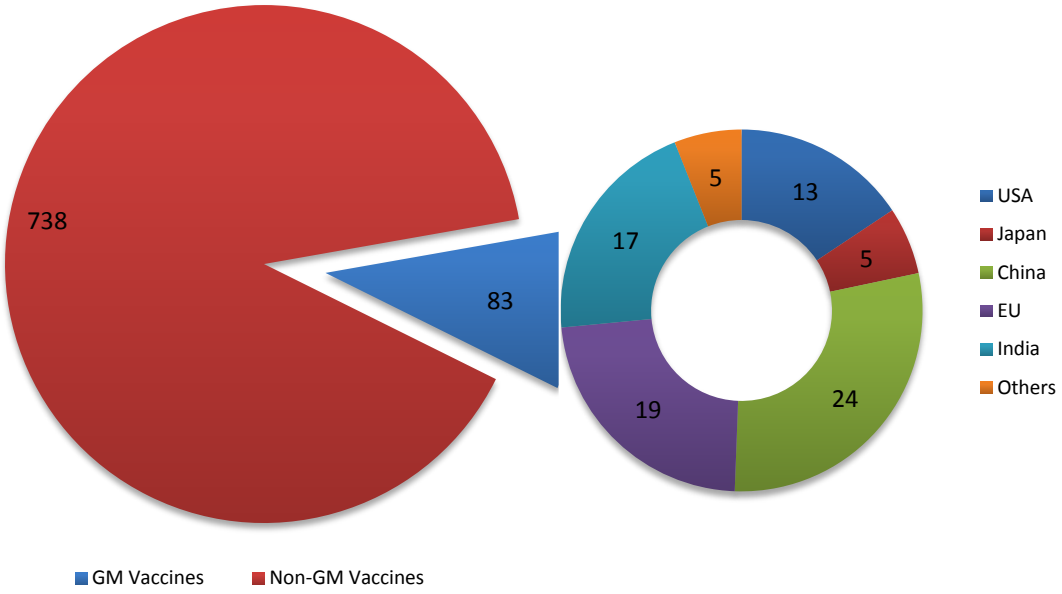


Figure 3 Registered human GM vaccines and subdivision of vaccines per region (USA, Japan, China, EU, India, Others)

Similarly, approximately 10% of veterinary vaccines (285 out of 2697) are genetically modified, Figure 4. In Europe, GM vaccines have to meet criteria and requirements of the EU pharmaceutical legislation for both human and veterinary applications in order to obtain marketing authorization within the EU.[7] These requirements are more stringent than requirements in countries and regions outside the EU. Consequently, only 19% of all available veterinary GM vaccines are being distributed in EU countries.

[7] Myhr AI, Traavik T (2012) Genetically Engineered Virus-Vectored Vaccines – Environmental Risk Assessment and Management Challenges. Genetic Engineering - Basics, New Applications and Responsibilities http://cdn.intechopen.com/pdfs-wm/25756.pdf

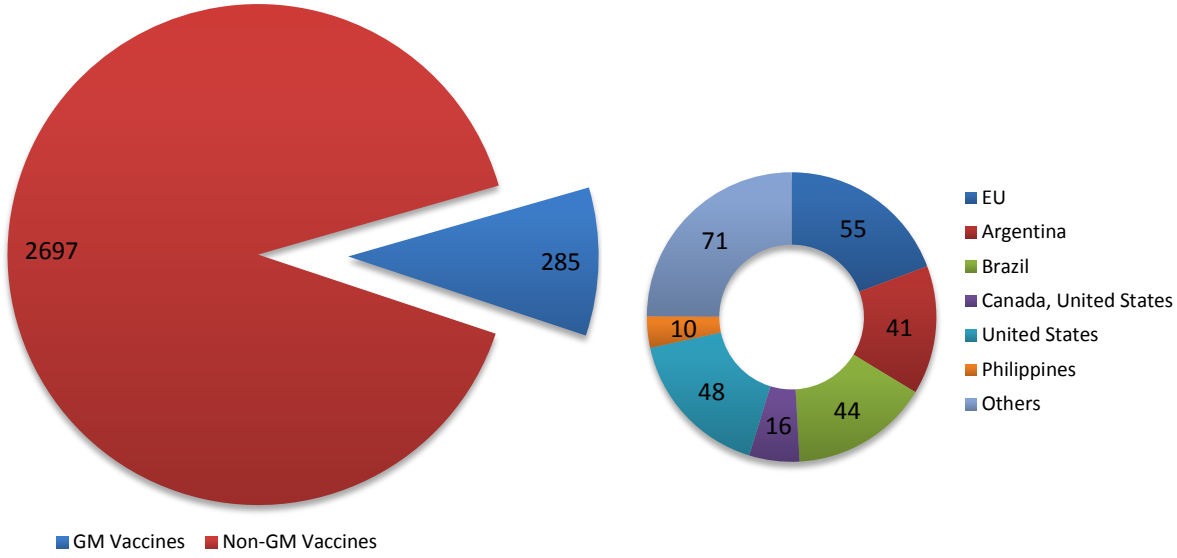


Figure 4 Registered veterinary (GM) vaccines and subdivision of vaccines per region (EU, Argentina, Brazil, Canada, United States, United States, Philippines, Others)

2.3 Vaccine target pathogens

Figure 5 illustrates the fact that GM vaccines for human use are predominantly developed against viral and bacterial infections. Also, combined vaccines are on the market, which induce immunity against both bacteria and viruses. For the veterinary field also parasitic vaccines are available. According to our data the large majority of both human and veterinary vaccines is for the prevention of viral diseases. Genetically modified vaccines, in particular live-attenuated vaccines are relatively easy to construct for viruses due to their unique properties. Viruses are relatively simple microorganisms containing a relatively small number of genes. They can be attenuated by passaging them through cell cultures over time. During adaptation to the cells they will lose their virulence and ability to replicate in human cells. Attenuation can also be achieved through recombinant DNA technology i.e. by deletion of particular genes. In contrast to viruses, bacteria have thousands of genes and a more complex presentation and thus it is much harder to characterize, control and modify them.[8] Despite the availability of techniques for attenuation of pathogens and the expression of recombinant antigens, it has proven to be extremely challenging to develop vaccines for human parasitic diseases. An illustrative example is that of Malaria for which there is still no registered vaccine available, even with all the major efforts and financial resources dedicated to Malaria vaccine development. The problem with parasitic diseases including Malaria is that we not yet fully understand the parasites and there antigenic and immunogenic properties.[9]

[8] (NIAID) TNIoAaID (2013) Type of vaccines. Vaccines.gov http://www.vaccines.gov/more_info/types/

[9] Mutapi F et al; Secor WE (2013) Infection and treatment immunizations for successful parasite vaccines. Trends Parasitol 29: 135-141.

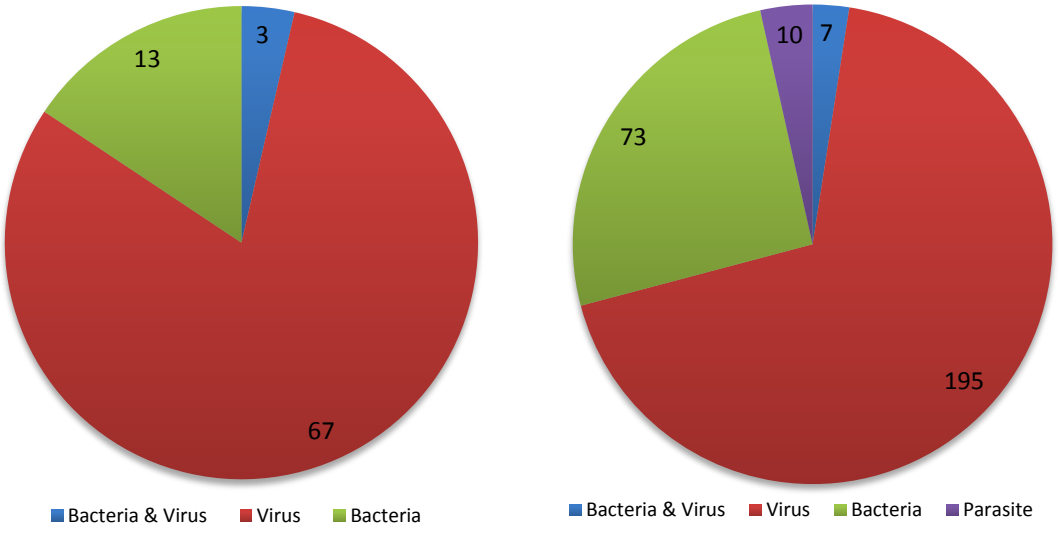


Figure 5 Human (left) and Veterinary (right) GM vaccines against various pathogens

2.4 Application fields

2.4.1 Human GM vaccines

All approved GM vaccines for humans are developed against infectious disease indications and can be categorized into 15 different disease areas (Figure 6). The very first GM vaccine was developed against hepatitis B virus and was approved by the FDA in 1986 and introduced on the market in 1987.[10] Our findings illustrate that GM vaccines are still mainly applied in the hepatitis B disease field. According to the World Health Organization (WHO), more than 780,000 people die every year due to the consequences of hepatitis B.[11] Thus hepatitis B is still an important infectious disease with a high disease burden for which vaccination remains the best preventive measure.

Human Papillomavirus (HPV) and influenza are the next major infectious disease fields in which GM vaccines are being applied. Both diseases have a devastating impact on public health, social, and economic issues. According to the WHO, HPV is the fourth most common cause of cancer in women with an estimated 266,000 deaths and 528,000 new cases in 2012.[12] Annual influenza epidemics are estimated at about 3 to 5 million cases of severe illness, and about 250,000 to 500,000 deaths.[13]

[10] (1987) Genetically engineered hepatitis B vaccine now available. CMAJ 137: 301.

[11] WHO (2014) Hepatitis B. http://www.who.int/entity/mediacentre/factsheets/fs204/en/

[12] WHO (2012) Human papillomavirus (HPV). Immunization, Vaccines and Biologicals http://www.who.int/immunization/diseases/hpv/en/

[13] WHO (2014) Influenza (Seasonal). Key Facts http://www.who.int/mediacentre/factsheets/fs211/en/

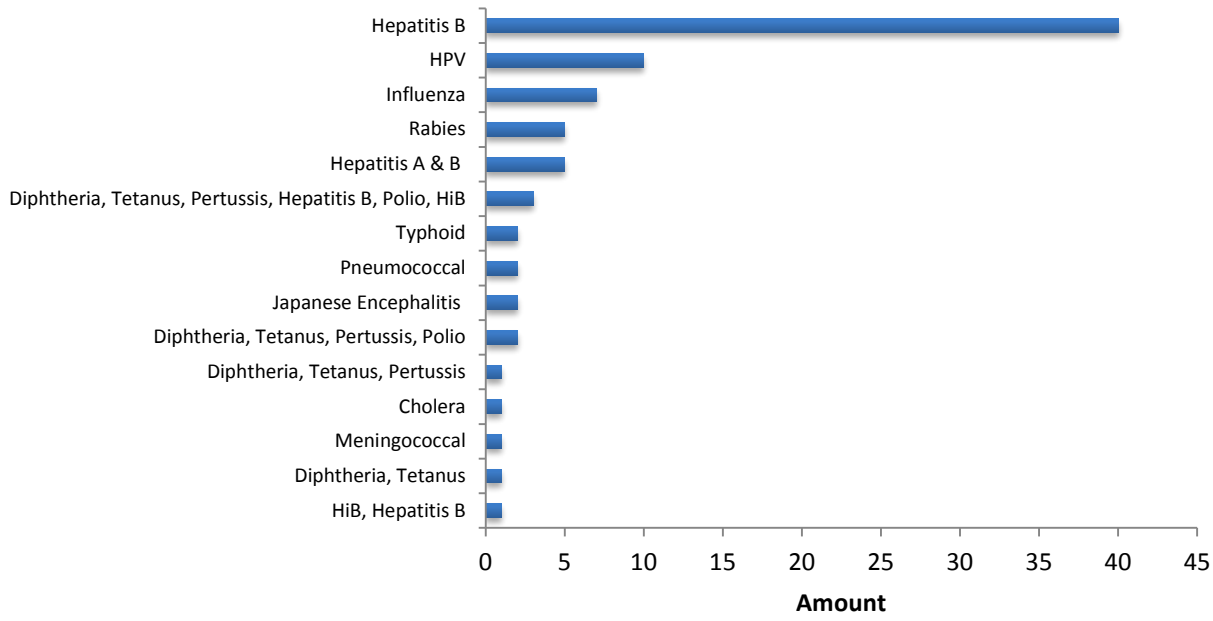


Figure 6 Human GM vaccines, application field (HiB: Haemophilus Influenza type B)

Successfully developed vaccines mostly target pathogens or toxins that can be neutralized by antibodies and have a stable antigen repertoire.[14] GM technology could be applied more in development of vaccines for diseases against which there are no effective vaccines available. Two of such targets that are pursued in vaccine research since decades are HIV and Malaria. For both targets there are still no vaccines available against these leading causes of death in the world. [15] HIV and malaria are considered challenging infectious diseases due to their antigenic variability and the requirement of T-cell immunity for protection.[16] Currently, there are several vaccine candidates in the clinical trial phase against challenging diseases including various types of cancer, HIV and malaria. Especially for HIV vaccine development strongly relies on recombinant technology.

2.4.2 Veterinary GM vaccines

The first veterinary GM vaccine was registered in 1982 by the Dutch company Intervet (MSD Animal Health).[17] This recombinant *Escherichia coli* (*E. coli*) vaccine for swine was the first-ever GM vaccine with the first human GM vaccine being registered only 4 years later. Now, over 30 years later, GM veterinary vaccines are applied into 73 different veterinary disease areas. The top 15 application fields are illustrated in Figure 7. Newcastle-, Infectious Bronchitis- and Avian Infectious Bursal diseases are the top three diseases in livestock for which GM vaccines are being applied. A complete list of the disease areas is provided in appendix 3.

[14] Rappuoli R, Aderem A (2011) A 2020 vision for vaccines against HIV, tuberculosis and malaria. Nature 473: 463-469.

[15] WHO (2012) The top 10 causes of death. WHO http://www.who.int/mediacentre/factsheets/fs310/en/index1.html.

[16] Rappuoli R, Aderem A (2011) A 2020 vision for vaccines against HIV, tuberculosis and malaria. Nature 473: 463-469.

[17] http://www.merck-animal-health.com/news-media/company-history.aspx

Newcastle Disease virus (NDV) is one of the most important pathogens of poultry affecting both domestic and wild birds. Newcastle disease can cause mortality up to 60% in livestock such as village chickens[18,19], consequently causing a huge socio-economic impact on the inhabitants of the region.[20] Infectious Bronchitis Disease is mainly a problem in the part of the world where poultry is being bred extensively enabling the pathogens to spread rapidly among birds, which can cause severe economic losses to the poultry industry.[21] Avian Infectious Bursal Disease is economically one of the most important diseases that affect commercially produced chickens worldwide.[22]

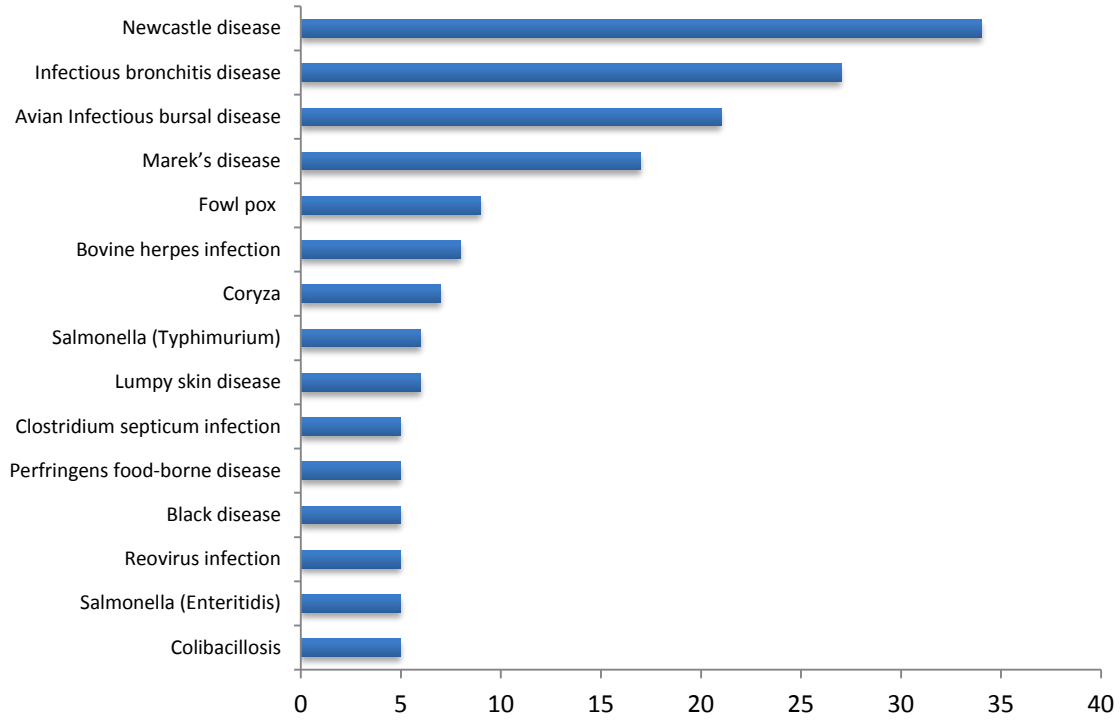


Figure 7 Veterinary GM vaccines, top 15 application fields

2.5 Vaccine development/production technologies

Recombinant proteins, prepared by diverse recombinant technologies such as baculovirus expression systems, are the most prevalent GM vaccines in humans. Veterinary vaccines are predominantly based on live-attenuated pathogens, Figure 8 (information on vaccine types is provided on page 8). According to our data, approximately 95% of the human GM vaccines are non-retrievable (do not contain a GMO). This percentage is much less for veterinary vaccines (41%). Almost 60% of the veterinary vaccines are co-injected vaccines (contain a GMO). Within the 5% of human vaccines that is co-injected, there is only

[18] Dortmans JC, Koch G, Rottier PJ, Peeters BP (2011) Virulence of Newcastle disease virus: what is known so far? Vet Res 42: 122.

[19] Bagnol B (2001) The Social Impact of Newcastle Disease Control. ACIAR PROCEEDINGS aciar.gov.au

[20] Jeřábková A, Juranová R, Rosenbergová k, Kulíková L, Hera A, et al. (2012) Detection of the Newcastle disease virus and its effect on development of post-vaccination immunity in a commercial flock of laying hens. ACTA VET BRNO 81.

[21] Sjaak de Wit JJ, Cook JK, van der Heijden HM (2011) Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol 40: 223-235.

[22] Muller H, Mundt E, Eterradossi N, Islam MR (2012) Current status of vaccines against infectious bursal disease. Avian Pathol 41: 133-139.

one registered viral vector vaccine (IMOJEV®) on the market. This is a vaccine against Japanese Encephalitis virus (JEV) based on a live yellow fever virus vector encoding the envelope proteins of an attenuated JEV strain (see also the highlighted box on the next page).[23]

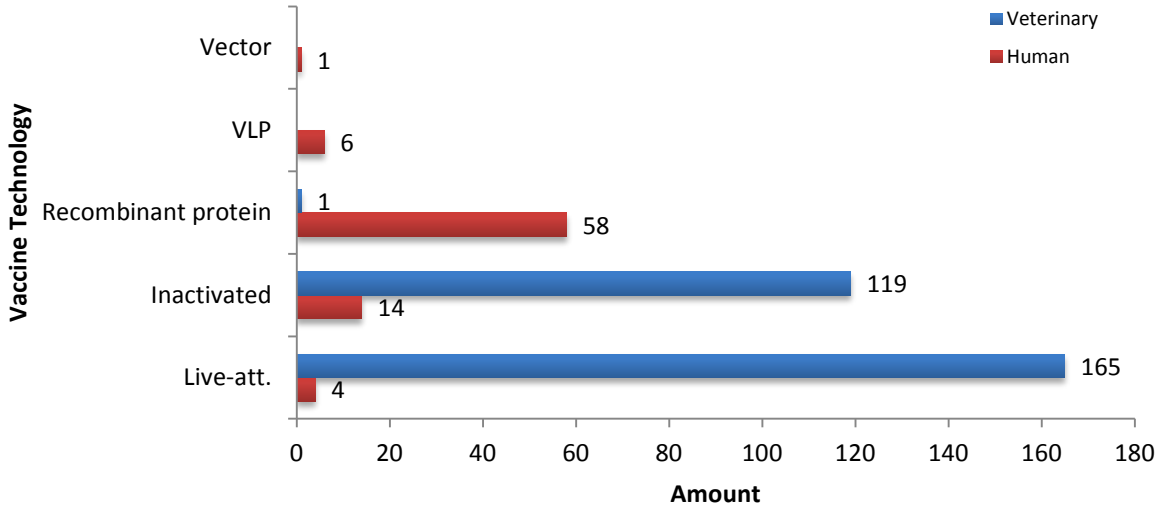


Figure 8 Vaccine technologies applied in human and veterinary GM vaccines[24]

All registered genetically modified HPV vaccines are based on Virus like particles (VLP) technology. GM viruses are being increasingly applied as live vaccine vectors in veterinary vaccines. Replication-competent Canarypox and Herpes viruses are the most common live virus vectors used in the veterinary field. EMEA has even developed a guideline for live recombinant vector vaccines for veterinary application.[25]

First viral vector vaccine in humans

IMOJEV® is the first registered vector based vaccine for humans. It is a vaccine against JEV based on a live yellow fever virus vector (YFV17D) encoding the envelope proteins of SA14-15-2, an attenuated JEV strain. This vaccine is a showcase for the possibilities and advantages of the use of genetic modification for the construction of vaccines. The conventional JEV vaccine was mouse brain-derived, inactivated and therefore also less immunogenic. With the IMOJEV® vaccine the JEV proteins are encoded by a live virus resulting in synthesis of the respective proteins in the target cells of vaccinees. This results in more pronounced immune responses.

[23] Newswire P (2012) Sanofi Pasteur Launches IMOJEV® First Single-dose Vaccine Against Japanese Encephalitis in Australia 2012. http://www.prnewswire.co.uk/news-releases/sanofi-pasteur-launches-imojev-first-single-dose-vaccine-against-japanese-encephalitis-in-australia-182744251.html

[24] Not all vector-based vaccines are represented in the Vetvac database and some were not retrieved from the database due to the limited search possibilities. Thus these results are an underestimation of the number of registered vector-based veterinary vaccines our results. The categories recombinant protein and Inactivated represent multiple vaccine formulations: polysaccharides, toxoids, subunit vaccines, whole-activated vaccines and others).

[25] Myhr AI, Traavik T (2012) Genetically Engineered Virus-Vectored Vaccines – Environmental Risk Assessment and Management Challenges. Genetic Engineering - Basics, New Applications and Responsibilities http://cdn.intechopen.com/pdfs-wm/25756.pdf

2.5.1 Vaccine technologies in humans

Our data demonstrate that recombinant protein-based technology is primarily used to produce human GM vaccines. There are in total 15 disease areas in which this technology has been applied. Figure 9 illustrates the top 5 disease areas in humans.

The world's first genetically engineered vaccine was against the hepatitis B disease, which is considered one of biotechnology's greatest triumphs.[26] HPV and influenza are the second and the third disease areas, respectively, in which recombinant protein technology plays an important role. Recombinant proteins are the most prevalent ingredients in human GM vaccines. Flublok is the world's first approved (in 2013) recombinant protein-based vaccine for prevention of seasonal influenza disease in humans. This vaccine is highly purified, does not contain any preservatives, egg proteins, gelatin or latex and it is only made using modern cell culture technology.[27]

Despite enhancements in novel vaccine technologies in the last decades, limited numbers of registered vaccines are based on GM technologies. Nevertheless, advances including improved formulations and delivery methods and optimization of vaccine vectors has currently resulted in many vaccine candidates in clinical trials.[28, 29]

2.5.2 Vaccine technologies in animals

Live-attenuated veterinary vaccines are applied in 73 different disease areas for livestock. Figure 10 demonstrates the top 5 disease areas in veterinary vaccines. Live-attenuated vaccines are predominantly applied in Newcastle disease, followed by infectious bronchitis disease and Marek's disease. When the number of GM vaccines is indexed per group of animals that it is registered for it becomes clear that most of the vaccines are meant for use in poultry (Figure 11).

[26] Deborah LI (1996) Pathbreakers: A Century of Excellence in Science & Technology at the University of Washington; Kwiram AL, editor: Office of Research, University of Washington, 1996.

[27] Sciences P (2014) Protein Sciences Adds Distributor for Flublok® Recombinant Influenza Vaccine to Increase Access. http://www.proteinsciences.com/PDF/pscp2.pdf

[28] Robinson HL, Pertmer TM (2000) DNA vaccines for viral infections: basic studies and applications. Adv Virus Res 55: 1-74.

[29] Ferraro B, Morrow MP, Hutnick NA, Shin TH, Lucke CE, et al. (2011) Clinical applications of DNA vaccines: current progress. Clin Infect Dis 53: 296-302.

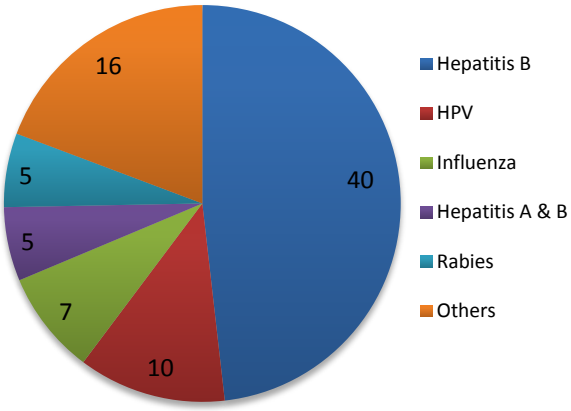


Figure 9 Top 5 disease areas for which recombinant protein-based vaccine technology is available

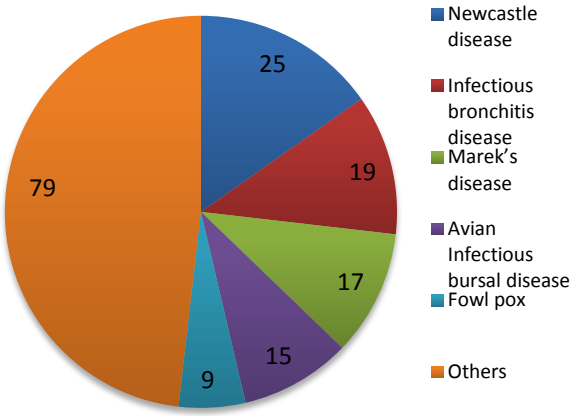


Figure 10 Top 5 veterinary disease areas for which live-attenuated vaccines are available

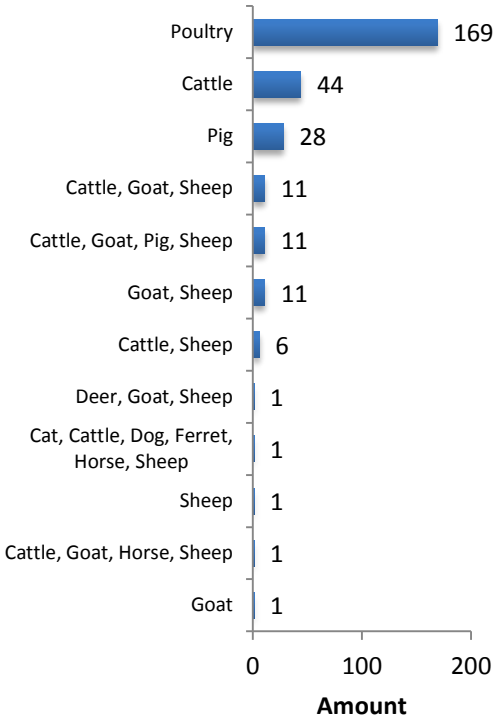


Figure 11 GM vaccines in livestock

2.6 Vaccines for companion animals

The results presented here predominantly focus on vaccines for livestock (poultry, goats, sheep, pigs, cattle). Apart from some livestock vaccines that are also used in companion animals the data do not reflect the available vaccines for companion animals, zoo animals and wild animals. For companion and zoo animals it is considered that vaccines can be more costly in contrast to livestock vaccines.[30] This is characterized by the availability of a wide-variety of GM vaccines, mostly vector vaccines. The most widely used vector is a Canarypox virus (RecombiTEK®, Merial, Sanofi) which is used as a platform for canine vaccines (Lyme, Canine distemper, Canine parvovirus and Canine coronavirus). The platform is also used in registered vaccines for horses against West Nile virus and Equine influenza virus.[31] Horses can be categorized as livestock but are often considered as companion animals (as indicated by

[30] Indicated by experts-in-the-field of veterinary vaccines that were interviewed for Chapter 7 Expert Opinions.

[31] http://www.equinewnv.com (Merial website).

interview candidates from the veterinary vaccine field, see Chapter 7). Also for horses the RecombiTEK®-based vaccines are available.[32,33]

2.7 Exotic animal vaccines (Wildlife & Zoo)

For exotic animals (e.g. foxes, prairie dogs) the most compelling example of the role of genetic modification is that of rabies vaccines. First, these were based on live-attenuated rabies viruses which harbored the risk of reverting to more pathogenic phenotypes. Second, more sophisticated tools became available to generate highly attenuated rabies viruses through a selection process. Now, with genetic modification the attenuation of the virus can be fully tweaked and pinpointed to targeted sites in the genome, allowing for pre-designed attenuation. To go one step further, the rabies antigen of choice could be produced as a recombinant protein or be encoded for by a viral vector that is used as a GM vaccine. The vaccines described can be used in the ORV (oral rabies vaccine) wildlife program that is successfully conducted in the EU to control rabies in the wildlife population (predominantly foxes).[34,35] When dedicated exotic animal vaccines are not available, which is the case for most pathogens, commercial vaccines for domestic species are often used as an alternative. An illustrative overview of vaccine recommendations for exotic animals is provided by the Merck Veterinary Manual.[36] RecombiTEK®-based Canine distemper vaccine is an example of a GM vaccine that was registered for use in companion animals (dogs) but could also be applied for animals in the wild or captivity; it is recommended for wolves and other Canidae species (fox, coyote, wild dog).

Regarding zoo animals, this is a niche application field with virtually no generally registered and applied vaccines. For many zoo animal species, domestic veterinary vaccines (e.g. canine vaccine for wolves) are applied, similar to wildlife animals. If these are not available, 'stable-specific' vaccines need to be developed in response to an emerging outbreak in a single zoo or a problem that turns out not to be restricted to a single zoo (e.g. Elephant herpes viruses).[37] For such vaccines genetic modification is an often used tool to design a vaccine based on the discovered pathogen. GM is the fastest and easiest, if not the only, way to create a vaccine against such exotic and recently discovered pathogens. Especially since culture and inactivation methods for the production of an inactivated vaccine to that specific pathogen in most cases do not exist yet and often take too much time to develop. GM technologies offer the possibility to design a vaccine based solely on the sequence of one or more of the pathogen's antigens. Such swift actions are often required for acute infectious disease outbreaks in zoos that can involve morbidity and mortality rates that do not allow for long development tracks as we see with normal vaccines. This requires also proper regulation to guideline such a fast-track vaccine development process.

[32] http://www.merial.ca/en/horses/products/Pages/recombitek-influenza.aspx

[33] http://www.merial.ca/en/horses/products/Pages/recombitek-wnv.aspx

[34] Freuling et al; 2013; The elimination of fox rabies from Europe: determinants of success and lessons for the future DOI:10.1098/rstb.2012.0142

[35] Klepac P, Metcalf CJ, McLean AR, Hampson K (2013) Towards the endgame and beyond: complexities and challenges for the elimination of infectious diseases. Philos Trans R Soc Lond B Biol Sci 368: 20120137.

[36] http://www.merckmanuals.com/vet/exotic_and_laboratory_animals/vaccination_of_exotic_mammals/overview_of_vaccination_of_exotic_mammals.html#v5637107

[37] http://www.houstonzoo.org/wp-content/uploads/2012/11/EEHV-Workshop-Houston-2011-final-report-17-July-2011.pdf

2.8 Highlights

- Currently, approximately 10% of licensed vaccines, human and veterinary, are GM vaccines (as defined in this report) and these are mainly focused on viruses
- Recombinant proteins and Live-attenuated are the most applied technologies in human and veterinary GM vaccines, respectively
- There is only one vector vaccine registered for human use (IMOJEV®) and multiple for use in animals
- There are no approved DNA vaccines for use in humans
- GM vaccines are applied in various disease areas

CHAPTER 3

Clinical Development

3. Clinical Development

To generate an overview of the status of GM vaccine technologies and their indications in the different phases of clinical development, a dataset was generated based on the WHO clinical trial registry that mirrors the entries of the major national and international clinical trial registries.

3.1 Methodology

Here, the methodology for collecting and analysing clinical trial data is described. As all registries of clinical trials per definition only include products for human application, this chapter will only concern human vaccines. Data on the development of veterinary vaccines is, to the best of our knowledge, not available and will therefore not be included in the analysis of vaccines in development. Table 3 summarizes the different steps in the collection of data on clinical trials of human vaccines.

Table 3 Methodology for clinical trial data collection

	Database	Search Terms	# of Vaccines		Total # of Vaccines	Variables
Clinical Trials	WHO International Clinical Trials Registry Platform	"Vector"	47	Deduplication: based on Trial ID number	1146	"Technology Class"
		"DNA"	180			
		"RNA"	26			
		"Recombinant Protein"	10			"Type of Organism"
		"Chimeric"	26			
		"Recombinant"	471			
		"Genetically Modified"	5			"Indication"
		"Genetically Engineered"	0			
		"Modified"	129			"Specific target"
		"Live-attenuated"	260			
		"Attenuated NOT Live-attenuated"	21			"Expression system"
		"VLP"	100			
		"Virosome"	12			
		"Engineered"	16			"Production system"
		"Genetic"	27			
		"Live"	403			
		All search terms were combined with the term "Vaccine"				"Development phase" (1, 2, or 3)

First, the raw data was extracted from the WHO International Clinical Trials Registry Platform[38]. By use of specific search terms in combination with the search term 'Vaccine', we compiled a dataset that should include all vaccines related to genetic modification. Table 3 provides an overview of search terms that were used, along with the number of trials found for each search term.

[38] http://www.who.int/ictrp/en/

Subsequent deduplication resulted in a data set containing 1146 clinical trials. For each clinical trial, we determined and/or included, several (independent) variables[39]:

- The technology class of the vaccine, which refers to the different types of vaccines related to genetic modification including: recombinant protein vaccines, Virus Like Particle (VLP) vaccines, vector-based vaccines, DNA vaccines, Virosomes, Live-attenuated vaccines, and inactivated vaccines.
- The type of organism, for which the vaccine prepares the immune system (e.g. Bacteria, Virus, Parasite, Plant).
- The indication and specific target of the vaccine in development. (e.g. cancer and Melanoma, or Infectious diseases and influenza).
- The development phase (1, 2, or 3) and the date of registration.
- The expression and/or production system used for the creation of the vaccine.
- In addition, other information such as the developing company, the country in which the trial is conducted, and primary trial outcomes were also provided by the databases.

These variables were subsequently analysed in relation to each other to identify and visualize trends regarding new technologies for specific markets.

In this chapter, we aim to present an explicit and complete overview of vaccines that are or have been in clinical development in the time period 1999-2013. After careful deduplication and categorization, our final dataset contained a total of 1146 clinical trials, including 447 Phase 1 trials, 353 Phase 2 trials and 255 Phase 3 trials (84 Phase 4 trials; 7 unspecified).

[39] Trial-specific data, such as the scientific description and if necessary additional online documents associated with the trial, were analyzed to retrieve information concerning the variables.

3.2 Indications

Not surprisingly, most vaccines in development target infectious diseases (82%) followed by cancer (17%), and the remaining 1% concerns vaccines for allergies, excluding 1 vaccine for Ricin (A toxin from castor beans) and 1 vaccine for rheumatoid arthritis (auto-immune). Of the vaccines for infectious diseases, 75% target viruses, 13% target bacteria, and 3% target parasites, mainly malaria parasites (83%). Analysis of additional variables, as mentioned in the previous section (methodology), will be presented hereafter.

Figure 12 shows the number of registered clinical trials over time for GM vaccines targeting the indications most prevalent in our dataset. There seems to be a significant rise in the number of new clinical trials for both influenza and HIV targeted vaccines in 2005. With current dataset it was not possible to explain this rise. For the other indications, the patterns appear to be quite static. However, for cancer and influenza there seems to be a decline in the number of trials in 2013. For the cancer vaccines this is mainly a decline in phase 2 trials, illustrating the difficulty of successfully developing an oncology vaccine product. It is difficult to distil the exact reason for the dip in the number registered clinical trials with influenza vaccines.

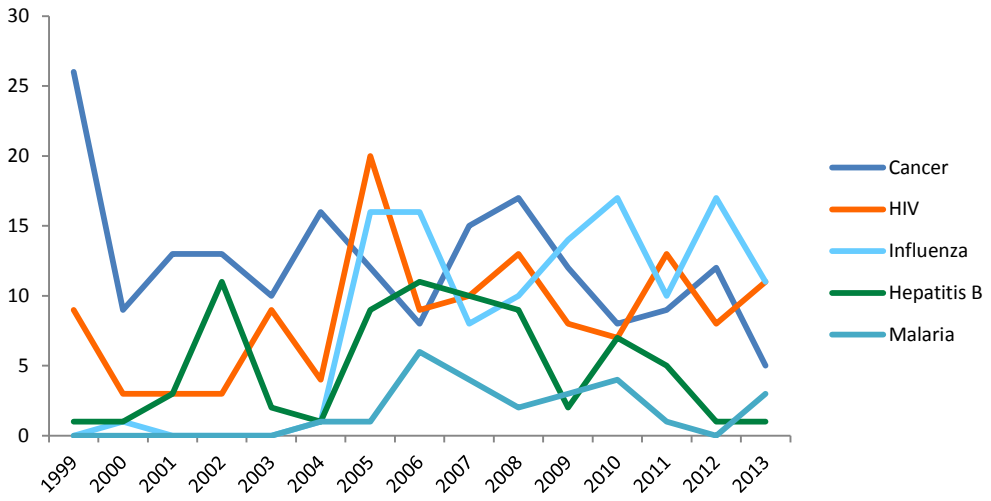


Figure 12 The number of registered clinical trials for main indications

It is apparent that most trials concern GM vaccines that are developed for viruses of which the top 3 are HIV, influenza and hepatitis B (see Figure 13 for distribution over clinical trials phases). Of these vaccines, most have been approved for hepatitis B (recombinant protein vaccines) and only one for influenza, which is FluBlok® (recombinant protein vaccine) in 2011.

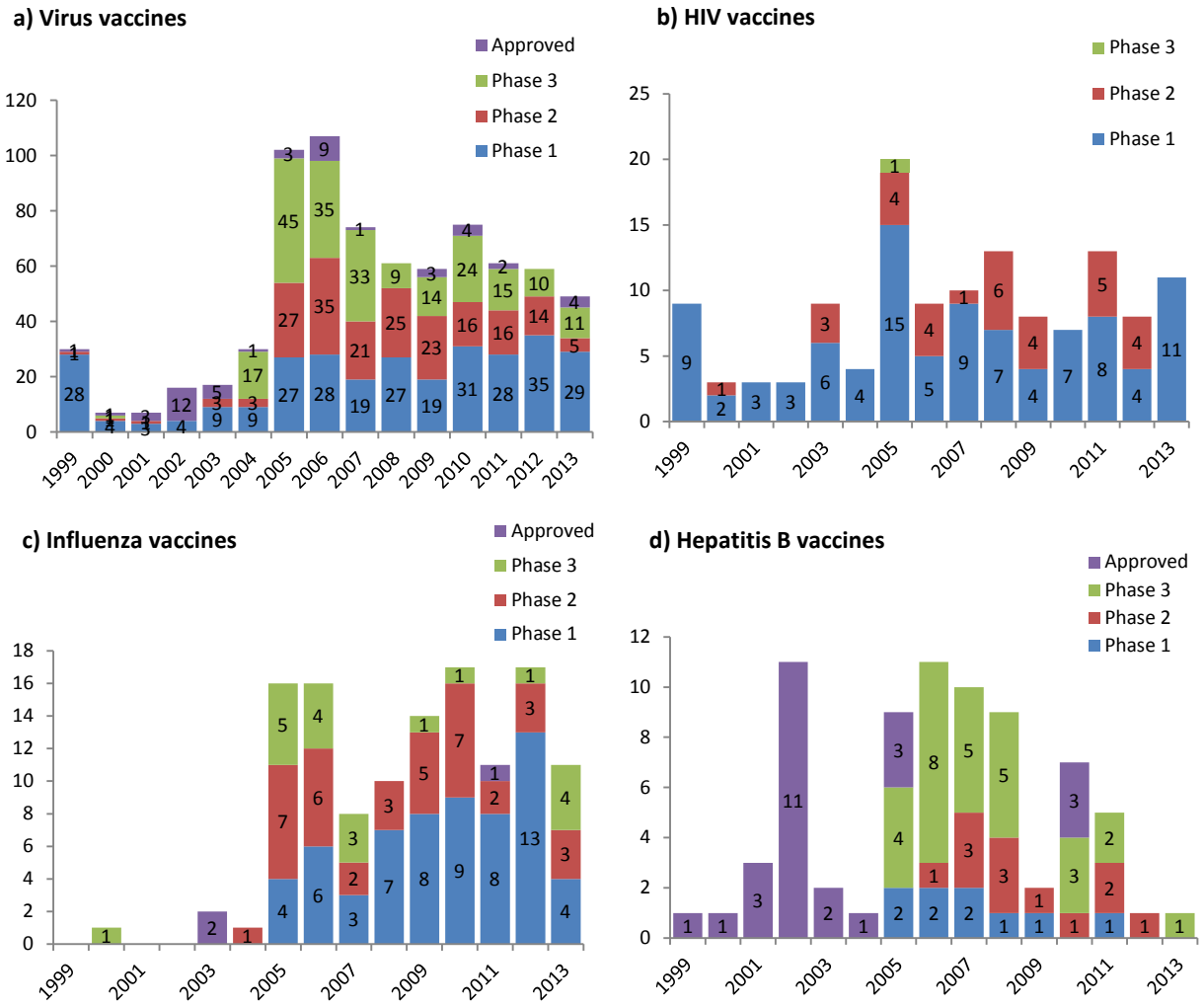


Figure 13 The number of Vaccines in development that target viruses

For cancer vaccines, we have found a total of 199 candidates that have entered clinical research phases, although none have been approved yet. Most cancer vaccines are still in clinical phase 1 and 2 (>95%) with only 8 cancer vaccines currently in phase 3 (Figure 14). Moreover, there seems to be an upward trend in bacteria vaccines, with a total of 5 approvals since 2010 (Figure 15a). The parasite vaccines (Figure 15b) mostly concern Malaria vaccines (83%).

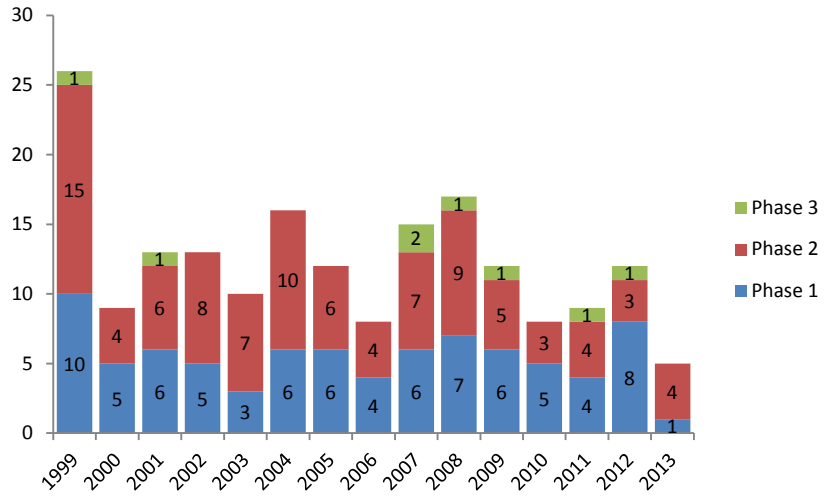


Figure 14 The number of cancer vaccines in development

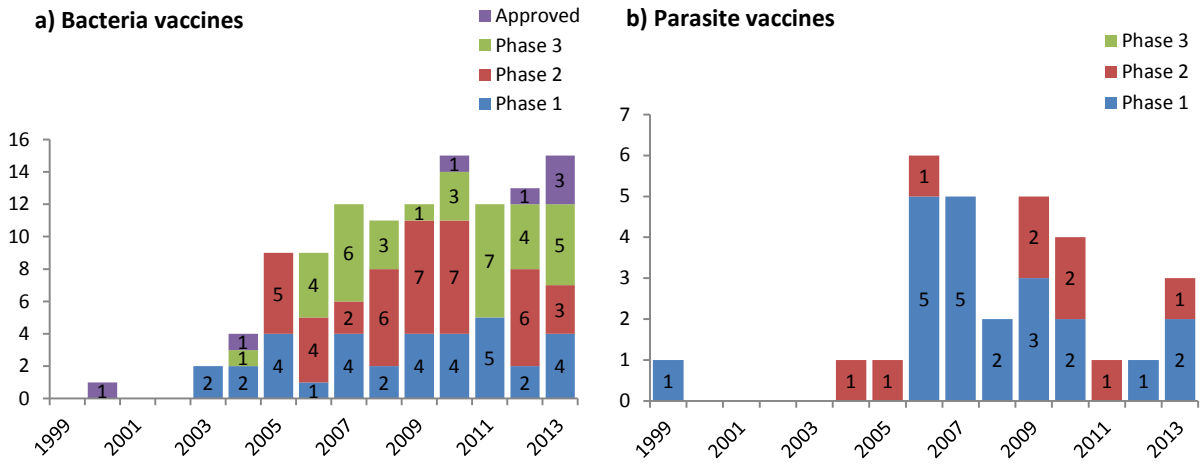


Figure 15 The number of Bacteria (a) and Parasite (b) vaccines in development

3.3 Vaccine technologies

In terms of technology types, most GM vaccines belong to one of four categories, being recombinant proteins, VLPs, Vectors, DNA vaccines (see page 7 for a delineation of genetically modified vaccine definitions and page 9 for a description of the vaccine types). However, we observe an increase of trials from 2004-2005, primarily of live-attenuated and recombinant protein vaccines (see Figure 16). There could be a link with the rise in influenza and HIV trials. Hereafter we present further analysis of the different types of technologies.

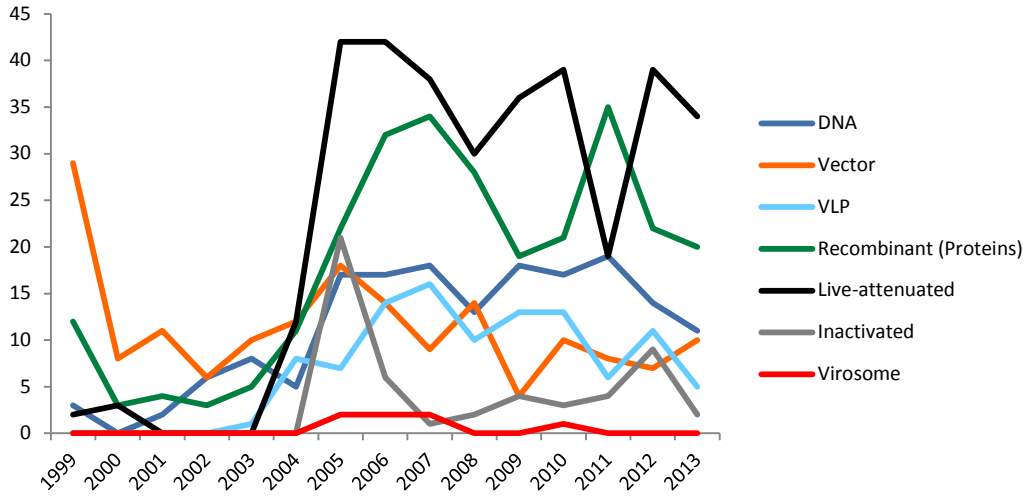


Figure 16 The number of registered clinical trials per technology type for GM vaccines

3.3.1 Recombinant protein/subunit vaccines

The first category concerns the direct products of recombinant DNA technology, which encompasses all recombinant proteins and subunit vaccines. This category includes most of the GM vaccines that are approved for the market (70% of total approved GM vaccines), while approximately 30% of recombinant protein vaccine trials have reached clinical phase 3. In Figure 17, the significant increase in phase 3 trials in 2006 is shown, which remains relatively high from then onward. This rise illustrates the progression of this technology in vaccine development.

Subsequently, we have differentiated the total amount of 270 recombinant protein/subunit vaccine trials for several indications and it seems that this technology approach is applicable for many indications (Figure 17). However, hepatitis B and cancer appear to be relatively the most targeted indications. 73 trials out of 270 (27%) target other indications than mentioned in this chart (e.g. meningitis, *Botulinum* toxins, certain *E. coli* infections, pollen allergy, other hepatitis viruses, RSV, varicella zoster, tuberculosis).

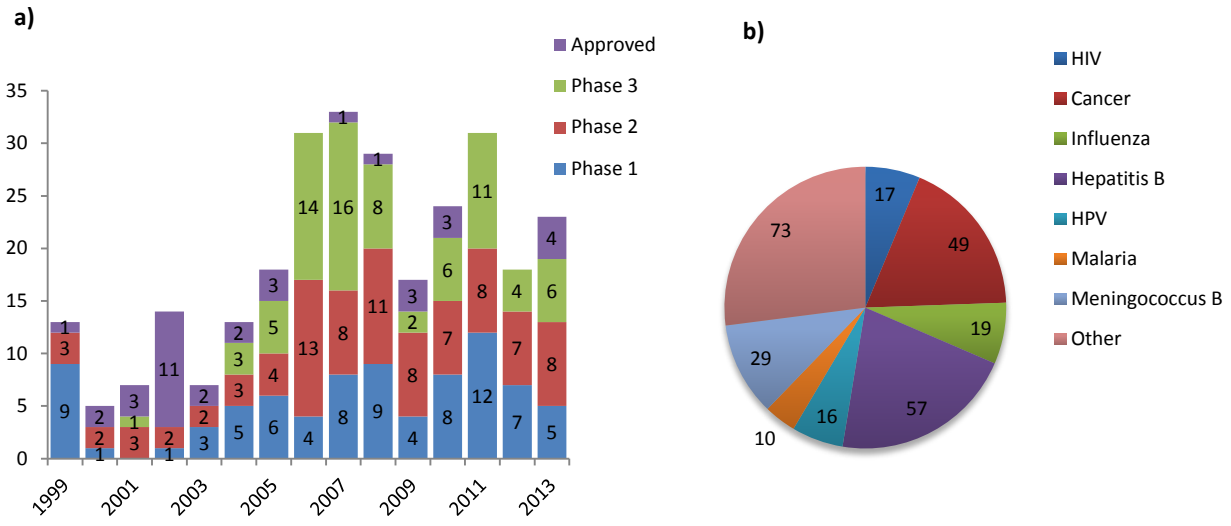


Figure 17 The number of Recombinant Protein/Subunit vaccine trials per phase (a) and per indication (b)

3.3.2 Virus Like Particle (VLP) vaccines

The second technology category, VLP, is less represented in our dataset with a total number of 105 registered clinical trials. Surprisingly, almost half of these are phase 3 trials (43%) of which the first seven started as early as 2004 (Figure 18a). Yet, there are only 6 approved VLP vaccines on the market, which equals almost 6% of the total number of VLP vaccines in clinical trials. There is a substantial difference when comparing this ratio for VLP vaccines (43% phase 3 trials versus 6% approved) and for recombinant protein vaccines (28% versus 13%). This indicates that obtaining approval for a recombinant protein vaccine might be less difficult, which could be explained by additional regulatory hurdles for some VLP vaccine technologies and possible difficulties with large scale production of VLP vaccines.[40] Although this cannot be determined based on the current dataset, the VLP vaccines tested in phase 3 trials thus far might not have been as efficacious as was expected based on previous results. Conclusively, thus far it is less likely that a VLP vaccine will proceed from clinical phase 3 to registration than a recombinant protein vaccine. In contrast to the spread of recombinant protein vaccines over different indications, VLP technology seems to be primarily used to address Human Papilloma Virus (HPV) as 73% of all VLP vaccines target this virus. Another 15% targets either influenza (Figure 18b) or different forms of cancer. 12% targets other indications, such as Norovirus and Chikungunya virus. Also, all 6 approved VLP vaccines are targeted at HPV.

[40] Warfield, K. L., & Aman, M. J. (2011). Advances in virus-like particle vaccines for filoviruses. *Journal of Infectious Diseases*, *204*(suppl 3), S1053-S1059.

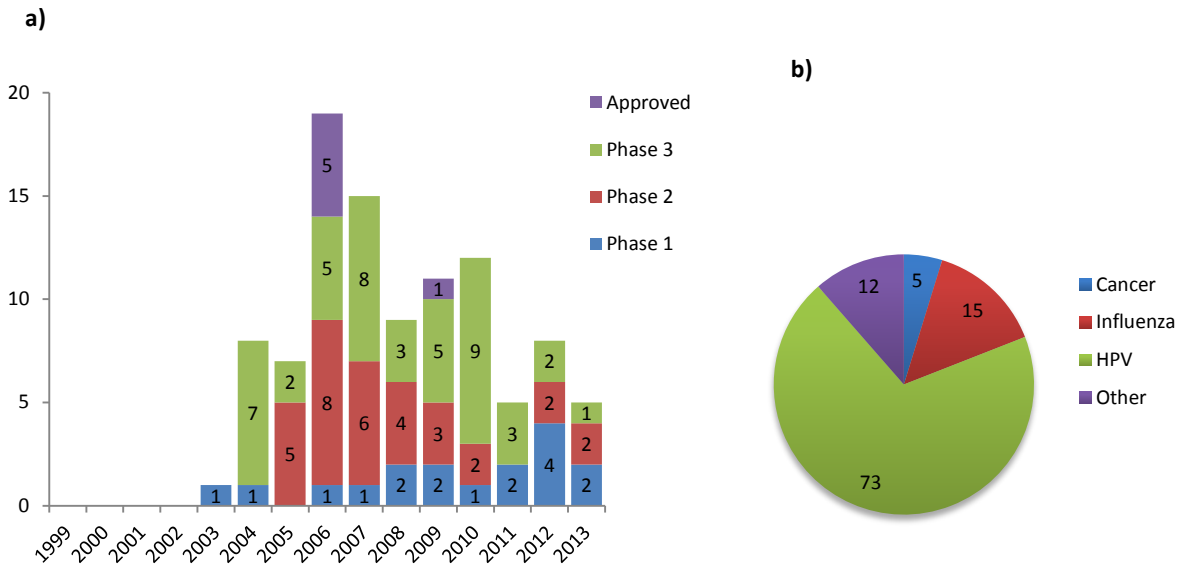


Figure 18 The number of Virus Like Particle (VLP) vaccine trials per phase (a) and per indication (b)

3.3.3 Vector vaccines

The third category of vaccine technologies concerns vector-based vaccines. In total we have found 178 registered clinical trials of vector vaccines, indicating that this technology is considered to be quite promising for the development of future vaccines. However, it does seem difficult to progress through clinical development, as only two vector vaccines have reached clinical phase 3 and just one has been approved (IMOJEV®, a vector-based JEV vaccine). Also, we observe no increase over time in the number of new registered phase 1 and phase 2 trials (Figure 19a), indicating that there is no rise in new pre-clinical investigational products. This could be an indication that the development path of vector vaccines is steep and challenging. In the production and development of vector vaccines different vector types are used which are usually modified versions of existing viruses. During analysis of the data we found that most vectors (61%) are either MVA, Fowlpox or Vaccinia (the latter two are often used in combination), which are often used together in therapeutic regimens, or Adenoviral vectors. However of the phase III trial vector vaccines, one concerns genetically modified allogeneic cells as a melanoma vector vaccine, and the other concerns an ALVAC (Canarypox) vector. The prime-boost vaccine Prostvac® (based on Vaccinia and Fowlpox) for the treatment of prostate cancer is also in clinical phase III, however this is categorized by its developer (Bavarian Nordic) as an immunotherapeutic drug and not as a vaccine.

The phase 2 trials in the database mostly concern autologous dendritic cell vectors (modified by e.g. transduction with a GM Adenovirus) (23%) and Fowlpox or Vaccinia vectors (18%). Whereas phase 1 trials mostly encompass Fowlpox or Vaccinia vectors (31%), MVA vectors (25%) and Adenoviral vectors (20%). This could indicate that the Technology Readiness Level (TRL) of using autologous dendritic cell vector vaccine technologies slightly surpasses viral vector vaccine technology. What makes the interpretation of these data difficult is that the autologous cell vector technology not always is categorized as a vaccine but also as immunotherapy, thus the dataset for this vaccine type is most likely incomplete. Figure 19b also shows 23 'Other' vectors, which include Yeast (*Saccharomyces cerevisiae*),

Salmonella typhi, Measles virus, and Lymphocytes. These vectors are applied in vaccines for prophylactic (against infectious diseases) and/or therapeutic (against various cancer types) indications.

A table with examples of vaccine vectors on the market and in development is provided in Chapter 8. It is difficult to predict the chances of registration of more vector vaccines that are based on one of the usual suspects (Poxviruses and Adenoviruses) or any of the other vectors. Although many of the vectors come with great technical possibilities for modification, that does not guarantee their success as a vaccine platform for use in humans and/or animals. Vectors seem to be a promising technology for cancer vaccines in particular, as 52% of all the viral vectors subject to clinical research are developed to target cancer. Of all cancers, it seems that melanoma (29%) is targeted most with vector vaccines (Figure 20).

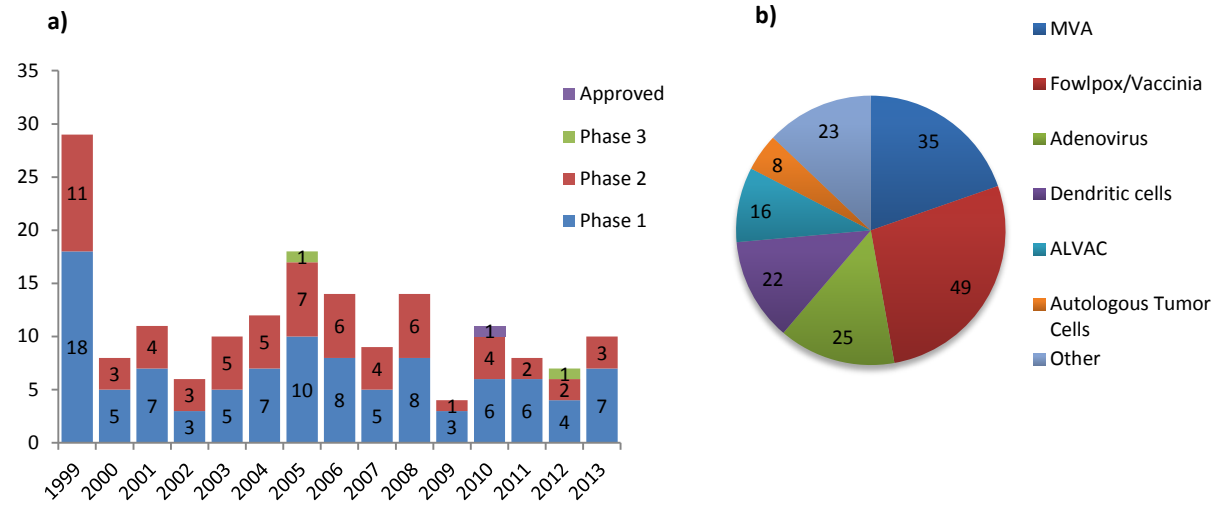


Figure 19 The number of Vector vaccine trials per phase (a) and per type (b)

By further specifying this for the most prevalent types of vectors we find that the combination of Fowlpox and Vaccinia vectors mainly targets cancers (63%), MVA vectors are mostly developed for HIV (29%) and Smallpox (20%), Adenoviral vectors mostly target HIV (52%), whereas dendritic cells in therapeutic vaccines are mostly applied for cancer and more specifically for treatment of Melanoma (36%) (Figure 21).

New HIV vaccine approaches

Between 2006 and 2009 several phase 2 trials commenced in which the safety, effectiveness and immunogenicity of a combination treatment (HVTN 505) of a multiclade HIV-1 DNA Plasmid Vaccine followed by a multiclade HIV-1 Recombinant Adenoviral Vector Vaccine was to be tested in HIV unaffected adults at risk for infection.[41] Both vaccines code for proteins from HIV subtypes A, B, and C, which together represent 75% to 85% of new HIV infections in the world. Adenoviral type 5-based vaccines have improved induction of HIV-specific CD8 cytotoxic T-lymphocyte cell responses, which correlate with lower HIV burden (viral load) and slower disease progression in primates and in HIV-1 infected people whose disease does not progress over the long term. The earlier studies have been withdrawn, yet the 2009 phase 2 trial is expected to be complete in early 2015.[42]

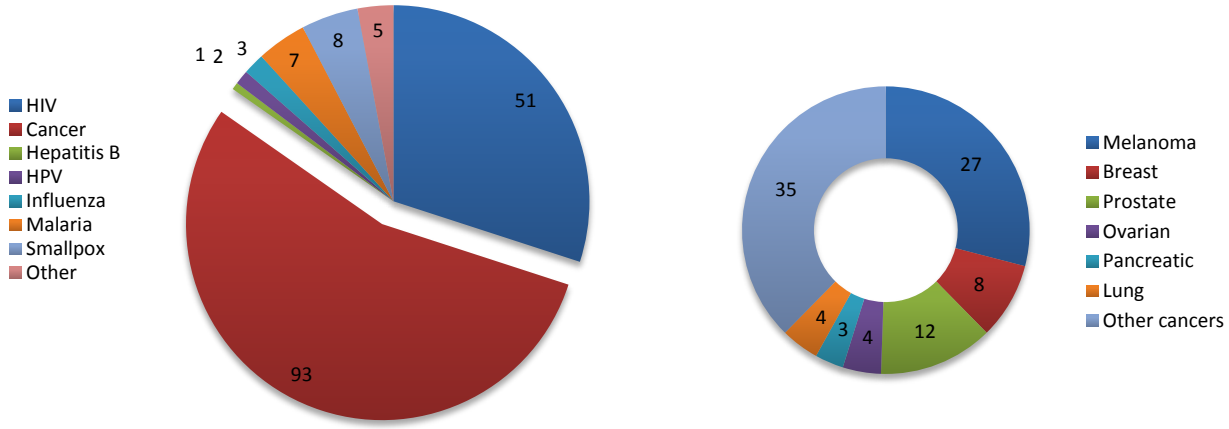


Figure 20 The number of Vector vaccine trials per indication and a specification of the number of trials per cancer

[41] http://www.niaid.nih.gov/news/QA/Pages/HVTN505qa.aspx

[42] Trial ID: NCT00865566; accessible at http://clinicaltrials.gov/show/NCT00865566

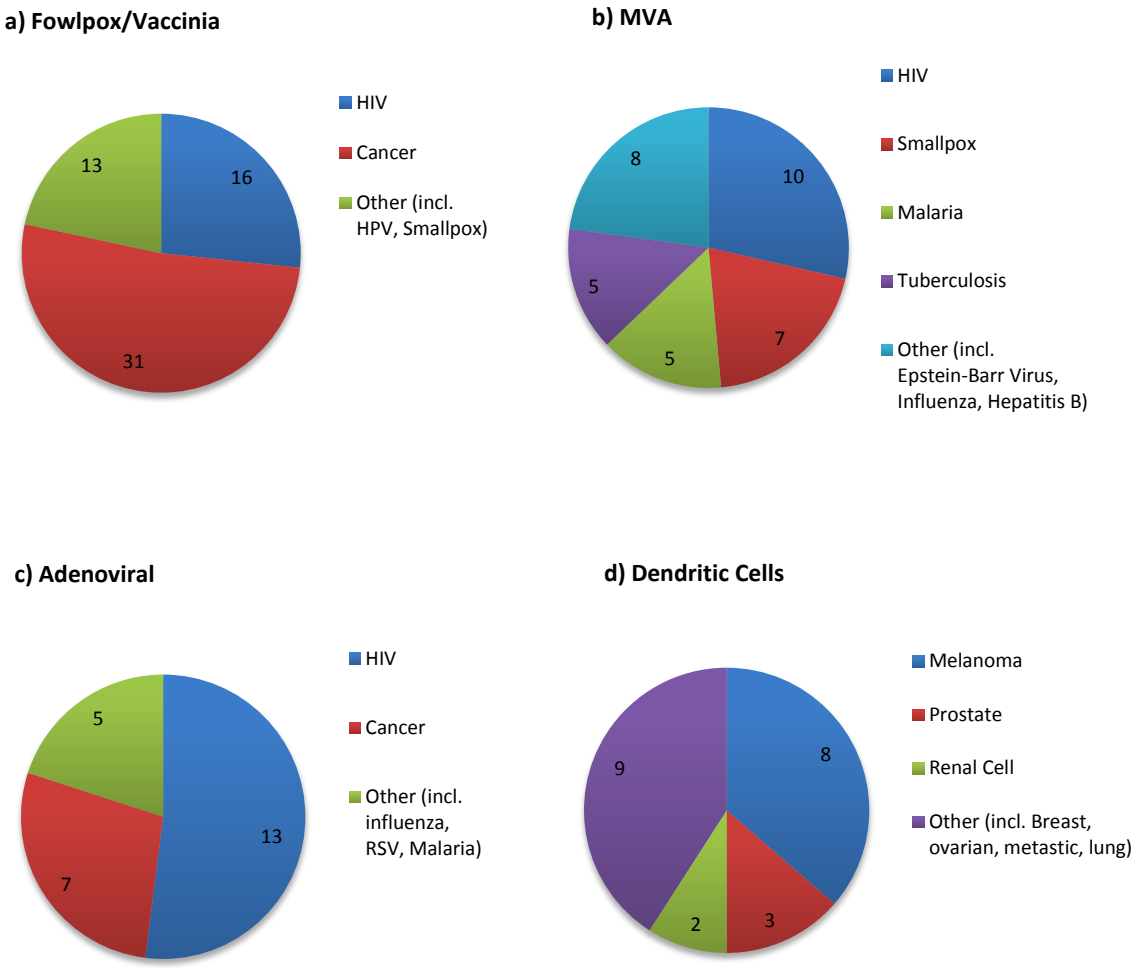


Figure 21 The number of Vector vaccine trials per indication for types Fowlpox/Vaccinia (a), MVA (b), Adenoviral (c), and Dendritic Cells (d)

3.3.4 DNA vaccines

Viral vectors as a technology platform seem to have the upper hand over DNA vaccines, of which the first phase 3 trial was initiated in 2013. Nevertheless, since 1999 there have been a total of 166 registered trials of DNA vaccines, of which 49% specifically targeted HIV (Figure 22b).

The fact that one out of 166 DNA vaccines has reached phase 3, which was only in 2013, indicates that there might be difficult regulatory [45] and clinical development barriers, as DNA vaccines have been disappointing in clinical trials [43]. In addition, the number of HIV targeted DNA vaccines are evenly distributed over phase 1 and phase 2 trials and the phase 3 trial DNA vaccine targets the

[43] Saade, F., and Petrovsky, N. (2012) Technologies for enhanced efficacy of DNA vaccines. *Expert review of vaccines*, 11(2), 189-209.

cytomegalovirus. Based on this data and considering average clinical trial phases[44], one could postulate that the first approval of a DNA vaccine for HIV is still 5 or more years ahead. It is also stated in literature that regulatory hurdles increase with the complexity of a technology such as DNA vaccines, in particular with regards to classification of the end product.[45]

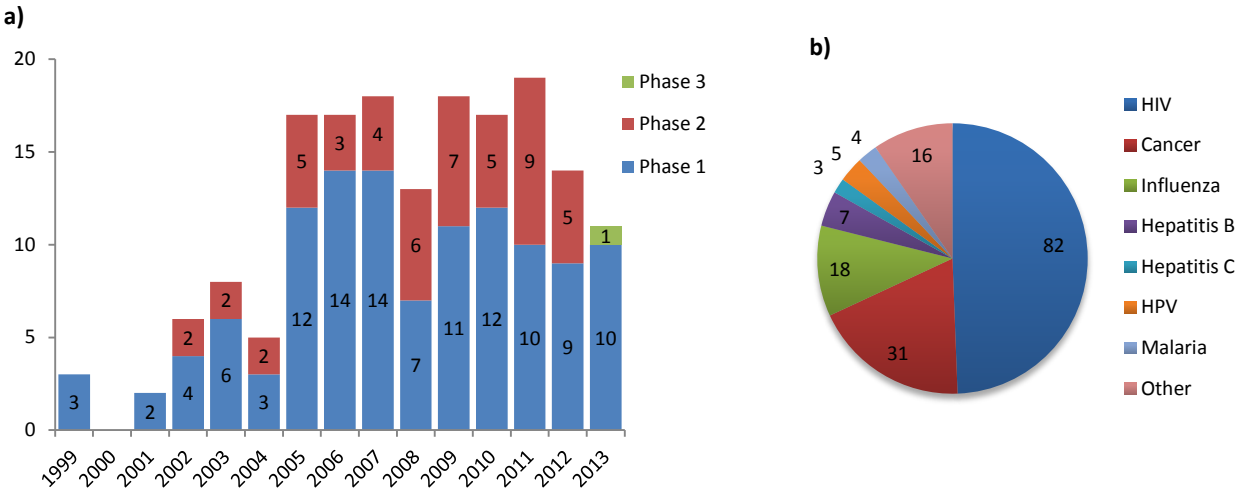


Figure 22 The number of DNA vaccine trials per phase (a) and per indication (b)

3.4 Vaccine developers

Interestingly enough, for both vector and DNA vaccines approximately 40% is developed by a select number of large institutes (see highlighted text box). It appears that the National Cancer Institute (31 vector vaccine trials) and the National Institute of Allergy and Infectious Disease (NIAID; 39 vector vaccine trials and 68 DNA vaccine trials) are investing most in vector vaccine development and the latter even more in DNA vaccine development.

MAJOR CLINICAL TRIAL SITES FOR VACCINE EVALUATION:

Vector vaccines:

- National Cancer Institute (NCI) (31 trials)
- National Institute of Allergy and Infectious Disease (NIAID) (39 trials)
- Bavarian Nordic (4 trials)
- Sanofi (4 trials)

DNA vaccines:

- National Institute of Allergy and Infectious Diseases (NIAID) (68 trials)
- Swedish Institute for Infectious Disease Control (6 trials)
- PowderMed (6 trials)

[44] Dimasi J., Hansen R, and Grabowski H. (2003) The price of innovation; new estimates of drug development costs. *Journal of Health Economics*, 22(151), 185.

[45] Donnelly, J., Berry, K., & Ulmer, J. B. (2003). Technical and regulatory hurdles for DNA vaccines. *International journal for parasitology*, *33*(5), 457-467.

3.5 Future expectations

Based on previous work from our research group, we have analysed the clinical trial data and calculated estimations of to be approved vaccines for different indications. Pronker et al. (2013) [46] have developed risk profiles for vaccines from preclinical development to registration. These risk profiles are based on applications of the formula of Dimasi et al. (2010) [47] to determine the transition probability of vaccines in different clinical development phases. Pronker et al. (2013)[46] show that fundamentally different risk profiles exist between vaccines for different indications for each step in clinical development. Based on these risk profiles, we have estimated the number of vaccines we can expect on the market and within which time period. These estimations are shown in Table 4.

Table 4 Estimations of new vaccines to be expected on the market, based on vaccine development risk profiles for key indications (based on risk profiles by Pronker et al. 2013[46])

	Currently approved	To be expected[a] (Percentage of total in development)	Of which GM products[a] (Percentage of total in development)	Expected within the time period
Hepatitis B	40	28 (58%)	24 (49%)	2-10 years
Influenza	7	88 (73%)	35 (29%)	2-6 years
HIV	0	7 (5%)	7 (5%)	7-15 years
Cancer	0	18 (10%)	18 (10%)	3-11 years
Malaria	0	2 (7%)	2 (7%)	2-6 years

For hepatitis B, the approval of an additional 28 vaccines can be expected within the next 2-10 years, of which 85% will be recombinant protein vaccines. Pronker et al. (2013)[46], reported an even more favourable risk profile for influenza vaccine development, which led to an estimation of as many as 88 vaccines being approved in the coming 2-6 years. However, more than 60% of these vaccines will be either inactivated or live-attenuated, which indicates that an estimated 35 new influenza vaccines will be products of genetic modification. Thus far, no vaccine products are approved for HIV. For certain cancers vaccine-related products are on the market; however it is difficult to discriminate if these are defined as therapeutic vaccines or immunotherapy as these terms are used alternately. For the future we do expect more vaccine products to be approved. For the analysis of cancer vaccines we have used the risk profiles as estimated by Pronker et al. (2013)[46] for therapeutic vaccines in general. For Malaria, we expect 2 vaccine approvals within the next 2 to 6 years. Currently, there is at least one Malaria

[46] Pronker, E. S., Weenen, T. C., Commandeur, H., Claassen, E. H., & Osterhaus, A. D. (2013). Risk in vaccine research and development quantified. *PloS one*, *8*(3), e57755.

[47] Dimasi, J.A. et al. (2010). Trends and Risks Associated With New Drug Development: Success Rates for Investigational Drugs. *Nature Clinical Pharmacology and Therapeutics 87*(3), 272-277.

vaccine that has proceeded to phase 3[48], this adjuvanted vaccine contains antigens produced on a GMO-based production platform (*S. cerevisiae*). This clinical trial was not included in our dataset because based on the clinical trial description it was not retrieved from the clinical trial database with the predefined search terms. Noteworthy, it is unfortunate that we could not make estimations for HPV vaccines, as there is no risk profile documented for this indication.

As shown in Chapter 2 (Registered vaccines), currently, 83 vaccines (10%) of all (821) registered human vaccines are products of genetic modification. Based on the risk profiles as estimated by Pronker et al. (2013)[46], we show that this number will double as we expect a total of 86 genetically modified vaccines being introduced on the market within the foreseeable future (2-12 years). Please note that in this estimated number only vaccines for the indications in table 4 are included and all live-attenuated vaccines are considered non-GM, suggesting that in reality this number might be higher.

Looking at the overall ratio, the data shows that most GM vaccines are co-injected vaccines (54%) versus non-retrievable (46%). This is substantially different from the ratio for currently approved vaccines (95% non-retrievable). Although most new vaccines for hepatitis B and influenza will be recombinant proteins, which are non-retrievable, based on this difference it does seem that the proportion of co-injected vaccines will increase. Therefore, biological containment fail-safe strategies regarding these types of vaccines will most likely become increasingly important for the future.

3.6 Highlights

- 70% of VLP vaccines are targeted at HPV, including the only 5 approved VLP vaccines
- 61% of vector vaccines being developed are either MVA, Vaccinia, Fowlpox, or Adenoviral vectors
- 52% of vector vaccines are developed for cancer and 49% of DNA Vaccines are developed for HIV
- Recombinant/Subunit Vaccines are evenly developed for a range of indications
- At least another 86 GM vaccines are expected to be introduced on the market within the next 2-12 years, doubling the current amount of registered GM vaccines.
- Based on the risk profiling, GM vaccines targeting influenza has the best chance to make it to the market in the forthcoming years

[48] The RTS,S Clinical Trials Partnership; 2012; A Phase 3 Trial of RTS,S/AS01 Malaria Vaccine in African Infants; doi: 10.1056/NEJMoa1208394

CHAPTER 4

Preclinical Development

4. Preclinical Development

To map the trends of genetically modified vaccines that are in the preclinical development phase we performed a literature search for reviews with specified terms aiming to cover the major vaccine technology platforms that make use of genetic modification for both human and veterinary applications.

4.1 Methodology

The literature search was performed with the use of search codes that were designed especially for this project (in collaboration with the Medical Library of the Erasmus MC, Rotterdam, The Netherlands). The search results are presented in table 4. The coding of the search terms for the different search engines is provided in Appendix 1.

Table 4 Literature search results

Database	Hits	Hits after removal of duplicates
Embase.com	945	940
Medline (OvidSP)	364	97
Web-of-science	323	123
PubMed publisher	8	4
Cochrane DARE	7	2
Google scholar	100	79
Total	**1756**	**1245**

First, the 511 duplicates were removed and of the remaining set of 1245 articles, relevant articles were selected for further analysis based on the following criteria:

- Exclusion of review articles that did not describe vaccine technologies (e.g. cancer reviews with no reference to therapeutic vaccines; meta-analysis of diagnostic methods),
- Exclusion of review articles that did not describe novel vaccine technology (e.g. meta-analysis of seroprevalance; acceptability of vaccines), the time frame was set for 2009-present (2014) with the assumption that review articles before that period describe vaccines that either proceeded into clinical trial or were unsuccessful.

The final set consisted of 87 review articles. Since each article describes one or more vaccines at different dosages and in different stages of development we provide observations made for the vaccine types and indications that were described in these articles. Examples are described for the major observations, which are based on one or more review articles and when considered to be illustrative, additional articles were referenced.

4.2 Results

In contrast to the vaccines on the market and in clinical trials, which mainly target viral diseases, the number of reviews on bacterial (e.g. TB, *E.coli*, *Streptococcus pneumoniae*) and parasite (Cutaneous

leishmaniasis and Malaria) vaccines is relatively higher than that of viral vaccines. There are two factors that play a role in this transition. First, the 'easy' and 'simple' vaccines (mainly based on inactivated virus preparations) have been developed, registered and marketed, thus the more difficult pathogens, mainly bacteria and parasites, remain. Second, genetic modification is especially suited for these targets since it allows for formulation of a vaccine based only on a DNA sequence, a single protein (recombinant) or a peptide. It also facilitates more specified attenuation of a pathogen if a live-attenuated vaccine is required to establish good immunogenicity. Malaria is an illustrative example of these advantages.

Malaria vaccine development

Malaria is caused by parasites of the Plasmodium family that are transmitted to humans by mosquitoes. Basically there are two types of vaccine developments ongoing for Malaria. One aims at the induction of immunity by vaccination with parasite components, be it in the context of a liposome, recombinant protein vaccine or another form.

