Chapter 4

DNA Vaccines: Experiences in the Swine Model

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Abstract

DNA vaccination is one of the most fascinating vaccine-strategies currently in development. Two of the main advantages of DNA immunization rely on its simplicity and flexibility, being ideal to dissect both the immune mechanisms and the antigens involved in protection against a given pathogen. Here, we describe several strategies used to enhance the immune responses induced and the protection afforded by experimental DNA vaccines tested in swine and provide with very basic protocol describing the generation and in vivo application of a prototypic DNA vaccine. Only time will tell the last word regarding the definitive implementation of DNA vaccination in the field.

Key words DNA vaccine, Genetic adjuvant, Antigen presentation, Antibodies, Cytotoxic T-cell responses (CTL), ELI, APC, Electroporation, Swine, Veterinary virology

1 Introduction

Immunization by means of what we know today as nucleic acid vaccination was described in the early 90s [1], opening a new and promising way in the vaccination field. The principle behind DNA vaccines (the most common nucleic acid vaccines are based on the inoculation of DNA plasmids) is based on a very simple, albeit smart, principle: the capability of the cells to in vivo uptake the DNA, intracellularly express the encoded antigen and, finally, the induction of protective immune responses. In order to obtain a fine expression of the encoded proteins, the gene construct is cloned under the control of a mammalian promoter, usually, the promoter from human cytomegalovirus (CMVp). DNA vaccines can be administered in many different ways, being the two most common the intramuscular injection and the intradermal inoculation. In the first case, the plasmid is primarily taken up by muscular cells whereas in the second case, the cells that receive the plasmid are dermis cells, and, among them, the Langerhans cells (a type of Ag-presenting cells). Independently of the immunization route, the success of DNA immunization relies on the final uptake of

Alejandro Brun (ed.), *Vaccine Technologies for Veterinary Viral Diseases: Methods and Protocols*, Methods in Molecular Biology, vol. 1349, DOI 10.1007/978-1-4939-3008-1_4,

DNA and/or the plasmid-encoded antigens by Professional Ag-presenting cells [2].

The advantages of DNA vaccines are many: chiefly, its safety. We do not have to worry at all about virulence, which is the main concern of attenuated vaccines. Also, depending on the DNA-construct used, we have the possibility of inducing humoral and/or cellular responses, being the lack of this latter possibility one of the main defaults of the inactivated vaccines, of importance while fighting intracellular pathogens. Last but not least, we have to keep in mind that DNA vaccines can be easily "à la carte"-designed (we can target the expressed antigens to induce different immune-responses) and, as other new generation subunit vaccines, they can behave as DIVA-vaccines (vaccines that allow us Differentiating Infected from Vaccinated Animals), an essential concept in veterinary medicine. DNA vaccination has been successfully developed in rodent-models, but results obtained in other animals showed to be contradictory. There are some commercial DNA vaccines available for fishes [3], for which DNA vaccines work extremely well, and for horses [4]. Albeit that, one of the major skepticisms generated by DNA vaccination in large animals relies in its low efficacy, in occasions attributed to the low efficiency of DNA transfection achieved in vivo. The most promising way to improve the efficiency of DNA delivery in vivo is the utilization of in vivo electroporation [5]. The use of other methods, such as biolistics or nanoparticles, to increase DNA-transfection efficacy have been also proposed. Besides the methodologies used to enhance the DNA uptake, research has provided many other strategies that have allowed enhancing the immune response induced and the protection afforded. Far from being able to present one "universal strategy" of vaccination, our experience have shown that vaccines should be tailored "à la carte", taking into account the target animal species and the pathogen to be fought.

2 Materials

1. DNA or RNA template containing the ORFs genes we want to immunize with.
2. Primers to amplify the aforementioned genes by means of PCR reaction (alternatively, synthetic ORFs can be used).
3. Plasmid DNA backbone (*see* **Note 1**).
4. Bacteria for transformation and large-scale production of plasmid DNA (*see* **Note 2**) and suitable media.
5. Mammalian Cell line for transfection, transfection reagent and suitable media.
6. Materials for Western-blotting.

7. Reagents and kits for plasmid ligation, purification of DNA (*see* **Note 3**).
8. The genetic adjuvant (*see* **Note 4**); if willing to use it.

3 Methods

As above mentioned, the first step prior to begin with the design of a DNA vaccine is to know what is relevant and what is not in protection against the virus we are working with. Which kind of immune response we are looking for? Shall we need production of antibodies or the induction of a cellular cytotoxic response? Maybe we should need both. Obviously, the answer to such questions lays in the nature of the pathogen antigens. For some viruses it may be simple, i.e., a DNA vaccine against E2 Ag from Classical Swine Fever Virus induced both humoral and cellular responses and conferred sterilizing immunity [6]. However, for many more complex pathogens, i.e., African Swine Fever Virus, is not that simple. Research from our group, based on DNA-vaccination strategy, found out that antibodies against certain proteins may even have deleterious effects [7].

The present chapter will review: (1) Methods used to enhance the efficacy of the DNA-delivery in the animal, focusing mainly on in vivo electroporation; (2) a discussion of strategies to enhance the immunogenicity of DNA vaccines (mainly focused in those approaches used in our laboratory that have been successfully used in swine); (3) a particular chapter dedicated to ELI immunization, an ideal protocol to search for protective antigens within complex pathogens; and (4) a final section dedicated to a simple protocol describing the steps involving the construction of a DNA vaccine and swine immunization and a brief consideration about prime-boost strategies.

3.1 Enhancing the DNA Delivery into Cells

One of the main criticisms of the commonly used methods of DNA immunization is the poor efficiency of in vivo transfection in animal cells. Some strategies, such as the use of electroporation, biolistics or, on the other hand, the delivery of the DNA-plasmid in formulations such as liposomes, have been proposed.

3.1.1 The Use of Liposomes

Liposomes are adjuvants of current use in traditional vaccines. Such compounds are able to entrap plasmid DNA and therefore facilitate the entrance of such DNA into the cell by penetrating the lipid bilayer of the cell membrane. Liposome-entrapped DNA has shown to enhance both humoral and cell-mediated immune response in a more effective way than naked-DNA. Such results could be explained by the ability of liposomes to protect their DNA content from local nucleases and direct it to APCs in the

lymph nodes draining the injected site [8]. Moreover, the use of liposomes to deliver DNA vaccines has opened the door to other administration routes for this type of vaccines, such as the oral route, playing the liposome a protecting role of the plasmid DNA against DNAse present in digestive tract. In mice model, the efficacy of a liposome-driven oral DNA vaccine has been successfully assayed [9] showing protection against Influenza challenge.

3.1.2 The Gene-Gun

The biolistics approach implies shooting the skin with plasmid-coated micron-sized particles (commonly made of gold) by means of ballistic devices such as the gene-gun. The particles are accelerated into skin tissue using the force of an electric discharge or compressed helium. Thus, DNA is delivered directly onto the cytoplasm of epidermal keratinocytes and thus, a very small amount of DNA is needed, compared to traditional DNA injection [10]. This particle-mediated DNA vaccine approach has shown to be effective in swine, inducing comparable CD8+ T-cell responses and superior antibody production with 100–1000 fold less DNA when compared to naked-DNA injection [11]. When epidermis cells are renewed, the transformed cells containing plasmid DNA will disappear, therefore stopping the production of Ag. Quite a different panorama of what happens with intramuscular DNA injection, where cells are able to produce the proteins for a longer period of time. Some authors [12] claim that, due to the limitations of the approach, it seems that the use of biolistics is slowing down, clearing a path to in vivo electroporation systems, the current most promising way to enhance DNA delivery into cells.

3.1.3 Electroporation

The principle behind electroporation is quite simple: to induce a temporary permeabilization of the cell membrane to allow the penetration of large molecules such as DNA. Briefly: Just after the injection of the DNA we will deliver in the plasmid-injection area a strong but short electric pulse followed by some other pulses a bit larger in duration but milder in voltage. The first pulse permeabilizes the cell membranes, whereas the following pulses induce a sort of in vivo electrophoresis, thus attracting the previously injected plasmid DNA into the temporary-permeabilized cells. Later on, the membranes recover its normal integrity. Optimal electroporation conditions result from a very subtle balance: If conditions are too aggressive, we will destroy the cells whereas if they are too mild, we will not induce the desired permeability. We can modify the following parameters: voltage (from 60 to several hundred Volts, depending on the tissue and type of electrode), pulse length (in milliseconds) and the number of pulses (ranging commonly from 2 to 12) [5] (*see* **Note 5**). On the other hand, some researchers suggest that mild tissue damage induced by the electric discharges may act as an adjuvant, inducing a release of danger signals (i.e., inflammatory mediators) in the affected zone,

enhancing the presence of APCs, as well as an increase of the release of the antigen proteins from injured cells, thereby improving antigen presentation [13].

There are various devices developed to perform in vivo electroporation in the market: TriGrid™ (Ichor medical systems), AgilePulse™ (BTX Harvard Apparatus), Cliniporator™ (IGEA), among others. Types of electrodes may vary from needle-free patch electrodes to multiple-needle array electrodes, depending logically on the chosen apparatus, but also on the tissue to be injected. The fact that animals must be anesthetized before the treatment, together with the bulky appearance of most devices, makes in vivo electroporation currently unfeasible for swine veterinary practice, remaining therefore to be employed in research or in small animal or human medicine. It is expected that a non too-distant future will bring us more portable devices, suitable to be used in massive vaccination as it happens in a regular swine farm.

3.2 Enhancing the Immunogenicity of the DNA Vaccine

Due to the complexity of the immune system, we should keep our experimental approach as simple as possible and therefore, we recommend choosing the adjuvant that better suit your interests taking into account both the animal species and the immune response willing to be induced. Here we summarize some of the most successful results described in the literature for DNA vaccines in swine.

3.2.1 The Use of Cytokines as Plasmid–Encoded Adjuvants

The use of plasmids encoding cytokines together with the DNA construct of interest has been proposed as one of the best adjuvant strategies for DNA immunization protocols. The main advantage of this strategy relies in the fact that after in vivo administration, the cytokines will act concomitantly and locally in the zone of Ag expression, therefore avoiding undesirable effects observed when they are systemically administrated and providing a more robust and long-term stimulation. The election of the cytokine to be included (IFN-γ, IL-18, IL-2, IL-12…) will depend on the type of response we want to elicit (*see* **Note 6**). Mostly of the reports of cytokines as DNA-adjuvant are carried out in mice models and, although there are not many reports on veterinary vaccination [14, 15], this strategy looks promising for the near future [12].

3.2.2 Targeting the Encoded Ag

From the many potential strategies to be used, this review will be focused in those successfully used for swine in our laboratory: (1) The employment of strategies aiming to drive the vaccine antigens to Antigen Presenting Cells (APCs); (2) to direct the vaccine-encoded antigens to the MHC-I pathway, in occasions avoiding the Ab production: and (3) the use of plasmid cocktails, an advantage of DNA immunization that allow even to immunize animals with thousands of plasmid-cocktails thus covering even large proteomes; an strategy first approached by Barry et al. [16] and named as ELI-vaccination.

Enhancing the Induction of CD4+ T-Cells and Antibodies: Directing the Viral Ag to APC

One of our favorite options is to target the viral Ag to the sites of the immune induction, a strategy first described in a mouse model with the use of CTLA-4 as a genetic adjuvant [17]. We have followed a similar approach, this time using as carrier the APCH1 molecule. APCH1 is a fragment of an Ab that recognizes an epitope of the Class II Swine Leukocyte Antigen (SLAII) molecule, highly expressed in swine APCs [18]. By fusing our DNA-construct to APCH1, the encoded fusion products were efficiently directed *in vitro* to SLAII positive cells and enhanced the *in vivo* induction of both specific antibodies and T-cell responses [7, 19]. Interestingly enough, the protection afforded by the vaccines totally varied depending on the antigens and pathogen used, going from the sterilizing protection observed in some pigs against Foot and Mouth Disease Virus (FMDV) challenge [19] to the viremia exacerbation observed in the case of the African swine fever virus (ASFV) challenge [7]. These results demonstrate once more that adjuvants are not universal and also that, in order to design a rational vaccine against a given disease, is absolutely required to have a deep knowledge about its pathogenesis.

The second choice we used in our laboratory was based on the so called sHA; extracellular domain of the ASFV Hemagglutinin, a molecule with important similarities to CD2 leukocyte molecule [20]. As described for the APCH1, fusion of antigens to the sHA, allowed the in vitro binding to APCs, most probably due to the expression on their surface to CD2 receptors. Also as described for APCH1, the in vivo reflection of this fusion allowed to exponentially enhance both the antibodies and the T-cell responses induced in pigs that again, did not result in any protection against ASFV lethal challenge [21].

Of course there are other strategies to target antigens to APC, but not many have been successfully used in swine [22]. In this way, the use of CD169 or CD163, two endocytic receptors mainly expressed on macrophages, resulted in a strong humoral response: either CD169 or CD163 could favor antigen uptake by subcapsular sinus macrophages, leading to the initiation and improvement of the humoral immunity [23]. The use of TLR-2, a member of the Toll-like receptors family, looks also promising in swine although the enhancement of antibody production was not as outstanding as the obtained with CD163 or CD169 [22].

Enhancing CTL Induction: Directing the Viral Ag to MHC I Pathway

The lack of success of our ASFV vaccines could be explained by either a failure in the induction of protective CTL responses, in view of the fact that specific CD8-T cell responses has been described as key players in ASFV protection [24], or simply due to the bad selection of the vaccine antigens (just 3 out of 150 antigens encoded by ASFV). In order to solve this “dilemma”, we decided to obtain a vaccine prototype encoding our favorite antigens as fusions with ubiquitin; a strategy successfully used in mice

to optimize the Class I antigen presentation of the encoded antigens, thus enhancing the CTL induced in vivo. Briefly, after the transcription, our DNA-construct results tagged with ubiquitin, which targets the protein to the proteasome. Hence, the protein is degraded by the proteasome and cleaved into short peptides that are carried via the "TAP" transporters to the endoplasmatic reticulum and there, such peptides are presented via MHC Class I to the specific cytotoxic CD8+ T-cells. Ten years later, also in our laboratory, we have been able to extend these studies to pigs by using DNA vaccines encoding the same previously mentioned ASFV antigens. Thus, the fusion of ubiquitin to the ASFV antigens not only enhanced the CTL induction, but also abrogated the antibody induction in vivo, as it was also described before for mice [25] and most importantly, allowed conferring partial protection ASFV against lethal challenge for the first time [21].

Once more, DNA vaccines provided with new lessons: One same antigen can induce from exacerbation to protection, depending on the immunological outcome that it provokes. Ubiquitin, as any other genetic adjuvant can fail to exhort its effects, as it happen in the case of the Aujeszky glycoproteins. Ubiquitination of such glycoproteins did not lead to an enhancement in the protection afforded, most probably due to the lack of efficient degradation in the proteasome as demonstrated in vitro [26] This result demonstrates once more the impossibility of designing universal vaccine strategies, depending always on the nature of the antigens to be used and also on the mechanisms involved in protection against the given pathogen.

Increasing the Number of Antigens in the DNA Vaccine: The Cocktail Option and the ELI-Strategy

As stated before, one of the major advantages of DNA vaccines is their flexibility, which allows us to blend specific cocktails for specific needs. I.e., we could design a DNA vaccine containing a mix of plasmids, each one specially designed following the different strategies explained so far. Such vaccine could contain, among others, a plasmid directing an Ag to MHC class II, thus inducing a CD4+ T-cell response and another plasmid containing the same Ag, but fused to ubiquitin, thus being directed to MHC class I pathway, enhancing a strong CD8+ CTL response. What is more: a single vaccine may include such blend of strategies, but repeated many times for different Ag. In addition, we have to keep in mind that plasmids used in DNA vaccines present less size limitation in comparison with other vectors used in vaccination field [27]. Nevertheless, we must take into account that the immune response is so complex and delicate that we should be really cautious when manipulating such response. Everything fits and works on paper, v we must always try on the animal to see if our idea works as expected: Immune system lays on a very fine-tuned balance with countless interactions and thus, some responses may inhibit others and vice versa.

Expression library immunization (ELI), a concept first described in mice [16] and later extended to many other target species, is a method for the systemic screening of any given genome to identify potential vaccine candidates. ELI strategy is in principle not specifically addressed to the generation of a vaccine including the whole genome, but a discriminating tool that will allow us to select antigens to build a rational plasmid cocktail to be used for vaccination purposes. The essence of this approach is that the entire genome of a pathogen can be cloned into genetic immunization vectors under the control of a eukaryotic promoter to create a library that would express all the open reading frames (ORFs) of a pathogen. We can associate such ELIs with APCH1, sHA, or Ub in order to target the Ag for obtaining the immune responses discussed before. Immunized animals can therefore be challenged with the viral pathogen to check which clones induced protective immunity. In our laboratory we have obtained partial protection against ASFV lethal challenge by means of ELI immunization [28].

3.3 A Prototypic Protocol to Obtain and Test a DNA Vaccine Specifically Designed for Swine

3.3.1 Construction of a DNA Vaccine

To build a typical plasmid-based DNA vaccine we need a plasmid backbone containing: (1) an origin of replication allowing for growth in bacteria, (2) an antibiotic resistance gene (it will allow to select plasmid-transformed bacteria); (3) a strong promoter for optimal expression in mammalian cells (most commonly, the promoter of human cytomegalovirus, CMVp); and (4) a polyadenylation signal sequence (that provides stability and effective translation) [29]. Moreover, some authors point out the fact that the plasmid itself has immunogenic properties due to its repetitive CpG motifs, which are able to induce strong B cell and T cell responses [30]. PCR products of the gene insert must be first cloned into the plasmid vector (Fig. 1), then used to transform bacteria and finally, the bacteria plated on medium containing the antibiotic for which resistance is encoded in the plasmid. Only bacteria with the plasmid incorporated will be able to grow. One bacterial colony containing the right insert will be large scale-growth and the obtained DNA-plasmid production must be purified in order to be free of bacterial toxins (if we are going to inoculate animals with such DNA).

DNA-Library Construction

The basic protocol to construct a DNA library will include the following steps:

1. Isolate the complete genome of the pathogen of interest.
2. Digest the genome with *Sau3A*I (New England Biolabs), a restriction enzyme that recognizes the $^{5'}$GATC$^{3'}$ sequence and cut commonly every 300–500 bp.
3. Clone fragments into the selected eukaryotic expression vector (*see* **Note 3**).

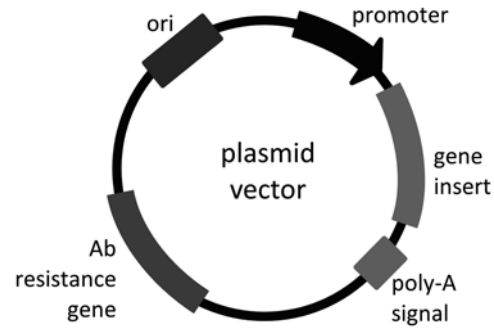

Fig. 1 Schematic representation showing essential components of a plasmid-DNA vaccine construct

4. Transform plasmids in selected bacteria (*see* **Note 2**) and afterwards plated in suitable medium in order to select the properly transformed bacteria.
5. Pick up a precise number of individual colonies for each restriction fragment and plasmid frame (*see* **Note 7**), to be individually inoculated into a 96-well culture plate.
6. Perform and store replicas of all plates at −70 °C with 15 % (v/v) of glycerol.
7. In order to obtain DNA material for vaccination purposes, a mixture of the generated plasmids must be carried-out (*see* **Note 8**) and therefore produce the DNA plasmid pool at large scale. Finally, the obtained DNA must be purified in order to be free from bacterial toxins.

3.3.2 In Vitro Assessing Ag-Expression

In order to prove that the obtained plasmids correctly express the inserted genes, it is recommendable to analyze its correct expression by Western blotting using specific polyclonal or monoclonal antibodies. Briefly, suitable cell lines shall be transfected with the vector, meanwhile control cells will be transfected with the void plasmid (or with the plasmid containing an irrelevant gene) using our favorite transfection protocol (electroporation, lipid based transfection, Calcium Phosphate based transfection…). Cells will be incubated for 24–72 h, and then harvested to evaluate its optimal in vitro expression kinetic. If everything is correct, the plasmid DNA is ready to be injected into the animal in order to elicit the desired immune response.

3.4 Swine Immunization

To finish with the present section dedicated to the production of DNA vaccines we include a simple and easy procedure to immunize swine currently used in our laboratory [21]. With this protocol we have achieved our best results: an immunization followed by a boost a fortnight after has given us a proper result and, in our experience working in ASFV, more boosts do not improve the

elicited immune reaction. Please remember, that since we will inject the produced DNA to animals it is compulsory to purify it from bacterial toxins.

1. Prepare a stock solution of 400 μg DNA/ml in commercial sterile saline-solution.
2. In an aseptic environment, put a sterile needle to a 2.5 ml sterile syringe. Charge one syringe per animal with 1.5 ml of the DNA stock solution. This is a DNA dose of 600 μg per pig (*see* **Note 9**).
3. Keep the charged syringes (with their cap on) in the fridge and transport them to the farm/animal facility into plastic bags inside a well protected polystyrene box filled with crushed ice (*see* **Note 10**).
4. Immobilize the animal according the animal welfare policies of your institution and clean and disinfect with absorbent paper imbibed with ethanol 70 % the injection points prior to the inoculation.
5. One third of the vaccine dose (0.5 ml) must be intramuscularly injected in the right rectus femoris quadriceps, one third must be injected in the right trapezius muscle of the neck and the last third must be subcutaneously injected in the right ear.
6. A fortnight after the first immunization, please repeat **step 5**, but performing the injections in the left side of the animal.

3.5 Prime Boost: The Hope for DNA Vaccines to Conquer the Market

DNA vaccination has gained a new impulse in the last years thanks to the exponential improvement of in vivo DNA transfection protocols both for human and veterinarian species (*see* Subheading 3.1.3): The maximal revolution seemed to arrive with the arrival of prime-boosting protocols. Even for the most skeptical, DNA electroporation clearly demonstrated to be the ideal protocol for immune priming, followed by boosting with recombinant viruses encoding same antigens or with recombinant proteins. Prime-boost strategies have improved the humoral immunity and also enhanced the DNA-primed CTL responses [31]. The most used viral vector platforms are, among others, the modified *Vaccinia* virus Ankara (MVA) and, specially, the adenoviral vectors [31]. The efficacy of both homologous and heterologous prime-boost strategies has been also tested in swine DNA vaccination with uneven results. Thus, optimal responses were obtained against Aujeszky disease by DNA priming—followed by a booster with an Orf virus recombinant-vaccine [32]. The efficacy of heterologous prime-boost regimes have lead to several human and nonhuman primates trials for important diseases such as HIV [33, 34], albeit in occasions homologous prime boost strategies have demonstrated to give optimal results [35]; insisting once more in the concept of individual vaccines for individual purposes. Independently

of the above mentioned results, DNA priming can greatly reduce the amount of booster vaccine needed as we have previously demonstrated for the Rift Valley Fever virus attenuated vaccine in sheep [36]. Similar concept was previously presented as an alternative to reduce the amount of booster vaccine needed at the time of influenza outbreaks, thus reducing costs and saving response time [37]. This concept could perfectly be extended to other diseases.

4 Notes

1. There are many options available in the market, having all in common the presence of a promoter capable to be recognized by the target species. In our case we used the pCMV plasmid from Clontech (Palo Alto, California), to express the vaccine-encoded product under the control of the immediate early promoter of human cytomegalovirus (CMVp). We also recommend the use of pVAX™200-DES (Invitrogen, California) which meets US Food and Drug Administration (FDA) guidelines for design of DNA vaccines.
2. We usually use electrocompetent *Escherichia coli* (ElectroMAX™ DH10B™ T1 Phage-Resistant Competent Cells, Invitrogen).
3. In order to clone the DNA insert inside the plasmid backbone we normally use the Quick Ligase Kit (New England Biolabs). To purify DNA products we commonly use Qiagen MinElute Reaction Cleanup Kit (Qiagen, The Netherlands). To purify from bacterial toxins the DNA-plasmid obtained by means of bacterial culture we usually use Endofree Plasmid Mega kit (Qiagen, The Netherlands).
4. The open reading frames (ORFs) encoding the antigens can be cloned into the plasmid backbone alone or as fusions with the ORFs encoding carrier molecules that act as genetic adjuvants. Some of the adjuvants (*see* Fig. 2) discussed in the present chapter are:
   - APCH1: the single chain of an antibody that recognizes the DR allele of the Class II Swine Leukocyte Antigen (SLAII) molecule.
   - sHA: the extracellular domain of the ASFV hemagglutinin (sHA), with homology to the CD2 leukocyte antigen.
   - Ub: a monomer of the mutated Ubiquitin (A76).
5. Please note that electroporation conditions must be optimized for each animal species.
6. Please remember that cytokines are species-specific to the host to be vaccinated.

