final report

Project code: B.CCH.2088

Prepared by: Peter Fennessy and Gertje Petersen

AbacusBio Limited

Date published: May 2014

PUBLISHED BY

Meat & Livestock Australia Limited

Locked Bag 991

NORTH SYDNEY NSW 2059

Review of approaches to generate a mucosal (antibody) response targeted at the ruminal Archaea

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

This publication is published by Meat & Livestock Australia Limited ABN 39 081 678 364 (MLA). Care is taken to ensure the accuracy of the information contained in this publication. However MLA cannot accept responsibility for the accuracy or completeness of the information or opinions contained in the publication. You should make your own enquiries before making decisions concerning your interests. Reproduction in whole or in part of this publication is prohibited without prior written consent of MLA.

Abstract

The potential to reduce methane production from ruminants is of major interest in Australia and internationally. As well as reducing the greenhouse gas production from ruminants, reducing methane production in the rumen could potentially enhance productivity and feed efficiency, as methane losses account for about 6-10% of gross feed intake. Many approaches are being evaluated but with recent progress in vaccine development, it is appropriate to once more review the possibilities for an immunization approach. There are precedents for a vaccination approach in that there are reports where vaccination against specific rumen microbes was effective in preventing lactic acidosis in cattle and sheep.

The purpose is to review the literature and propose a protocol for evaluation to induce a mucosal secretory IgA and IgG response. The review assumes that appropriate target (Archael antigen) motifs are available (this area is the subject of research projects within Australia and New Zealand). The options are considered and recommendations highlight some particular approaches to evaluate a vaccination approach. The approach involves use of a Virus-like Particle vaccine incorporating the target antigens (VLPs are self-assembling protein complexes derived from recombinant systems expressing viral proteins; while they lack a viral genome, they have the immunogenic competence of actual viruses).

It is recommended that the approach is evaluated in rats and targets the sub-lingual salivary gland (not the parotid which is the likely target of a nasal vaccine) using two archaeal (model) antigens; the response criteria would include both the serum antibody response and the mucosal antibody response (per a lung lavage sample). Should the protocol be successful, it would then be evaluated in sheep in terms of both the immunogenic (antibody) response and the impact on methane production.

Executive summary

The potential to reduce methane production from ruminants is of major interest in Australia and internationally. As well as reducing the greenhouse gas production from ruminants, reducing methane production in the rumen could potentially enhance productivity and feed efficiency, as methane losses account for about 6-10% of gross feed intake. Many approaches are being evaluated but with recent progress in vaccine development, it is appropriate to once more review the possibilities for an immunization approach. There are precedents for a vaccination approach in that there are reports where vaccination against specific rumen microbes was effective in preventing lactic acidosis in cattle and sheep.

The review provides a survey of the literature and proposes a protocol for evaluation to induce a mucosal secretory IgA and IgG response. It is recognized that a reliable vaccine to lower methane emissions will need to target a wide array of methanogens and induce a mucosal, and potentially a systemic, response. The review assumes that appropriate target motifs are available (this area is the subject of a number of projects within CSIRO and in New Zealand).

IgG is the predominant immunoglobulin class in serum, but the main immunoglobulin in bovine saliva is IgA and this is the effector group of the mucosal immune response. Since IgA antibodies, specifically secretory IgA (S-IgA), have a relatively high tolerance to proteolytic enzymes, they have a better chance of surviving in the ruminal environment and hence are the preferred target for a vaccination solution.

The use of prime-boost vaccination regimes enhances the efficacy of vaccination. