WORKSHOP

TargetFish industry forum on DNA vaccination: where do we stand and what's next?

G. F. Wiegertjes[1]*, M. Forlenza[1], N. Lorenzen[2], B. Collet[3], U. Fischer[4], C. Tafalla[5], Ø. Evensen[6], P. Smith[7], P. Christofilogiannis[8] and N. H. Henriksen[9]

[1]Wageningen University & Research, The Netherlands; [2]Aarhus University/ Danish Technical University, Denmark; [3]Marine Scotland, United Kingdom; [4]Friedrich Löffler Institut, Germany; [5]Spanish National Institute for Agricultural and Food Research and Technology, Spain; [6]Norwegian University of Life Sciences, Norway; [7]Tethys Aquaculture Limited, United Kingdom; [8]AQUARK, Greece; [9]Organisation of Danish Aquaculture, Denmark

Summary

Maybe most characteristic of the TargetFish[1] project, which kicked off some five years ago with 30 partners from 10 EU member states, two associated countries (Norway, Israel) and one international cooperation partner country (Chile), has been the close cooperation between research groups and enterprises; more or less equally represented in this large consortium. In this respect, TargetFish has been revolutionary validating by this close cooperation fundamental knowledge for the development of next generation vaccines and different routes of vaccine administration. TargetFish had the ambition to demonstrate market applicability of improved vaccines or new prototype vaccines that would come forward from the project. Via frequent joint meetings of its partners, be it research group or enterprise, TargetFish aimed to drive vaccine development in an industrial applicable way. This could facilitate adoption of new intellectual property and stimulate the presentation of new fish vaccines on the market. The industry forum has been a platform for a continuing validation of the applied potential of the research outcomes. Workshops were organised at the different EAFP meetings to communicate the validation process to those not directly involved with the project but interested in the fish vaccine market. After a kick-off meeting during the EAFP in Tampere, Finland fours years ago and a second meeting at the EAFP in Las Palmas, Spain, two years ago, at the present EAFP in Belfast, Northern Ireland a final meeting was organised. This report is a summary of the 'Industrial Forum workshop' held at the EAFP in Belfast 2017 and provides a short overview of the highlights presented to, and discussed with, those present and interested in DNA vaccine development, policies and laws, production and delivery routes.

[1]TargetFish is a large collaborative project funded by the European Commission under the 7th Framework Programme for Research and Technological Development (FP7) of the European Union (Grant Agreement 311993) aiming to 'improve fish vaccination strategies to help prevent important diseases in the European aquaculture industry'.

* Corresponding author's email: geert.wiegertjes@wur.nl, targetfish.cbi@wur.nl

DNA vaccines

Traditionally, many fish vaccines have been based on inactivated bacteria or viruses, and although several of these have been extremely (cost) effective, not all disesases caused by fish pathogens can be prevented with these relatively simple forms of vaccines. More recently, a new approach to vaccination has been developed, involving the direct introduction into appropriate tissues of a DNA plasmid encoding the protective antigen of the pathogen causing the disease to be prevented. Upon administration into the host (usually muscle) tissue, some cells will take up the DNA vaccine and express the pathogen protein. The host will recognise the protein as non-self and mount a protective immune response through stimulation of both innate and adaptive immune mechanisms.

Regulatory requirements for the authorisation of DNA vaccines for fish in the EU

DNA vaccines are classified as immunological veterinary medicinal products under EU legislation and are thus subject to the relevant directive (EC Directive 2001/82/EC). As vaccines developed by recombinant technology they are subject to authorisation through the European Medicines Agency (Regulation (EC) 726/2004). Further, the manufacture of DNA vaccines should comply with the rules on good manufacturing practice (GMP) for veterinary medicinal products (EC Directive 91/412). Additionally, there are some specific regulatory guidelines that are applicable to DNA vaccines, for example the guideline on 'DNA vaccines non-amplifiable in eukaryotic cells for veterinary use' (EMA/CVMP/IWP/07/98). There are also some specific regulatory issues for DNA vaccines in addition to those required for more conventional aquaculture vaccines, which address potential safety concerns. Important points to consider include: 1) the definition of a DNA vaccine in the context of the legal framework and whether such products could be classified as genetically modified organisms (GMOs) and subject to the relevant legislation (EC Directive on the deliberate release into the environment of genetically modified organisms 2001/18/EC), 2) safety for the consumer, environment and target species, 3) proof of efficacy in laboratory and - unless justified - supported with field studies, 4) a benefit-risk assessment.