7. The number of colonies to be picked up in order to ensure the representation of all *Sau*3AI fragments in the three possible frames was calculated following a formula that takes into account the length of each original viral DNA restriction fragment and the number of fragments generated by the complete *Sau*3AI digestion.

$$N = 2\left(\frac{\ln(1-P)}{\ln(1-f)}\right) \quad f = \frac{m}{L}$$

where:

N: Number of colonies to be picked up.
P: Probability (=0.9).
m: average length of fragments generated by *Sau3A*I.
L: full length of digested vector.

8. We prepare a pool by taking 0.5 μl from each individual clone. This pool is used as starter culture to inoculate 1 l of the proper

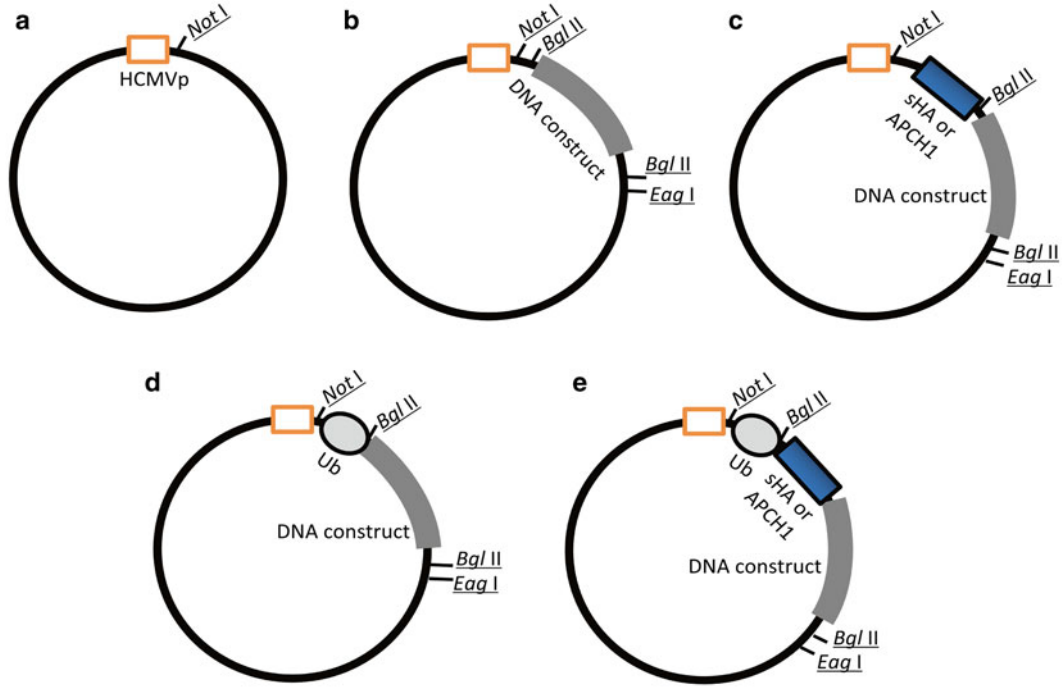

Fig. 2 Schematic representation of plasmids used for DNA-immunization. (**a**) Control plasmid, with no insert gene; (**b**) plasmid with a DNA insert; (**c**) plasmid containing the DNA construct fused to the ORF encoding for APCH1 or sHA; (**d**) plasmid containing the DNA construct fused to the ORF encoding for Ub; (**e**) plasmid containing the DNA construct fused to the ORF encoding for Ub and APCH1 or sHA. The plasmids contain the correspondent ORF within the unique *Not*I cloning site and contain their initiation AUG codon in a Kozak context for optimal transcription and with a *Bgl*II unique site in their 3′ for downstream in frame cloning of the target sequence

broth culture (we normally use LB medium), supplemented with the appropriate antibiotic.

9. We prefer to prepare the vaccine-doses the same day than the inoculation. If this is not possible, please keep the syringes under refrigeration (4 °C) until its use. If different DNA-constructs are going to be used, it is highly recommendable to mark the syringes with different color-tapes in order to avoid confusions during the immunization.
10. Please allow to reach room temperature the DNA doses. If the injection is to cold it may cause undesired additional pain to the animals.

Acknowledgment

This work was supported by the Spanish Government (Project reference number: AGL2010-22229-C03-01).

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