The other approach aims at the use of attenuated sporozites to immunize a person.[49] This attenuation was traditionally performed by treatment with chemicals but increasing knowledge on the composition and different stages of the malaria parasite allows for more specified attenuation through the use of genetic modification, although this does not necessarily guarantee full attenuation.[50] Attenuation can also be used to prevent the parasite from spreading from human to human through mosquito transmission. If the two alterations described here could be combined, the attenuated sporozite could not only protect the person in which it is injected but could also reduce transmission within the population.

The Malaria Research group of Prof. Robert Sauerwein at the Radboud Institute for Molecular Life Sciences (Nijmegen, the Netherlands) is one of the leading teams worldwide when it comes to development and evaluation of new Malaria vaccines and other countermeasures. They were among the first to apply genetic modification to malaria parasites in order to remove liver stage genes, hereby attenuating the parasite and making it suitable for vaccination.[51,52]

Some indications that stand out are Cutaneous leishmaniasis and, of particular interest for the Netherlands (regarding the recent outbreak in the east of Noord-Brabant): Q fever. For the first pathogen vaccines are described based on different GM vaccine platforms ranging from recombinant protein to DNA and live vaccines.[53,54]

[49] Guilbride et al; 2010; Why functional pre-erythrocytic and bloodstage malaria vaccines fail: a meta-analysis of fully protective immunizations and novel immunological model; doi: 10.1371/journal.pone.0010685

[50] Annoura et al; 2012; Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates.; doi: 10.1016/j.vaccine.2012.02.010

[51] Nganou-Makamdop K and Sauerwein RW; 2013; Liver or blood-stage arrest during malaria sporozoite immunization: the later the better?; doi: 10.1016/j.pt.2013.03.008

[52] Van Schaijk et al; 2008; Gene disruption of Plasmodium falciparum p52 results in attenuation of malaria liver stage development in cultured primary human hepatocytes; doi: 10.1371/journal.pone.0003549

[53] Kedzierski et al; 2006; Leishmania vaccines: progress and problems; doi: http://dx.doi.org/10.1017/S0031182006001831

[54] Khanjani et al; 2009; Vaccines for preventing cutaneous leishmaniasis; doi: 10.1002/14651858.CD007634

Q-fever vaccine development

Although Q-fever, caused by *Coxiella burnetii*, is not a new indication (first vaccines originate from the 1960's)[55], however the recent outbreak in the Netherlands has raised the discussion of the necessity and benefit of vaccination of people at risk (occupational or geographical). Interestingly, for Q fever, most vaccines that are described are based on conventional technology, illustrating that new indications do not necessarily require novel vaccine platforms.[56] The most described vaccine Q-vax (based on killed pathogen) causes a relatively high rate of side effects.

A quick scan of the recent research articles on Q fever vaccines indicates that indeed most vaccines are based on conventional technology. Safer alternatives are discussed that are also based on conventional technology and are meant for human application, e.g. a peptide based vaccines. Peng et al. describe such a vaccine based on a peptide (non-retrievable but nevertheless produced using genetic modification).[57] A reason to switch to such a vaccine platform could be one from an environmental and biological safety standpoint: large scale production of a pathogen, such as *Coxiella burnetii*, that is harmful to animals and humans, could be something that one would like to circumvent by using the peptide platform that allows for vaccine production without having to produce the live wild type pathogen first.

Furthermore, Q fever vaccines described in literature are mainly developed for human application, however veterinary vaccines against this disease could be of great benefit for the health of goats and greatly reduce the incidence of miscarriage in these animal populations.

As postulated above, genetic modification can be used in vaccine production for a reason of practical nature: it allows for safer production platforms than when working with the wild type pathogen. An illustrative example is that of Melioidosis, a disease caused by *Burkholderia pseudomallei*, a pathogen that is indexed in the US as a Category B select agent (potential bioweapon).[58] This pathogen is considered a bio-threat but is also a threat to public health: it is endemic in Thailand and Northern Australia. Protein-based, DNA and vector vaccine (dendritic cells) technologies are currently in development for this disease.[59] Another example is that of a recombinant protein vaccine that is developed for Anthrax, a disease that is caused by *Bacillus anthracis*, a bacteria that is transmitted occasionally from cattle to humans but is also used as a biological weapon. The latter application is the main reason for vaccine development against this pathogen. Anthrax vaccines are available and these are based on live spores or acellular formulations that contain the Protective Antigen (PA) protein,

[55] O'Neill et al; 2013; A Systematic Review and Meta-Analysis of Phase I Inactivated Vaccines to Reduce Shedding of Coxiella burnetii From Sheep and Goats From Routes of Public Health Importance; doi: 10.1111/zph.12086

[56] O'Neill et al; 2014; The effectiveness of Coxiella burnetii vaccines in occupationally exposed populations: a systematic review and meta-analysis; doi: 10.1111/zph.12054

[57] Peng et al; 2012; Development of a lipopolysaccharide-targeted peptide mimic vaccine against Q fever; doi: 10.4049/jimmunol.1201622

[58] http://www.bt.cdc.gov/agent/agentlist-category.asp#b

[59] Peacock et al; 2012; Melioidosis vaccines: a systematic review and appraisal of the potential to exploit biodefensevaccines for public health purposes; doi: 10.1371/journal.pntd.000148

purified from *Bacillus anthracis* cultures. New recombinant variants are in development to overcome safety issues with the currently registered vaccines.[60,61]
For example for live-attenuated vaccines, several risks can be identified: reversion to virulence, risk of transmission through shedding of the vaccine strain and risk of disease in immune-compromised subjects. One of the reasons to use genetic modification to attenuate a pathogen for vaccine purposes could be the reduction of such risks.

Of the vaccine types described in the reviews, GM vaccine technologies that are often discussed are recombinant proteins and viral vectors. DNA vaccines are only a minority. We speculate that this could be the result of the disappointing results with some DNA vaccines in clinical trials that might have dampened interest in the initiation of new DNA vaccine based platforms.[62] Recombinant proteins (non-retrievable) already form the largest portion of the registered genetically modified vaccines in humans and have a minimal risk in terms of biological containment. Viral vectors (co-injected) however are, apart from the single vector vaccine registered in Australia, still in the development phase. In the review articles these are underrepresented with only a few reviews on adenovirus and poxvirus based vectors. A possible explanation for this is that many of the vaccine vectors are explored in a limited number of studies, making it difficult to cover these vectors in review articles. As indicated throughout this report there are various vaccine vectors under development and information regarding the biological containment of these vaccine platforms is of importance. Therefore the established and novel vector technologies are also addressed in Chapter 8 of this report (Discussion).

Interestingly, new vaccine indications, of which autoimmune diseases are a prominent example, are not well represented in our literature dataset. There are two possible explanations for this. Firstly, their development is still in an early phase without relevant preclinical/clinical data to be reported in a review. Secondly, terminology that is used for immunomodulating approaches for autoimmune diseases and other indications, e.g. allergies, is not straightforward. Some call it immunotherapy others call it vaccination. A recent review by Van Hage et al illustrates the latter, in this single article both definitions are exchanged constantly.[63] For future registration of such products proper definition and guidelines have to be in place to facilitate the regulatory process.

In conclusion, genetic modification is employed for the development of especially bacterial and parasite vaccines currently in preclinical development. Since the dataset consisted of reviews that discussed varying numbers of research articles per vaccine type it was not possible to quantify these results or to provide actual numbers per vaccine type in this context. Furthermore the search terms were aimed to retrieve only reviews discussing vaccine technologies involving genetic modification and not what vaccines are in development based on 'conventional' technologies. Therefore it cannot be discussed whether or not at the preclinical stage the ratio of GM vaccines is higher compared to 'conventional'

[60] Campbell et al; 2007; Safety, reactogenicity and immunogenicity of a recombinant protective antigen anthrax vaccine given to healthy adults; Sep-Oct;3(5):205-11.
[61] Donegan et al; 2009; Vaccines for preventing anthrax; doi: 10.1002/14651858.CD006403.pub2
[62] Saade, F., and Petrovsky, N. (2012) Technologies for enhanced efficacy of DNA vaccines. *Expert review of vaccines*, 11(2), 189-209.
[63] Van Hage et al; 2014; New vaccines for Mammalian allergy using molecular approaches; DOI: 10.3389/fimmu.2014.00081

vaccines. Veterinary vaccines were underrepresented in the literature dataset, which is most likely the result of the fact that preclinical and clinical studies of veterinary vaccines often remain unpublished, especially when conducted by a commercial entity. We also noticed that, despite the specified search terms, review articles were retrieved that did not describe any GM vaccine technologies or only referred to them as a future option. A more specified search on one single technology would allow including research articles that provide detailed information on vector generation, handling and safety. Inclusion of research articles in our structured search was not feasible due to the large amount of available articles. Therefore we had to limit the search to review articles only, which explains why we could only present a birds-eye view of the preclinical stage of GM vaccines in this chapter. For future literature searches on GM vaccines we would advise to conduct these with a very specific research question in mind, focusing on a single indication (e.g. malaria) or a single vaccine technology platform (e.g. Adenovirus vectors (preferably even with the specific serotype e.g. Ad5)). This will give a more detailed insight in the status of that particular GM vaccine technology field and allows for evidence-based risk assessment when it concerns a co-injected vaccine type.

4.3 Highlights

- Bacterial and parasite vaccines are well represented in review articles (2009-2014)
- Vector vaccines and recombinant proteins are the most abundant GM vaccine types
- Vaccines for novel vaccine indications were not represented in the dataset, most likely due to the ambiguous terminology that is used for therapies that are developed for these novel indications
- The discrimination between immunotherapy and vaccines is vague when it comes to therapies for autoimmune diseases and especially allergies
- DNA vaccines are only a minority of the GM vaccines described in recent reviews

CHAPTER 5

Latest Trends

5. Latest Trends

In order to map the latest developments in vaccine technology and be informed with unpublished data and new vaccine concepts, one GM-focused and two vaccine-oriented conferences were visited (Table 5). For each conference the highlights were summarized and these are discussed in this part of the chapter.

Table 5 Vaccine conferences attended in the context of the GM vaccine project

	Organizer	Focus	Background Attendees
NVGCT Spring Symposium 2014	NVGCT Nederlandse Vereniging van Gen- en Celtherapie	Genetically modified vaccines and gene therapy	Academia / Industry / Regulatory
World Vaccine Congress 2014	Terrapinn	Vaccine development, production, marketing	Industry / Government
Vaccine Technology V	ECI Engineering Conferences International	Vaccine development	Academia / Industry / Government

5.1 NVGCT Spring Symposium 2014 - 14th of March 2014

Whereas the majority of the work discussed here was aimed at gene therapy, genetic vaccines had a dedicated session at this meeting. The majority of the work considered alphaviruses. These are small single-stranded RNA viruses of which Semliki Forest virus is most applied as viral vector for both prophylactic and therapeutic purposes. The first were aimed at induction of immunity against hepatitis C virus and Human papillomavirus and the second were used as anti-cancer vaccine in combination with local tumor irradiation. This work is all conducted at the UMCG (Groningen, the Netherlands), an indication that the Netherlands are at the frontline of novel vaccine technologies. Also an MVA-based influenza vaccine was discussed, which has been evaluated in a phase 1/2a clinical trial at the Erasmus MC (Rotterdam, The Netherlands). MVA-based influenza vaccines have been described first in 1994[64] and since then have evolved into a promising vaccine platform for influenza with multiple vaccines in phase 1/2a clinical trials aiming either at the induction of virus-neutralizing antibodies[65] or virus-specific T cells.[66]

5.2 World Vaccine Congress 2014 - 24-26th of March 2014

The conference, fully dedicated to vaccine development, manufacturing, marketing and registration provided a good overview of these four pillars and the indispensible connection between them. One of the main issues brought forward at this meeting was the necessity for a mind shift in the vaccine field.

[64] Sutter et al; 1994; A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus; Vaccine. 1994 Aug;12(11):1032-40.

[65] Kreijtz et al; 2014; Safety and immunogenicity of an MVA-based influenza A/H5N1 vaccine in healthy adults. A randomized phase I/IIa clinical trial; Submitted for publication.

[66] Gilbert; 2013; Clinical development of Modified Vaccinia virus Ankara vaccines; DOI: 10.1016/j.vaccine.2013.03.020

'Map what you know about vaccines then turn it upside down and look at it again with a fresh perspective' (quote from a conference speaker)

By means of vaccinomics, a directed rather than an empiric approach (Dr. G. Poland, Mayo Clinic, Rochester, United States) and the related concept of reverse vaccinology (Dr. R. Rappuoli, Novartis Vaccines, Italy) we could design better vaccines based on pre-defined antigens.[67,68] These can be presented in the form of a recombinant protein, DNA vaccine or vector-based vaccine. This would also allow for personalized vaccinology where gender, age, race/ethnicity and genetic polymorphisms can be taken into account when deciding which vaccine should be selected for the respective person. It was postulated that we should abandon the 'one size and dose fits all vaccine approach'. The generation of recombinant proteins, DNA vaccines (or maybe even mRNA vaccines, supposedly two groups worldwide are working on that at the moment, one in Germany and the other is Novartis) and recombinant vectors is only possible with the use of genetic modification. Regarding the latter, although there is already a wide variety of vaccine vectors available, new vectors were introduced at this meeting as well: e.g. Triatoma virus (TrV) a virus derived from arthropods that can also be applied for generation of VLP vaccines (Guérin et al, Unidad de Biofisica, Bizkaia, Spain). It was also suggested that viruses from plants could be used as vaccine vector. What is indicative for these examples is that vaccine developers are thinking out of the box to come up with solutions for one of the main issues raised against vector vaccines: immunity to the vector, which is an issue for adenoviruses but also for other human virus-based vectors. When using a vector that is not derived for humans and does not have a human analogue, this circumvents the issue of pre-existing immunity to the vector. However, the environmental risk assessment could be complicated when the vector's original host is an insect or a plant. Apart from recombinant proteins and DNA and vector vaccines, genetic modification can also be used to specifically attenuate a pathogen such as malaria sporozites. This technology was described to be filed with the FDA in 2017, with mass vaccination starting 2019 (Sanaria Inc, Rockville, United States). This is an example of how the use of genetic modification for pathogen attenuation can and in the future most likely will replace 'natural' attenuation (e.g. through passaging) since the GM-attenuation enables exact mutations/alterations to the genome.

Regarding vaccine production, the upcoming GM production platforms are based on existing and novel cell lines and plants. The latter promises high yield per kilo and should be able to facilitate production of 10 million doses of influenza vaccine in 30 days (Medicago, Québec, Canada). The platform can be used for various infectious diseases since it makes use of the VLP technology: after infiltration of DNA in the plant it produces VLPs.

Vaccine development has always aimed at infectious disease indications. In the last decade therapeutic vaccines for different types of cancer have been developed. Several conference speakers indicated that non-infectious disease targets will be the future. It will be challenging but it would be great to have such vaccines since there are large public health issues to counter such as Alzheimer, type 1 Diabetes and Rheumatoid Arthritis.

[67] Poland et al; 2011; Vaccinomics and Personalized Vaccinology: Is Science Leading Us Toward a New Path of Directed Vaccine Development and Discovery?; DOI: 10.1371/journal.ppat.1002344

[68] Rappuoli; 2001; Reverse vaccinology, a genome-based approach to vaccine development; DOI: 10.1016/S0264-410X(00)00554-5

5.3 Vaccine Technology V - 8-13th of June 2014

This meeting was fully dedicated to novel vaccine approaches and technologies. One of the main outcomes of the meeting was the need for vaccines for new indications, mainly in, but not restricted to, the field of infectious diseases. To name a few: Candida infections, hepatitis C and hepatitis D. Among the non-infectious disease targets were Multiple Sclerosis, Rheumatoid Arthritis, bee venom allergy and type I Diabetes. Also hepatitis B DNA vaccines were presented that induced stronger cellular immune responses than adjuvanted hepatitis B virus core antigen (protein-based) vaccines (Karolinska University Hospital, Stockholm, Sweden). Although DNA vaccines have been in development a long time, they do not seem to make it to the market. It was indicated that this is because DNA prime vaccination induces poor serology and CD4+ T cell responses which could be improved in 3 ways: better gene expression (through gene optimization), more cells transfected (better delivery technology), excite direct presentation to the immune system. The first option is only possible with genetic modification.

- ***The term antigen-by-design was much heard during the conference –***

In the field of influenza vaccine research, the development of a universal influenza vaccine, protecting against all influenza A virus subtypes is the Holy Grail. By means of antigen-by design (computationally optimized broadly reactive antigen (COBRA)), a hemagglutinin was developed that could evoke broadly neutralizing antibodies based on its conserved stem region (University of Pittsburgh, Pittsburgh, United States). Furthermore, antigen-by-design does not only apply to improvement of vaccine immunogenicity but also to vaccine safety. A significant example was given; Rift valley fever virus (RVFV) vaccine development is complicated because of the pathogenicity of the virus. An alternative technology was described in which specifically designed RVFV genes were cloned in cDNA plasmids that were then used to generate attenuated RVFV virus and establish a DIVA effect, allowing for the discrimination of infected and vaccinated animals (CDC, Atlanta, United States). The attenuated virus has excellent environmental containment since it has been downscaled as a BSL-2 agent making production much more convenient.