Highly relevant to some sectors of aquaculture are the regulatory requirements in the guideline for immunological veterinary medicinal products intended for minor use or minor species (MUMS)/limited market (EMA/CVMP/IWP/123243/2006-Rev.3). The data requirements for authorisation of a DNA vaccine that is eligible for classification as a MUMS application are reduced. Thus, this applies when the vaccine is intended for use in a minor species (most fish species) only, or when a disease is relevant in a major species but for minor use, or if the respective vaccine has a limited market. This was the case for a DNA vaccine for Atlantic salmon recently authorised for use in the EU (Clynav, against Salmon Pancreatic Disease, SPD). Key regulatory points on DNA vaccines include:
DNA vaccines are new innovative veterinary products for regulators. DNA vaccines are not classified as GMO. The DNA plasmid vaccine by itself cannot be considered a GMO, since it is a construct and not a replicating living organism. However, this may not apply to all DNA vaccines should the vector be designed to integrate into the genome of the target species.

Target species safety studies should address the requirements under legislation. The safety studies should be carried out in the target species and the dose to be used for the safety study should be the dose recommended for use. The product should be produced according to the manufacturing process.

Although DNA vaccines are not classified as live vaccines it is considered prudent to approach some of the safety studies for such products. The safety study could be conducted as a 10-fold overdose and studies should include dissemination of the DNA in the target animal with consideration for the potential integration into relevant organs and tissues including gonadal tissue.

The safety for the user and consumer should demonstrate a negligible risk for humans exposed to the vaccine.

An environmental risk assessment (ERA) should be extensive depending on the potential environments the product may be used in and include quantitative assessment with consequence analysis.

Potential integration into the genome of the vaccinated animal is a critical safety issue and should be thoroughly investigated through the relevant laboratory safety studies and environmental risk assessment.

Why are DNA vaccines not GMOs?

A DNA plasmid vaccine by itself cannot be considered a GMO, since a plasmid is a construct and not capable, on its own, to replicate or transfer genetic material into the genome of the fish. Therefore, a plasmid cannot be regarded as a biologically viable entity. Yet, even if not a GMO, there is the question as to whether fish vaccinated with the plasmid become GMOs, where the latter are defined as organisms in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. Although the position for future DNA vaccines still remains unclear, the answer appears to be no-they-are-not from the recent decision of a GMO authority for fish vaccinated with the first DNA vaccine authorised in the EU.

The pros and contras of DNA vaccination against SPD for Atlantic salmon culture in Norway

The Norwegian Medicines Agency (NOMA) has voiced an opposition or divergent position against the Clynav plasmid vaccine to be used to protect Atlantic salmon against PD caused by subtype 3 of SPDV. NOMA's opposition is based on lack of real duration of immunity (DOI) studies, where current DOI is only 2 months. It is NOMA's standing this (short duration of protection) cannot outweigh the risks related to animal safety, potential lack of protection beyond 2 months, potential interaction with other vaccines currently used in Norway and potential reduction in fillet quality as a consequence of intramuscular injection of the vaccine. The volume injected is also considered high given it will go into the epaxial muscle or the back loin of the fish. The latter concern comes from the observation that local and general reactions to the injected plasmid solution were seen under laboratory testing.

Yet another concern that was raised initially was the GMO issue, i.e. that vaccinated fish would be considered GMOs and thus labelling of the

final product would be required. Based on an EFSA assessment where it was concluded that "the actual integration rate is likely to be orders of magnitude lower than the upper estimated integration rate calculated in the context of the worst-case scenarios" (EFSA Journal, doi: 10.2903/j.efsa.2017.4689), this should be understood in such a way that the likelihood of integration is considered negligible. On this basis the conclusion from the Norwegian side is that Atlantic salmon vaccinated with Clynav will not be considered GMO. This is a decision made on a case-by-case basis and not a general standing of the Norwegian authorities.

Evaluating the level of genomic integration after DNA vaccination

As discussed above, DNA vaccines are generally not considered GMOs, unless the plasmid was specifically engineered to integrate and maintain genomic integration. If not the case, integration should be considered a random occurrence and would not present any additional risk as compared to e.g. the rate of natural spontaneous mutation. Yet, integration studies may be required for future applications on DNA vaccination. Stimulated by the DNA vaccine guidance, TargetFish 'travelled down this road' by experimentally addressing genomic integration of plasmid DNA after intramuscular injection. In TargetFish we designed an experimental DNA vaccine based on the structural protein of subtype 1 of SPDV to study genomic integration in muscle samples from DNA-vaccinated Atlantic salmon collected for several days post-vaccination at the site of i.m. injection. To evaluate the limit of detection, genomic DNA from unvaccinated salmon muscle tissue was mixed with increasing amounts of DNA from a recombinant salmonid cell line with the DNA vaccine construct integrated into its genome. These data mimicked different levels of genomic integration and were used to establish a calibration curve. Finally, 1) the purification of high molecular weight genomic DNA excluding free plasmid followed by 2) an enrichment step for fragments containing the DNA vaccine sequence and 3) deep sequencing, will help identify and count sequences with both salmon genome and plasmid DNA in order to estimate the integration rate using the calibration curve. This approach to study integration after DNA vaccination will be useful also long after TargetFish is finished.