Novel production substrates were also introduced such as a protozoa from lizards: *Leishmania tarentolae* which is in development as a platform for production of influenza A virus hemagglutinin (Sanofi Pasteur, Marcy L'Etoile, France).[69] Furthermore a cell-based platform was described using a combination of two existing cell lines: Vero cells and CHO cells. Evaluation of high density production of MVA vaccines on a relatively new cell line AGE1.CR.pIX was also described (Probiogen, Berlin, Germany). This cell line derived from Muscovy ducks could provide a solid alternative for the production of MVA virus on Chicken Embryonated Fibroblasts.[70]

[69] Pion et al; 2014; Characterization and immunogenicity in mice of recombinant influenza haemagglutinins produced in Leishmaniatarentolae; doi: 10.1016/j.vaccine.2014.07.092

[70] Lohr et al; 2009; New avian suspension cell lines provide production of influenza virus and MVA in serum-free media: studies on growth, metabolism and virus propagation; doi: 10.1016/j.vaccine.2009.05.083

- Registration and licensing of IMVAMUNE is expected to boost MVA-based vaccine development –

The fast developments in the field of vector vaccines were characterized by the fact that IMVAMUNE (MVA-based smallpox vaccine)(Bavarian Nordic, Kvistgaard, Denmark) is delivered to the BARDA-initiated US vaccine stockpile. It is expected that this vaccine will be licensed within the next 2 years. Although the vaccine itself is not a GM and does not function as a vector for this particular application, MVA is widely used as a vaccine vector in numerous clinical vaccine candidates.[71]

5.4 Highlights

-Two prominent viral vector platforms used for vaccine development in the Netherlands, are based on alphaviruses and poxviruses

-The mind shift in vaccine development has led to new insights and possibilities for future vaccines through implementation of reverse vaccinology and vaccinomics

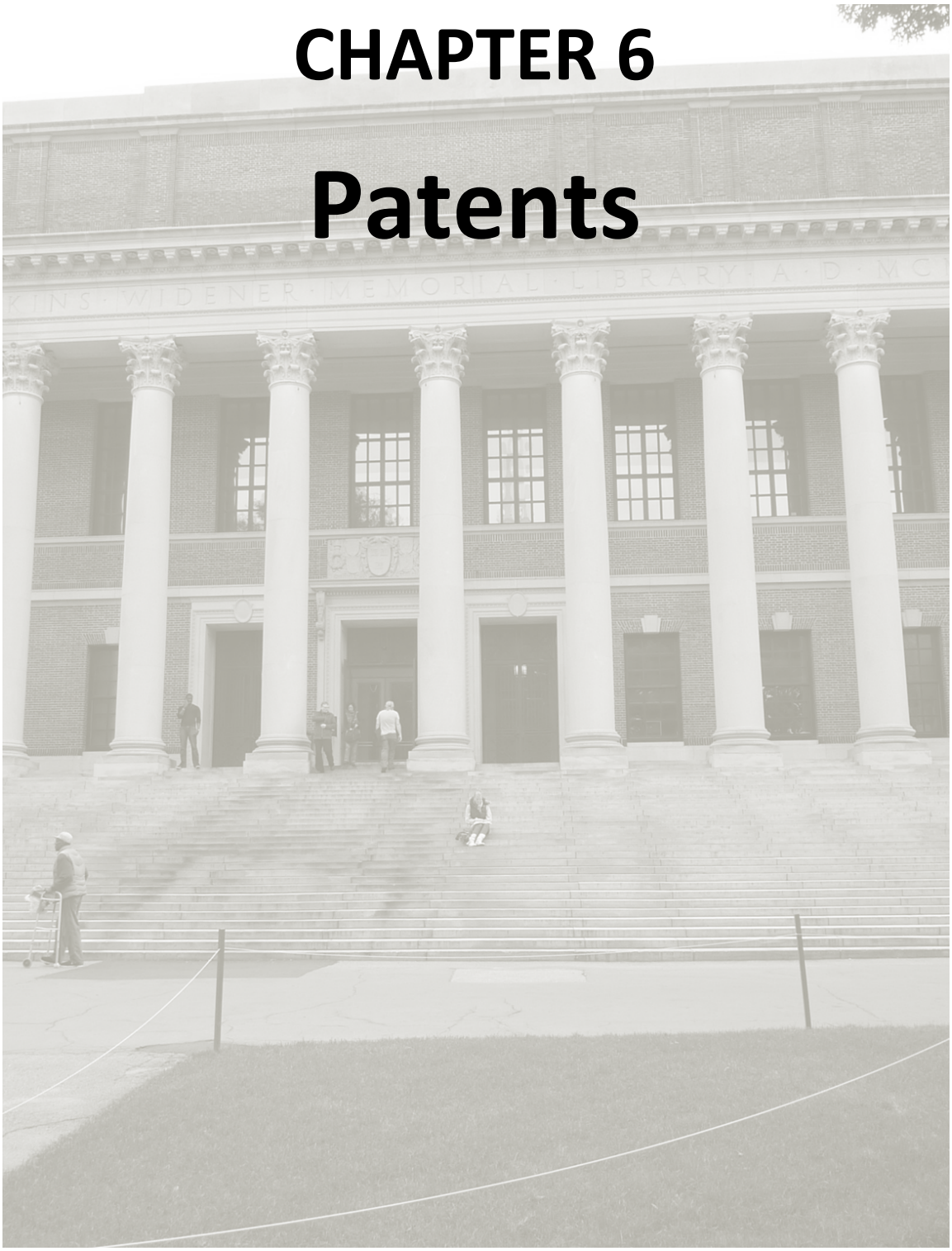
-New production substrates are upcoming such as plant-based protein production and advanced cell lines based on genetically modified cells

-New vaccine indications, based on non-infectious disease targets, such as autoimmune diseases and Alzheimer's disease are difficult targets but it would be of great benefit to have vaccines available for these diseases that have an increasing impact on public health

[71] Kreijtz et al; 2013; Poxvirus vectors; DOI: 10.1016/j.vaccine.2013.06.073

CHAPTER 6

Patents



6. Patents

Here we present the analysis of the new vaccine concepts as these are described in the patent literature to provide an overview of new technologies that can be expected in the next 5-10 years in preclinical and clinical vaccines studies with GM vaccines. The patents cover both human and veterinary vaccines, as many vaccine concepts are applicable to one or more pathogens for multiple hosts.

6.1 Methodology

Espacenet was used as the main database for the patent search (for a full list of the patent issuing organisations in Espacenet please refer to Appendix 2).[72] The most effective way to find patents relating to a specific technological subject is by using CPC codes.[73] CPC codes are added to patent documents by reviewers, to classify the content of the patent. This enables users of the database to also retrieve patent documents on which different keywords were used to describe the invention.

The current research focuses on genetically modified vaccines, leading us to use the CPC codes as defined in Table 6. The complete query was defined as: (A61K2039/00 OR A61K39/00) AND C12N15/00 OR C12N2015/00). The search retrieved all patent documents that were filed with this combination of CPC codes (16.745 patent documents). Since this resulted in many duplicates in patent documents, priority numbers were used to deduplicate the results.[74] Whenever a patent document had a priority number that corresponded to a priority number on another patent document, both documents were considered to be part of the same family. When all patent documents were analysed, the document with the oldest application and publication date was considered to be priority document and mother patent and was used for subsequent analysis (2897 patent documents). Choosing the mother patent resulted in consistency throughout the entire analysis, since all patent documents have a priority document but not all recently patent applications have extended on their primary patent document.

Table 6 CPC code search

	Database	Search term	# of patent documents	Deduplication on the basis of priority numbers
Patents	Worldwide. espacenet. com	Medicinal preparations containing antigens or antibodies (A61K2039/00 OR A61K39/00) AND Mutation or genetic engineering; DNA or RNA concerning genetic engineering, vectors, e.g. plasmids, or their isolation, preparation or purification; Use of hosts therefore (C12N15/00 OR C12N2015/00)	16.745 patent documents	**2897 unique patent families**

[72] www.worldwide.espacenet.com
[73] http://ep.espacenet.com/help?locale=en_EP&method=handleHelpTopic&topic=cpc
[74] The priority number is the number of the application in respect of which priority is claimed, i.e. it is the same as the application number of the claimed priority document. By using priority numbers, patents belonging to the same patent family can be identified.

Only patent families of which the first patent was applied for after 01-01-2009 were included in this study, leading to a reduction of the sample to 589 unique patent families. These patents were characterized based on their CPC codes. All found patents had the CPC codes that were used in the query, but other CPC codes were also used to characterize the invention. All CPC codes in the sample were defined and classified into a group of codes. Not all CPC codes referred to information relevant for the current study. The CPC codes that entailed information that was relevant for the current research questions were classified in the categories as shown in Table 7.

Table 7 CPC codes considered relevant for characterization of patents

Relevant CPC codes	
Application field	Indication
Class	Organism
Co-injected / Non-retrievable	Production system
Containment measures	Target
Expression Systems	Exclusion criteria

Furthermore, the current study specifically did not intend to look at therapies or agronomic applications. Therefore, all patents describing therapies (gene therapy, immunotherapy, non-vaccines) or agronomic applications were excluded, leaving 309 unique patent families.

Each patent family was characterized based on the CPC codes on the mother patent. Afterwards, rechecking patent titles, and sometimes even abstracts with characterizations, allowed for some refinements in patent definitions.

Finally, for each patent family, all downloaded patent documents were identified based on their publication number and kind codes. [75] This allowed for an *indication* of the number of countries in which a patent was applied for or granted.[76]

[75] The first two letters of the publication number on a patent refer to the geographical region for which patent protection is applied, e.g. a patent that starts with NL concerns patent protection in the Netherlands. The publication number ends with a code between brackets, i.e. the kind code. This code refers to the type of document that is being published. This may for instance be a patent application, a (preliminary) search report or a granted patent.

[76] Espacenet automatically groups patent documents in the search results that belong to the same patent family, although this information is not downloadable. Due to downloading restrictions (maximum of 50 patent documents per download), search terms were constantly adapted to ensure the search did not yield more than 50 patent documents at once. This was done by restricting date of publication, going back a month / half a month at a time. When patents belonging to the same patent family were published in the same time period, Espacent grouped these documents into one family and one version was downloaded, thereby leaving certain publication numbers out of our initial results. When the same patents were published in different time periods, all these documents were found in our initial results. The number of countries in which a patent was applied for or granted thus constitutes a *minimum.*

6.2 Indication and Targets

As shown in Figure 23, the vast majority of patents are related to infectious diseases (62%), with multipurpose (20%)[77] and cancer (13%) applications being the other two large indication groups. The remaining 5% of patents were divided over autoimmune / immunosuppressant (e.g. allergies and autoimmune diseases); nervous system (e.g. MS or Alzheimer's) and other (e.g. addiction).

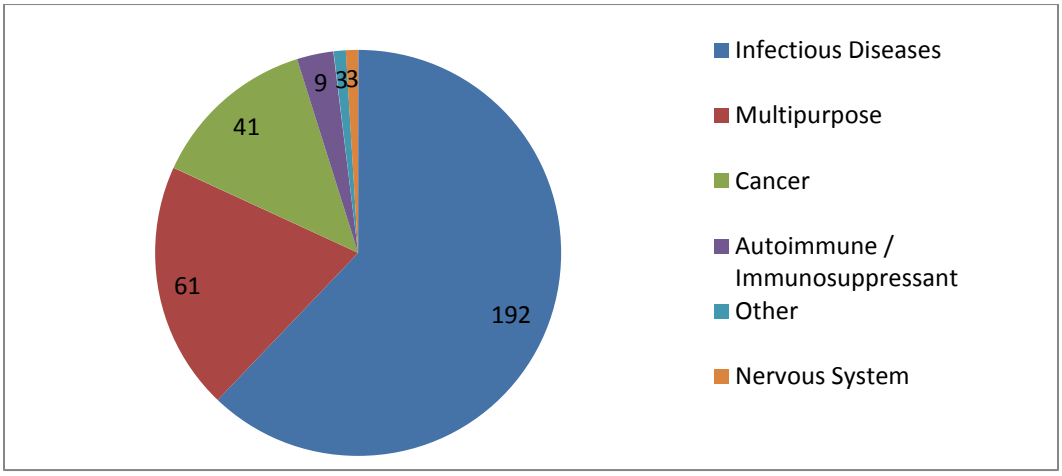


Figure 23 Indication fields of patents

Within the patents that were related to infectious diseases, the majority of patents describe vaccines targeting viruses (66%). Bacteria are the second largest target (15%). The remaining 19% is divided over multiple types (e.g. viruses and bacteria), protozoa (e.g. malaria) and mites / parasites / worms. An overview of the targets is provided in Figure 24.

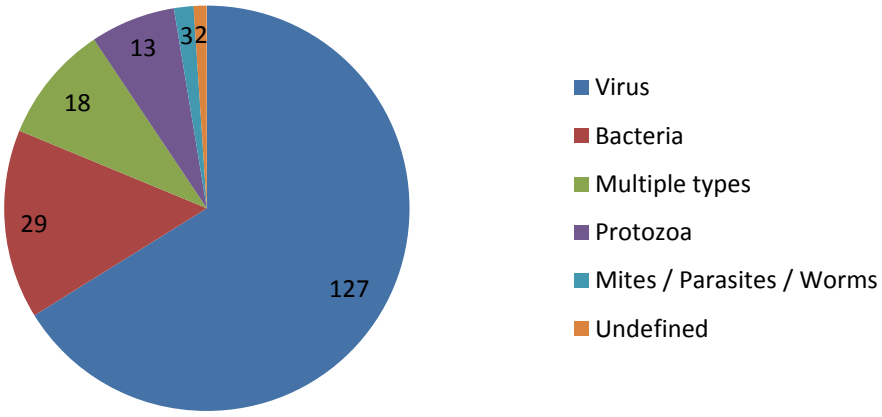


Figure 24 All vaccine targets within infectious diseases

[77] Multipurpose: a platform technology that could be applied for vaccines against indications from the different sets described; for example (fictional) a viral vector that can be used as the basis for a prophylactic influenza A virus vaccine but also as the backbone for a therapeutic vaccine against Rheumatoid Arthritis.

When looking more closely at the viral targets (see Figure 25), it was found that most patents relate to multiple viruses (29%) as target. This is illustrative of the 'broad patenting' approach that enables the development of new findings in multiple directions (e.g. one vaccine vector platform that can be employed for vaccines against various infectious diseases (e.g. viral or bacterial infections). These patents are written as 'broad' as possible while still adhering to patent guidelines that only allow patenting of findings for which a proof of principle exists. In those patents that indicated a single viral target the vaccines within the respiratory virus area (*Orthomyxoviridae* and *Paramyxoviridae*) are most widely represented (22%). Furthermore, a large set of new patents is filed in the field of *Retroviridae* (e.g. HIV) (9%), *Herpesviridae* (e.g. Cytomegalovirus (CMV)) (8%), *Rhabdoviridae* (e.g. Rabies virus) (4%) *Flaviviridae* (e.g. Dengue virus) (4%), *Togaviridae* (e.g. Equine Encephalitis virus and Rubella virus) (3%) and *Papillomaviridae* (e.g. Human Papillomavirus) (3%). This could indicate a high unmet medical need for vaccines targeting these pathogens or refer to commercial opportunities in these vaccine fields.

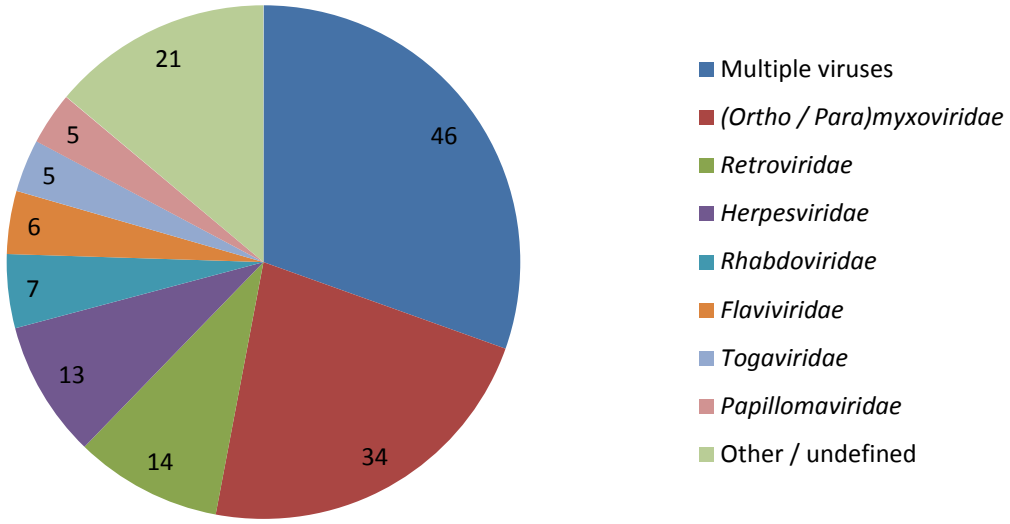


Figure 25 Viral targets within infectious diseases

In the remaining group (other / undefined) the following virus families are found (with number of found patents between brackets): *Reoviridae* (3), *Parvoviridae* (3), *Hepadnaviridae* (2), Alphatorquevirus (2), Hepatitis virus (2), *Poxviridae* (2), *Picornaviridae* (2), *Filoviridae* (1), *Bunyaviridae* (1), *Arterivirus* (1), *Comoviridae* (1) and *Hepeviridae* (1).

6.3 Technology

When taking all the patents in the period 2009 – 2013 together, almost half of the found patents relate to vector-technology (46%). The second largest technology type is DNA / RNA vaccines (20%) with the third largest group being vaccine being VLP (11%). Peptide / protein vaccines account for 8% of patents and live-attenuated for 5% of patents.

Although the proportion of each technology class has fluctuated over the last five years (see Figure 26), no major changes have occurred. The results show that in current vaccine development the focus is

placed on techniques that are more complex than the attenuation of pathogens or simple purification of antigens, since the majority of vaccines in development are manufactured using VLP, DNA/RNA or vector technology.

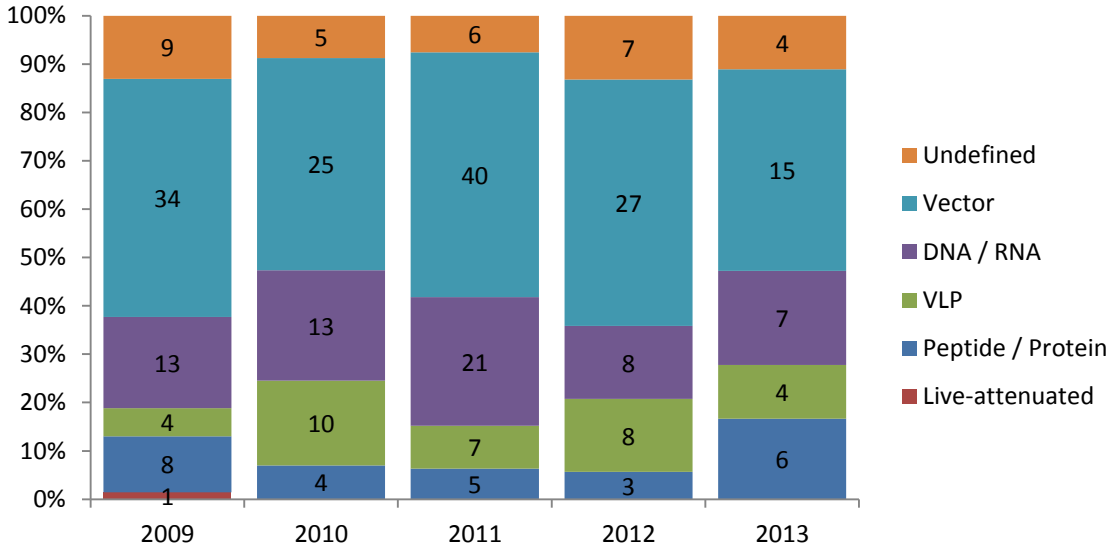


Figure 26 Number of patents based on specific technology classes and their relative proportion over the years

6.4 Geography

6.5.1 Applicants

Looking at the applicants of the analysed 309 patents, it was found that the majority of applicants were based in the United States: 145 patents (47%) were applied for by a US applicant. Other countries with many applicants were Germany (20 patents), France (16 patents), Canada (15 patents) and Japan (15 patents). Applicants based in the upcoming BRIC countries applied for 5 (Brazil), 0 (Russia), 1 (India) and 6 (China) patents. Table 8 provides an overview of all the number of patent families with an applicant from a specific country.

Table 8 Number of patent families with main applicant from the specified countries

Country	*Nr. of patent families with country-based applicant*	***Country***	*Nr. of patent families with country-based applicant*
[US]	145	**[CN]**	6
[DE]	20	**[BR]**	5
[FR]	16	**[SE]**	4
[CA]	15	**[AU]**	4
[JP]	15	**[Unknown]**	3
[CH]	14	**[TW]**	3
[GB]	12	**[PL]**	2
[ES]	10	**[ZA]**	2
[KR]	8	**[MX]**	1

[NL]	8	**[IN]**	1
[DK]	7	**[AT]**	1
[IT]	6	**[IE]**	1

6.4.1 Patent Protection

Based on the kind codes (see also footnote 75 and 76) of all downloaded patents belonging to the 309 analysed patent families, an indication can be given of those markets that are considered to be commercially viable by the applicants. Again, it is important to stress that this is just an indication, since not all patent documents belonging to the analysed patent family are downloaded due to the INPADOC feature of Espacenet (see also footnote 76). The results are shown in Table 9. At least 226 of the 309 analysed patent families applied for a worldwide patent, 159 for a US patent and 118 for a European patent. The following two largest countries for which patent protection was applied were both Asian: China (at least 74 patent families) and Japan (at least 70 patent families). Interestingly, although none of the applicants were based in Russia, for 9 patent families patent protection was applied for in Russia.

Table 9 Geographical areas in which patent protection was applied for

Geographical area	*Nr. of patent families applying for protection*	***Geographical area***	*Nr. of patent families applying for protection*
WO	226	**TW**	7
US	159	**CO**	6
EP	118	**BR**	4
CN	74	**DE**	3
JP	70	**ES**	3
CA	41	**IT**	3
AU	40	**FR**	2
MX	39	**GB**	2
KR	35	**UY**	2
SG	18	**DO**	1
NZ	10	**PE**	1
AR	9	**PL**	1
RU	9	**SE**	1
EA	8		

For correct interpretation, also see footnote 75 and 76.

6.5 Trends

Over the 5 years that have been included in this analysis, distribution of patents over the different indications has remained similar (Figure 27). Infectious diseases make up for more than half of the patents and the two other major groups are multipurpose and cancer vaccines. Within infectious diseases the majority of patents focus on viral vaccines, although in recent years a small decline in the number of patents focusing on viral targets can be observed (see Figure 28). This may be due to the fact

that vaccine development for viral targets is increasingly difficult now that all the low-hanging fruits (viruses that are 'easily' targeted with vaccines) have been picked.

Example of an innovative technology: iDNA vaccines for RNA viruses

A patent filed by Medigen (US) describes the use of immunization with DNA as a vehicle for *in vivo* expression of RNA viruses.[1] This invention is a "best of both worlds" scenario, combining the benefits of DNA vaccines with those of live attenuated vaccines while at the same time circumventing the limitations of both. DNA vaccines are known for their genetic and chemical stability, safety, ease of production, activation of innate pathways and their lack of need for a cold chain e.g. during transport and storage. They have not reached the human market yet, however, primarily due to their limited immunogenicity.

Live attenuated vaccines are highly immunogenic but also encompass risks, such as the possibility of reversion to wild type. This is especially the case with RNA vaccines. Live attenuated vaccines furthermore are less easy to handle (bio-containment during preparation, cold chain requirements).

The present invention incorporates genomic RNA from an RNA virus into the genome of a plasmid under the control of an eukaryotic promoter. Vaccination with this (DNA) plasmid leads to the *in vivo* transcription of viral RNA and the assembly of the attenuated RNA virus. Since the attenuated virus no longer needs to be grown in a cell culture the risk of reversal is limited, while the vaccination process has shown to be highly immunogenic in mice.[2]

1 Patent: Idna vaccines and methods for using the same; WO2010008576A3
2 Tretyakova et al; 2013; Novel vaccine against Venezuelan equine encephalitis combines advantages of DNA immunization and

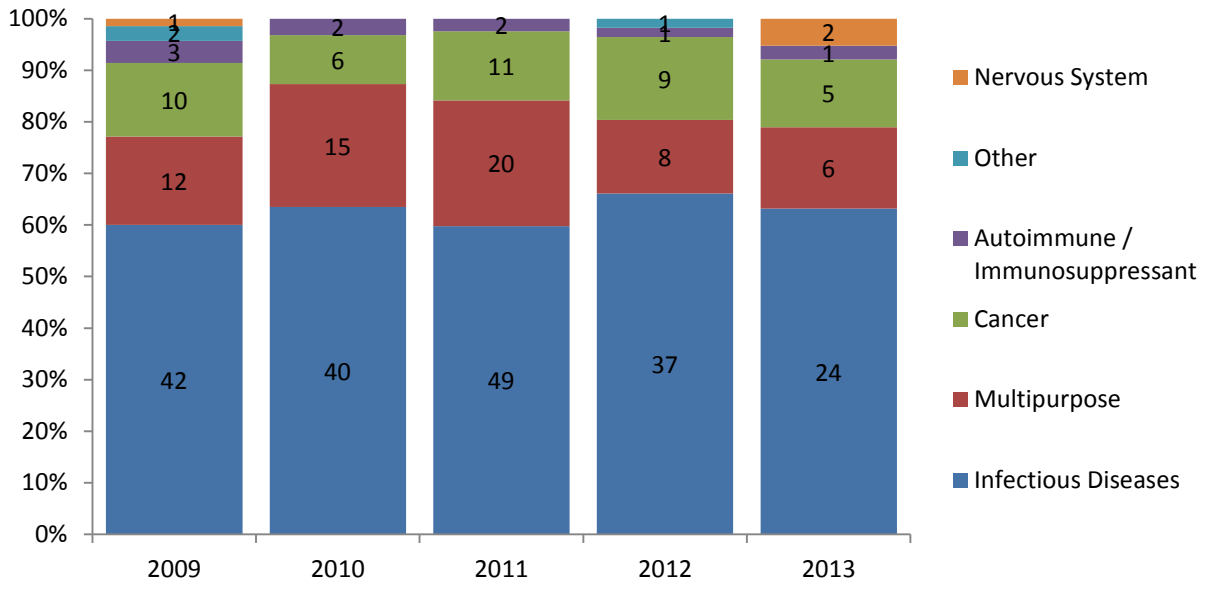


Figure 27 Number of patents on different indications and their relative proportion over the years (absolute numbers)

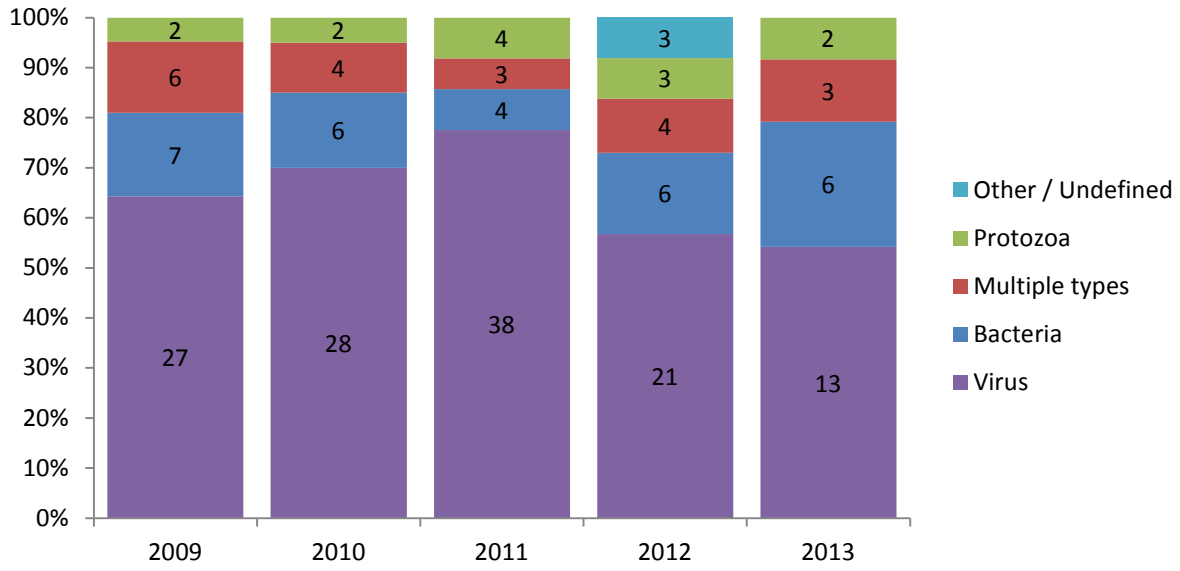


Figure 28 Number of patents targeting infectious disease types and their relative proportion over the years (absolute numbers)

6.6 Highlights

- Patents on GM vaccines mainly focus on infectious diseases, and more specifically on viruses

- The two other main patent categories are cancer vaccines and multipurpose vaccines

- The vast majority of patented inventions concern technically more complicated vaccines with the less complicated protein and live attenuated vaccines being the small minority

- The proportion of viral vaccines has been decreasing in recent years, which may be indicative of the saturation of vaccines for the 'easy' targets

- Most applicants originated from the US, Germany or France. In Asia the major applying countries are China and Japan

CHAPTER 7

Expert Opinions

7. Expert Opinions

To provide context and relevance of the data presented in the previous chapters, experts-in-the-field of vaccine technology were interviewed and asked for their insights and opinions on the role of genetic modification in vaccine development.

7.1 Methodology

Candidates for the interviews were selected and invited from Academia, Industry, Regulatory authorities and others. We aimed for at least 2 to 3 candidates per segment. Apart from the regulatory segment for veterinary vaccines two candidates responded in each group (see Table 10). Once they had accepted the invitation they received an introductory document together with the questions upfront. Once the candidates agreed to participate, they received the introductory document together with the questions in advance.

Table 10 Interviewees from key sectors in vaccine development

	Human	Veterinary
Academia	2	2
Industry	2	2
Regulatory	2	1
Others*	2	

Indicated in this table are the number of candidates per sector that accepted the invitation of the interview and contributed with an interview.

** Entrepreneur / Subsidiary*

Questions were deducted from the research questions as described in Part B. Although each expert has his own field of expertise be it antigen design, production or vaccine registration, all questions were asked to every candidate. Most interviews were either held by phone or video conference; one candidate answered the questions in writing. The interview questions are listed in Table 11.

Table 11 Interview questions

1	**Vaccine development**
	What is the technical potential of genetic modification in vaccine development?
2	**Vaccine manufacturing**
	a. What is the large scale production potential of genetically modified vaccines? Is this different from 'traditional' vaccines?
	b. *What is the market potential of genetically modified vaccines? Is this different from 'traditional' vaccines?*
3	**Vaccine registration**
	a. What is the registration potential of genetically modified vaccines? Is this different from 'traditional' vaccines?

		b. What is the application potential of genetically modified vaccines? Is this different from 'traditional' vaccines?
		c. *Do current rules and regulations cover these novel vaccine technologies?*
4	**Hurdles**	
		a. What are the hurdles in the development pipeline of genetically modified vaccines?
		b. *How can these hurdles be overcome?*
5	**General questions**	
		a. How come that in contrast to the food sector, where GM is clearly labeled, GM in the vaccine field is rather anonymous?
		b. What should be done to make sure that novel vaccine technologies (GMO or using GM) will evolve from 'best next thing' to 'bench' to 'bedside'?
		c. *What are your future perspectives for the role of genetic modification in the vaccine field?*

7.2 Results

7.2.1 Vaccine Development

What is the technical potential of genetic modification in vaccine development?

All candidates are convinced that the use of genetic modification has high potential for smart vaccine design (e.g. reverse vaccinology, vaccinomics and DIVA vaccines) in order to create safer and more efficacious vaccines. Furthermore they indicate that GM allows for construction of vaccines against difficult pathogens and tumor-derived antigens.

'This is where the cutting edge is. Where the state of the art is!' (Industry, human vaccines)
'We can make a vaccine from an email' (Industry, human vaccines)

7.2.2 Vaccine Manufacturing

a. What is the large scale production potential of genetically modified vaccines? Is this different from 'traditional' vaccines?

Large-scale production of GM vaccines will definitely not be more difficult/laborious than that of conventional vaccines. It is plausible that GM vaccine production will be more efficient and safer since it is based on platform technology and the pathogen to be grown can be modified so that it can be grown at a lower biosafety level (e.g. Classical Swine Fever). With regard to vector vaccines, a new

component in a vector might affect yield but this is not the case per se.

b. What is the market potential of genetically modified vaccines? Is this different from 'traditional' vaccines?

The market is good and does not differ that much from the market for traditional vaccines. In addition, GM vaccines provide the opportunity to open up new markets for which no traditional vaccines are available. Although the production of GM vaccines is often more costly than that of traditional vaccines, the scalability of this production process is higher and compensates for this difference. In the veterinary field a striking example is the Porcine circovirus 2 (PCV) vaccine, a VLP generated via GM that has the highest market value in animal health vaccination worldwide (460 million dollars).
In conclusion, the overall market potential of GM vaccines is good.

'In the veterinary field vector vaccines are the most rapidly growing vaccine type in terms of sales' (Industry, veterinary vaccine)

7.2.3 Vaccine Registration

a. What is the registration potential of genetically modified vaccines? Is this different from 'traditional' vaccines?

In general GM vaccines are slightly more difficult to register, mainly based on the more stringent requirements to show safety, stability and efficacy. This primarily has an effect on the budgets for clinical evaluation of such vaccines, which need to be even higher than the budgets for traditionally produced vaccines. Furthermore, the regulations differ between geographical areas. The FDA (United States) already has strict regulations when it comes to GM vaccines. The EMA is even stricter and regulations in Japan are considered to be most stringent. In contrast, Australia is considered to be one of countries with more lenient regulations regarding GM vaccine registration.

'Traditional vaccines: use "natural" mutations. With GM we know better what we are doing' (Industry, human vaccines)

b. What is the application potential of genetically modified vaccines? Is this different from 'traditional' vaccines?

Although public opinion might hamper acceptance a bit, willingness to take GM and GM produced vaccines will eventually not be different taking into account the following conditions:
- there is a clear need for vaccines that target pathogens for which traditional methods have failed to produce safe and effective vaccines.
- the use of GM for vaccine construction/production is beneficial for safety and efficacy and these advantages are well communicated to the public

This might be slightly different for DNA vaccines because there the sentiment is different based on the history of DNA and gene technology and their use in the genetic modification (also referred to as genetic manipulation) of food. In the end, once more and more GM vaccines are registered the add-on effect is expected to result in more general acceptance.

'At the end of the day it's the way to go' (Academia, human vaccines)

'The medical world is reluctant to step away from the traditional technology. Good data convinces everyone in the end' (Industry, human vaccines)

c. Do current rules and regulations cover these novel vaccine technologies?

Yes, guidance documents are available for vector and DNA vaccines and rules and regulations are in place. Often, vaccine developers take an even more strict interpretation of these rules than would scientifically be necessary. Generally speaking, rules and regulations are a bit behind the state-of-the-art technology that has to prove itself for registration by generating a convincing data set on safety and efficacy.

'New technologies are often in a gray regulatory area' (Regulatory, human vaccines)

7.2.4 Hurdles

a. What are the hurdles in the development pipeline of genetically modified vaccines?

The majority of interviewees indicated that costs were the major hurdle for the development of vaccines and GM vaccines in particular. This money is needed for the extensive safety testing, stability testing, generation of stable cell substrates / transfected cells, establishment of internal competence (for industry) and last but not least efficacy testing. These issues apply to vaccines in general; however, since the low hanging fruits have been picked the more difficult targets remain that require more investment of time, knowledge and money.

'In French Cuisine the main ingredient is Butter, Butter, Butter; for development of GM vaccines it is Money, Money, Money' (Other)

b. How can these hurdles be overcome?

Funds (e.g. through Public-Private Partnerships), generate extensive datasets on safety of the vaccine that is to be registered.

'Furthermore, harmonization of rules and regulations internationally will contribute to faster availability of GM vaccines' (Regulatory, human vaccines)

7.2.5 General questions

a. How come that in contrast to the food sector, where GM is clearly labeled, GM in the vaccine field is rather anonymous?

Labeling of GM vaccines is considered to be unnecessary. It might be good to communicate on the strengths and benefits of the technology but not through labeling on the product, the interviewees indicate that this could evoke misuse or incorrect risk-perception (GM as a danger signal instead of modern technology that is used for the sake of better vaccines). Although in the food sector this is a common procedure, the interviewees consider food and vaccines to be fundamentally different; whereas consumers have a broad buffet of choices when it comes to food, the availability of vaccines is scarce while the need is high. Furthermore, GM vaccines, though not many, have been on the market for years and there has been no furor on this from the public.

'A vaccine is already a drug and its risks do not per se come from the fact that it's a GMO' (Industry, Human vaccines)

b. What should be done to make sure that novel vaccine technologies (GMO or using GM) will evolve from 'best next thing' to 'bench' to 'bedside'?

Overall the interviewees indicated that nothing special needs to be done to stimulate the development of GM vaccines, since they are the future and that will not change. They did provide suggestions on how to speed up development, registration and acceptance: resources for R&D and public-private partnerships and starting the discussion with the regulatory authorities as early as possible, were considered key success factors. Furthermore, addressing high priority unmet clinical needs first will favor early registration and acceptance.

'Invest in these technologies and the knowledge that comes with it --> never change a winning horse' (Academia, Human vaccines)

c. What are your future perspectives for the role of genetic modification in the vaccine field?

The technology is now already widely applied and will take over most of the conventional vaccine production technologies. It is said that in the future all human vaccines will be constructed, optimized and/or produced by using genetic modification. For veterinary vaccines many conventional vaccines will stay (easy and cheap to produce and the veterinary vaccine field, livestock in particular, is highly economically driven). For new targets, GM vaccines will be developed when there is a technological need from a market, regulatory and/or production perspective.

'The sky is the limit' (Academia, Human vaccines)
'Negative attitude against GM vaccines could be considered criminal' (Academia, Veterinary vaccines)
'This is the future, full stop.' (Other)

7.3 Highlights

- GM vaccines and GM vaccine production facilitate antigen-by-design and safe production
- Their Research & Development should go hand in hand with establishment of rules and regulations
- Costs are the major hurdle in bringing these vaccines to the market
- For human application GM vaccines will be the only way forward, for veterinary application both GM vaccines and conventional vaccines will be developed

CHAPTER 8

DISCUSSION

8. Discussion

Here we combine the data from all chapters to come to an integrated view of general insights, trends and recommendations. This chapter is divided in different paragraphs dedicated to the questions that have risen during the course of this project.

8.1 What is available now and what can we expect?

While the first-ever recombinant vaccine was introduced on the market over 30 years ago (Recombivax HB®)[78] the proportion of GM vaccines in both the human and veterinary field is still limited to approximately 10%.[(this report)] GM vaccines on the market for human use are virtually all recombinant proteins whereas for veterinary use, multiple vector vaccines have been registered and marketed. It can be concluded that the low hanging fruits have been picked in both the human and veterinary vaccine field. The 'easy' pathogens are covered, mainly by conventional vaccines (e.g. purified antigens or live-attenuated pathogens), leaving the more 'difficult' pathogens (e.g. Malaria, HIV, tuberculosis) and other indications open to be covered by new vaccine technologies that use genetic modification, as is indicated by the expert interviews discussed in Chapter 6.[[this report]] This is underscored by an analysis of the patent landscape (Chapter 5), the literature (Chapter 4) and the clinical trial registrations (Chapter 3). Patents on GM vaccines mainly focus on infectious diseases, and more specifically on viruses. The proportion of viral vaccines, however, has been decreasing in recent years, while the absolute number of patents focusing on bacterial and other targets has remained the same.

Cancer vaccines are an upcoming market with a range of vaccine candidates in clinical trials up to phase 3.[[this report]] Furthermore, novel vaccine indications are explored by many research groups in both academia and industry. Autoimmune diseases, allergies, addiction (nicotine, cocaine) and other indications are also a target for vaccine development, however these field are rather underrepresented in the data sources that were consulted for this report. Correspondingly, it was indicated at the vaccine conferences (see Chapter 5) that indeed these new targets are of great interest but are challenging to address. Although data are still scarce, anti-addiction vaccines for example are studied such as anti-nicotine-addiction vaccines that reached up to phase 3 clinical trials (results indicated no beneficial effect).[79,80]

Genetic modification could also be employed to improve existing vaccines, constructed with conventional technology, with regard to safety, immunogenicity (e.g. in immune-compromised subjects) and/or to improve short-term production capacity of the antigens. From the literature and clinical trial analysis it can be concluded that the majority of new GM vaccines are based either on DNA or vector vaccine technology with each in different stages of development. These platforms allow for antigen-by-design through synthetic biology: e.g. optimizing antigens for processing and presentation to the immune system, combining different immunodominant epitopes in one antigen, designing an antigen

[78] http://www.fda.gov/downloads/biologicsbloodvaccines/vaccines/approvedproducts/ucm110114.pdf

[79] Nabi Biopharmaceuticals Announces Results of First NicVAX(R) Phase III Clinical Trial. Smoking Cessation Immunotherapy Failed to Meet Primary Endpoint http://phx.corporate-ir.net/phoenix.zhtml?c=100445&p=irol-newsArticle&ID=1586001&highlight=

[80] Shen et al; 2011; Anti-addiction vaccines; DOI:10.3410/M3-20

that induces broadly-reactive humoral responses, etc. Especially the vector vaccine technology has been explored rather extensively in the last years and is well represented throughout this report. To provide more insight in the variety of vectors that are currently in development we compiled Table 12 with some examples of vectors that are used as vaccine platform. Vectors are virtually always genetically modified organisms, each with their own requirements for biological containment. To focus on this aspect it would be of great interest to conduct a study fully dedicated to vector vaccines, allowing also exploring the more conceptual vector technologies that could raise new regulatory challenges.