Applied aspects of DNA vaccination

Some of the most efficient DNA vaccines described to date are those against fish rhabdoviruses. By mediating expression of the viral glycoprotein (G) in vaccinated fish, these vaccines induce rapid and long lasting protection against the respective viruses. A DNA vaccine known as Apex-IHN has been used commercially for protection of sea-reared Atlantic salmon against infectious haematopoietic necrosis (IHN) in British Columbia, Canada, since 2005. To date, no outbreaks of IHN have been reported among the vaccinated fish. A related disease in European rainbow trout is called viral haemorrhagic septicaemia (VHS), or "Egtved disease" named after the village in Denmark where it was first recognised. Although historically, VHS may have been associated mostly with aquaculture of freshwater salmonids in Western Europe to date the virus is known to affect over 80 freshwater and marine fish species worldwide. It may be clear that vaccination against VHS can indeed be highly relevant, both in Europe or worldwide, in freshwater or marine environments.

There is ample evidence of the effectiveness of DNA vaccination against VHS, at least when based on the i.m. injection of G-protein encoding plasmids. The advantages of DNA vaccines for VHS are multiple and include 1) high safety – no risk of disease, 2) rapid and long-lasting protection, 3) no additional adjuvant needed, 4) high stability and 5) simple to produce. Given the discussion above on legislation, commercialisation of DNA vaccination against VHS may become a realistic option in the near future, although there will be local restrictions related to the disease status. In Denmark and some other countries in Europe the virus has been eliminated from the freshwater by, e.g. replacing earthen ponds for intensive recirculation systems and by stamping out procedures. However, VHSV is still present in the marine environment, also in Denmark. In Europe, VHS is a notifiable disease and falls under Council Directive 2006/88/EC: Member States shall ensure that vaccination against the non-exotic diseases listed in Part II of Annex IV is prohibited in any parts of their territory declared free of the diseases in question, or covered by a surveillance programme. Although vaccination against notifiable diseases is generally prohibited, member states may allow vaccination in parts of their territory not declared free from the diseases in question, or where vaccination is part of an eradication programme.

New routes of delivery for DNA vaccines

There are several (experimental) DNA vaccines that when injected i.m. protect very well against a subsequent lethal challenge and provide values for relative percentage survival (RPS) >90% for months after a single administration of plasmid. Often, a local inflammatory response in the muscle tissue is observed immediately after injection and may be responsible for triggering a first period of innate immunity, followed by a highly specific response after a few weeks (depending of course on the temperature) correlating with occurrence of specific antibodies induced locally (IgM, IgT) and/or systemically (IgM).

Of specific interest are attempts to protect fish against disease with an oral DNA vaccine, with successes reported for infectious pancreatic necrosis virus (IPNV) in rainbow trout. High protection with RPS values of 85% were obtained, albeit based on much higher dosages of plasmid than needed for i.m. vaccination. In addition, oral DNA vaccination of several fish species against bacteria has been reported with RPS values of 60-70% after challenge, suggesting DNA vaccination by oral route could become a reality in the near future. However, time course studies and specificity of the protective mechanisms still remain to be determined for several of these vaccines. On the other hand, DNA vaccines based on the G-protein of rhabdoviruses, so extremely efficacious when injected i.m., have so far yielded limited success when applied orally. Here, despite active transcription of the G protein and induction of local immune responses, serum antibodies and protection appear mostly absent when the DNA vaccine is provided orally. Possibly, the optimal route for induction of protection by DNA vaccines could be linked to the nature of the actual disease, including parameters such as the natural site of pathogen entry and systemic versus local propagation of the infection. Further studies are required to clarify these aspects and to determine how several parameters such as encapsulation method, adjuvant effect, vaccine concentration and delivery regime might require individual

optimisation for oral (mucosal?) DNA vaccines. This puts forward a plethora of questions to address in the near future, including aspects of oral tolerance.

What's next?

Clearly, DNA vaccines can be highly efficacious and safe, do not require adjuvants, have high stability and are relatively simple to produce. Some DNA vaccines can induce rapid and long-lasting protection when injected i.m., while others are able to trigger a protective response (also) when administered orally. The regulatory requirements and extent of data needed to support an application for registration of a DNA vaccine are strongly reduced when the application qualifies for use in minor species, or disease in major species which are of minor importance, or have a limited market (MUMS). This might facilitate commercialisation of DNA vaccines for use in aquaculture. Although in the light of a growing importance of aquaculture and animal welfare connected with disease outbreaks and their eradication it cannot be excluded that the MUMS issue for farmed fish might be revised in the future, with a DNA vaccine for Atlantic salmon authorised for use in the EU in 2017, the future of DNA vaccination for disease prophylaxis in farmed fish is brighter than ever before. Yet, there are several key regulatory issues that remain to be further explored such as genomic integration and risk to the environment which, along with practical challenges like plasmid vector design and determination of optimal route of (oral?) administration, will remain key issues for future research.