Table 12 Examples of vaccine vectors for human and veterinary applications[81]

Family / Strain	Organism	Types	Indication	Stage	Ref
Poxviruses	Virus	Fowlpox	Veterinary: H5	Registered	82
		Canarypox (ALVAC)	HIV	Phase 3	83
			Veterinary: Multiple indications	Registered (RecombiTEK®)	84
		Modified Vaccinia virus Ankara (MVA)*	Prophylactic (Multiple) Therapeutic (Multiple)	Phase 1-3	85
			Veterinary: Prophylactic (Multiple)	Phase 1-3 - Registered	86
Adenoviruses	Virus	Human Adenoviruses: Ad4; Ad5; Ad26; Ad35; Modified variants (to render them replication deficient)	Multiple indications	Preclinical Phase 1-3	45
		Simian Adenoviruses: Chimp Ad3; Chimp Ad6; Chimp AdV63; Bonobo PanAd3; Gorilla AdC46	Multiple indications	Preclinical Phase 1-3	45 87 88

[81] These are examples of the wide range of vaccine vectors that are in development. These examples were selected based on their track-record and/or potential for the future. One indicative reference is provided for each example. There are various other vectors, however many of these have major drawbacks: e.g. Lentiviruses have the potential to integrate in host cell and measles virus suffers from strong pre-existing immunity in humans.

[82] http://www.offlu.net/fileadmin/home/en/meeting-reports/pdf/OFFLU_Beijing_2013/XU_W._Vaccines_of_today_and_products_needed_for_the_short__intermediate_and_long_term.pdf

[83] http://www.who.int/immunization/research/meetings_workshops/Oct2013_viral_vector_meeting_comments.pdf

[84] http://www.equinewnv.com/Pages/index.aspx

[85] Kreijtz et al; 2013; Poxvirus vectors; DOI: 10.1016/j.vaccine.2013.06.073

[86] Alberca; 2014; Vaccination of horses with a recombinant modified vaccinia Ankara virus (MVA) expressing African horse sickness (AHS) virus major capsid protein VP2 provides complete clinical protection against challenge; DOI: 10.1016/j.vaccine.2014.04.036

[87] http://gmoinfo.jrc.ec.europa.eu/bsnifs-gmo/B-ES-12-09.pdf

[88] Vitelli et al; 2013; Vaccination to Conserved Influenza Antigens in Mice Using a Novel Simian Adenovirus Vector, PanAd3, Derived from the Bonobo *Pan paniscus;* DOI: 10.1371/journal.pone.0055435

Newcastle Disease Virus (NDV)	Virus	Newcastle Disease virus (NDV)	Veterinary: Multiple indications	Registered (NDV-AI H5; Vectormune HVT-AI)	89
Yellow Fever Virus	Virus	YFV17D	JEV	Registered (IMOJEV®)	90
Vesicular Stomatitis virus	Virus	rVSV	Multiple indications (predominantly filoviruses: e.g. Ebola and Marburg)	Preclinical	91
Salmonella	Bacteria	Salmonella Typhimurium mutant-1	Veterinary: Campylobacter	Preclinical	92
Lymphocytic Choriomeningitis virus (LCMV)	Virus	Recombinant LCMV[93]	Multiple indications	Preclinical	45

It is difficult to predict when these novel GM vaccines will actually reach the market, however, it is expected to happen within the next 5-10 years based on the progression of GM vaccines in clinical trials and the risk profiles for several indications, as discussed in Chapter 3. This quantitative analysis, however, does not correspond to the qualitative analysis on expert opinions as summarized in Chapter 7. Whereas the experts postulate that in the future all vaccines will be GM based, our estimation data indicate that it would increase from approximately 10% to 13.6% in the coming decade. A plausible explanation for this discrepancy could come from the level of genetic modification that is applied to vaccines. From a technological perspective (represented by the expert opinions) it is likely that genetic modification is applied in the construction and/or production of every novel vaccine in the future, at levels varying from mutations to improve yields of wild type pathogen to antigens that are designed from scratch. For the first class it does not necessarily mean that they would be classified as actual GM vaccines (as represented by the estimations provided in Chapter 3). The latter indeed will be classified as genetically modified and the concept of antigen-by-design, as also discussed extensively at the vaccine conferences (Chapter 5), is in line with the new approaches of vaccine development that strongly rely on the use of genetic modification.

Two illustrative vaccine concepts are 'reverse vaccinology' and 'vaccinomics', as referred to in Chapter 5. The first was introduced by Rino Rappuoli and is based on analysis of the genetic code of the pathogen in order to identify vaccine antigens.[94] The second concept was illustrated by Gregory Poland at the World Vaccine Congress with the following statement: *We define drug regimens for a person based on gender, age, weight and pre-existing conditions, why should we not also do this for vaccines and vaccination regimens? The application of vaccinomics could facilitate development of personalized vaccines.* As these concepts were born from the perspective of human vaccines, they can also be applied for veterinary vaccine development. The use of genetic modification in the veterinary vaccine field is acknowledged and described by the World Organization of Animal Health (OIE) in an informative guidance document that they published in 2012.[95]

[89] http://www.vectormune.com
[90] http://products.sanofi.com.au/vaccines/IMOJEV_AUS_CMI.pdf
[91] Geisbert et al; 2011; Recombinant Vesicular Stomatitis Virus–Based Vaccines Against Ebola and Marburg Virus Infections; DOI: 10.1093/infdis/jir349
[92] Saxena et al; 2013; Strategies to reduce Campylobacter colonisation in chickens; doi: 10.1016/j.provac.2013.06.008
[93] Vector is replication deficient in mammalian cells.
[94] Rappuoli; 2001; Reverse vaccinology, a genome-based approach to vaccine development; DOI: 10.1016/S0264-410X(00)00554-5
[95] http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/GUIDE_3.3_VACCINES_NEW_TECH.pdf

8.2 Changes in the vaccine industry landscape

The number of mergers and acquisitions in the vaccine industry has accumulated in the last few years.[96] It is hard to keep track of the different technologies, patents and production platforms that are exchanged, sold and licensed in these deals. The fact that large pharmaceutical companies acquire small companies, once these have brought a vaccine technology from the preclinical to the clinical development phase, is a result of the contraction in R&D at the large companies that was initiated in the past decade.[97] It is likely that these changes will have some effect on the chances of new vaccine technologies to make it to the market. However at this moment it is too early to predict what the influence of these changes will be on the development of GM vaccines.

8.3 Production platforms

Apart from the conventional platforms for vaccine production (embryonated chicken eggs, Vero cells, bacteria and others) vaccine developers are looking into new production platforms. Genetic modification allows for the generation of new production substrates that are specifically designed for vaccine antigen production. The new platforms are based on one or more of the following pillars: high yield in low production substrate volumes, low costs, ease of use, ease of technology transfer (e.g. to developing countries) and reproducibility. In terms of GM production platforms, the baculovirus expression systems in insect cells have proven themselves in the past years.[98] Furthermore, cell lines can be genetically modified to optimize production of recombinant proteins and or complete pathogens for vaccine production, e.g. the engineered Procell92 cell line. This cell line was obtained through genetic modification of Human Embryo Kidney (HEK) 293 cells to allow for efficient production of adenovirus vectors.[99]

The novelties such as plant-based production (e.g. *Nicotiana benthamiana* as used by Medicago USA) and algae (diatom), as described in patent WO2013063388 enable fast production of vaccine antigen with high yields.[100,101] For plants and algae the antigens can be purified from the plant material. However the antigens do not necessarily have to be extracted and purified from the substrate, the leaves and algae themselves could be part of the vaccine, as is indicated in the diatom patent. From that perspective, the genetically modified plants and algae would then become part of the vaccine and thus these organisms could then be considered as vaccine vectors instead of vaccine production platform.

8.4 Environmental risks of genetically modified vaccines

There are multiple options on how to deal with the environmental risks of GM vaccines and their containment. Regarding vaccines for human application, it is observed that the risks are mainly managed by manners of proper risk assessment and precautionary measures during handling, distribution and administration. This reflects the notion that for vaccines for human application, safety and efficacy are the most important factors. When vaccines for either human or veterinary applications contain or consist of genetically modified organisms, extensive Environmental Risk Assessments are mandatory

[96] http://www.gsk.com/media/press-releases/2014/gsk-announces-major-three-part-transaction-with-novartis.html
[97] http://www.investor.jnj.com/releasedetail.cfm?ReleaseID=515096
[98] http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm335891.htm
[99] http://www.okairos.com/e/inners.php?m=00084
[100] http://www.medicago.com/English/Medicago-USA/MedicagoUSA/default.aspx
[101] Patent: Diatom-based vaccines; WO2013063388

before market authorization is granted. In cases where vaccines are used for animals that are part of a food chain, these aspects play an even greater role.

This is a reason why in the veterinary field, more than in the human field, vaccines are modified in order to limit spreading of the GMO. Alternatively, the antigen can be designed to function as a DIVA vaccine, e.g. the HIPRABOVIS® IBR MARKER LIVE vaccine that is used in cattle to protect against Infectious Bovine Rhinotracheitis (IBR) caused by Bovine herpes virus type 1 (BoHV-1).[102] By deletion of two genes in the vaccine virus, vaccinated animals can be differentiated from animals infected with the natural BoHV-1. In less developed countries the distribution, handling and administration of GM vaccines and the resulting GM waste is an issue that needs to be taken into consideration when vaccines are developed for these markets.

[102]http://www.hipra.com/wps/portal/web/inicio/nuestrosProductos/!ut/p/c4/04_SB8K8xLLM9MSSzPy8xBz9CP0os3gDU8dASydDRwMLpwADA09PC2cXA3MnAwtDM_3g1Dz9gmxHRQAF0D0V/?WCM_GLOBAL_CONTEXT=/productos_en/hipra/secciones/nuestrosproductos/00/400225_00

8.5 Summarizing Statement

The vaccine field is evolving and although 'conventional' vaccine technologies still have a solid development and market potential, development of new vaccines can benefit from the implementation of genetic modification. From the perspective of vaccine developers it is a natural process of innovation to use genetic modification, however one must not forget that public perception of such new technologies could be different and thus communication on GM vaccine development and proper risk-assessment of the actual GM vaccines is essential. Furthermore the use of genetic modification must not be a goal by itself. It is a tool to:

1) Improve existing vaccines

2) Modify pathogens to allow for higher vaccine production yields

3) Facilitate production at lower biological safety levels

4) Design antigens for new vaccines, e.g. through reverse vaccinology or vaccinomics

The latter two approaches are not possible without the use of genetic modification. In the near future GM vaccines will not replace conventional vaccines, they will co-exist and genetic modification will be applied to an increasing extent in the construction and improvement of vaccines.

Genetic modification can thus be seen as one of the sharpest tools in the shed of vaccine developers.

8.6 General Recommendations

These recommendations to stimulate novel (GM) vaccine technologies apply to:

Scientists, vaccine manufacturers, regulatory authorities and other disciplines within the vaccine field

- Stimulate public private partnerships to ensure sufficient funding
- Facilitate communication between vaccine developers and regulatory authorities early in the development pipeline
- Think three steps ahead: will this novel vaccine be:
  1) safe and efficacious?
  2) applicable?
  3) affordable?
- Build a wide clinical network to enable large efficacy studies
- Improve technology transfer processes to streamline the development pipeline and speed up the process from bench to bedside

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Appendix 1

Search term coding

Embase.com 945

('genetic immunization'/de OR 'DNA vaccine'/de OR 'live vaccine'/de OR 'virosome vaccine'/de OR 'recombinant vaccine'/de OR 'virus like particle vaccine'/de OR (((gene* OR live OR protein*) NEAR/3 (vaccin* OR immuni*)) OR ('virus like' NEXT/1 particle*)):ab,ti OR (('DNA modification'/de OR dna/exp OR rna/exp OR 'genetic recombination'/exp OR 'recombinant protein'/exp OR 'virus vector'/exp OR 'bacterial vector'/de OR virosome/de OR (genetic* OR attenuat* OR enigneer* OR modif* OR DNA OR rna OR vector* OR recombin* OR chimeric* OR virosom*):ab,ti) AND (vaccine/exp OR Vaccination/exp OR immunization/exp OR (vaccin* OR immuni*):ab,ti))) AND ('systematic review'/de OR 'meta analysis'/de OR ((systematic NEAR/3 review*) OR (meta NEXT/1 analy*)):ab,ti)

Medline (OvidSP) 364

(exp "Vaccines, Synthetic"/ OR "Vaccines, Attenuated"/ OR "Vaccines, Virosome"/ OR (((gene* OR live OR protein*) ADJ3 (vaccin* OR immuni*)) OR ("virus like" ADJ particle*)).ab,ti. OR ((exp dna/ OR exp rna/ OR exp "Recombination, Genetic"/ OR exp "Recombinant Proteins"/ OR exp "Genetic Vectors"/ OR Virosomes/ OR (genetic* OR attenuat* OR enigneer* OR modif* OR DNA OR rna OR vector* OR recombin* OR chimeric* OR virosom*).ab,ti.) AND (exp vaccines/ OR exp Vaccination/ OR exp immunization/ OR (vaccin* OR immuni*).ab,ti.))) AND ("meta analysis".pt. OR ((systematic ADJ3 review*) OR (meta ADJ analy*)).ab,ti.)

Cochrane DARE 7

((((gene* OR live OR protein*) NEAR/3 (vaccin* OR immuni*)) OR ('virus like' NEXT/1 particle*)):ab,ti OR (((genetic* OR attenuat* OR enigneer* OR modif* OR DNA OR rna OR vector* OR recombin* OR chimeric* OR virosom*):ab,ti) AND ((vaccin* OR immuni*):ab,ti)))

Web-of-science 323

TS=(((((gene* OR live OR protein*) NEAR/3 (vaccin* OR immuni*)) OR ("virus like" NEAR/1 particle*)) OR (((genetic* OR attenuat* OR enigneer* OR modif* OR DNA OR rna OR vector* OR recombin* OR chimeric* OR virosom*)) AND ((vaccin* OR immuni*)))) AND ("systematic review*" OR "meta analy*"))

PubMed publisher 8

((((gene*[tiab] OR live[tiab] OR protein*[tiab]) AND (vaccin*[tiab] OR immuni*[tiab])) OR (virus like particle*[tiab])) OR (((genetic*[tiab] OR attenuat*[tiab] OR enigneer*[tiab] OR modif*[tiab] OR DNA[tiab] OR rna[tiab] OR vector*[tiab] OR recombin*[tiab] OR chimeric*[tiab] OR virosom*[tiab])) AND ((vaccin*[tiab] OR immuni*[tiab])))) AND (((systematic review*[tiab]) OR (meta analy*[tiab]))) AND publisher[sb]

Google Scholar

"genetic|DNA|live|attenuated|virosome|recombinant|engineered|modified vaccine|vaccines|immunization|immunisation "systematic review"|"meta analysis"

/de = description (term, not what is categorized below)
/exp = explode (term plus everything that is catergorized below)
*/ab are free text words

Appendix 2

Patent issuing organisations in Espacenet

1 Albania (AL)	47 Liechtenstein (LI)
2 ARIPO (AP)	48 Lithuania (LT)
3 Argentina (AR)	49 Luxembourg (LU)
4 Austria (AT)	50 Latvia (LV)
5 Australia (AU)	51 Morocco (MA)
6 Bosnia and Herzegovina (BA)	52 Monaco (MC)
7 Belgium (BE)	53 Moldova (MD)
8 Bulgaria (BG)	54 Republic of Montenegro (ME)
9 Brazil (BR)	55 Former Yugoslav Republic of Macedonia (MK)
10 Canada (CA)	56 Mongolia (MN)
11 Switzerland (CH)	57 Malta (MT)
12 Chile (CL)	58 Malawi (MW)
13 China (CN)	59 Mexico (MX)
14 Costa Rica (CR)	60 Malaysia (MY)
15 Czechoslovakia (CS)	61 Nicaragua (NI)
16 Cuba (CU)	62 Netherlands (NL)
17 Cyprus (CY)	63 Norway (NO)
18 Czech republic (CZ)	64 New Zealand (NZ)
19 German Democratic republic (DD)	65 OAPI (OA)
20 Germany (DE)	66 Panama (PA)
21 Denmark (DK)	67 Peru (PE)
22 Algeria (DZ)	68 The Philippines (PH)
23 Eurasia (EA)	69 Poland (PL)
24 Ecuador (EC)	70 Portugal (PT)
25 Estonia (EE)	71 Romania (RO)
26 Egypt (EG)	72 Republic of Serbia (RS)
27 European Patent Office (EP)	73 Russia (RU)
28 Spain (ES)	74 Sweden (SE)
29 Finland (FI)	75 Singapore (SG)
30 France (FR)	76 Slovenia (SI)
31 Great Britain (GB)	77 Slovakia (SK)
32 Gulf Cooperation Council (GC)	78 San Marino (SM)
33 Georgia (GE)	79 Soviet Union (SU)
34 Greece (GR)	80 El Salvador (SV)
35 Hong Kong S.A.R (HK)	81 Tajikistan (TJ)
36 Croatia (HR)	82 Turkey (TR)
37 Hungary (HU)	83 Chinese Taipei (TW)
38 Indonesia (ID)	84 Ukraine (UA)
39 Ireland (IE)	85 United States of America (US)
40 Israel (IL)	86 Uruguay (UY)
41 India(IN)	87 Viet Nam (VN)
42 Iceland (IS)	88 World Intellectual Property Organization (WO)
43 Italy (IT)	89 Former Serbia and Montenegro (YU)
44 Japan (JP)	90 South Africa (ZA)
45 Kenya (KE)	91 Zambia (ZM)
46 Korea (South) (KR)	92 Zimbabwe (ZW)

Appendix 3

Veterinary GM vaccine disease areas

1 Newcastle disease	38 Avian encephalomyelitis
2 Infectious bronchitis disease	39 Leptospirosis
3 Avian Infectious bursal disease	40 Pseudorabies
4 Marek's disease	41 *Brucellosis* abortus
5 Fowl pox	42 Rabies
6 Bovine herpes infection	43 Avian infectious bronchitis
7 *Coryza*	44 Porcine enzootic pneumonia
8 Lumpy skin disease	45 *Mycoplasma synoviae* infection
9 *Salmonella (Typhimurium)*	46 *Haemophilus somni*
10 *Colibacillosis*	47 Tetanus
11 *Salmonella (Enteritidis)*	48 Rinderpest
12 Reovirus infection	49 Bluetongue disease
13 Black disease	50 Orf
14 Perfringens food---borne disease	51 *Chlamydophila* abortus
15 *Clostridium septicum* infection	52 Avian rhinotracheitis
16 Swine herpes	53 Anthrax
17 Bovine viral diarrhoea	54 Epizootic haemorrhagic disease
18 Parainfluenza infection	55 Pneumonia
19 *Mycoplasma mycoides* infection	56 Turkey rhinotracheitis
20 Foot and mouth disease	57 Coronavirus infection
21 Avian or fowl cholera disease	58 Botulism
22 Blackleg disease	59 Intestinal coccidiosis
23 *Clostridium sordellii* infection	60 Gallid herpes
24 Bovine respiratory syncytial infection	61 Avian metapneumo infection
25 Avian influenza	62 *Moraxella bovis* (pinkeye)
26 Ovine rinderpest	63 *Erysipelas*
27 *Bordetella*	64 Classical swine fever
28 Porcine parvovirus infection	65 Pigeon pox
29 Rift valley fever	66 Porcine reproductive and respiratory syndrome
30 Porcine circoviral disease	67 Avian pneumo infection
31 Egg drop syndrome	68 *Bacillary hemoglobinuria*
32 Avian viral Arthritis	69 *Campylobacter fetus* infection
33 Coccidiosis (*Eimeria acervulina*)	70 Leptospirosis
34 Coccidiosis (*Eimeria maxima*)	71 Bovine coronavirus infection
35 *Eimeria tenella*	72 Bovine rotavirus infection
36 Meleagrid herpes	73 *Pasteurella somnus* infection
37 Mycoplasma gallisepticum infection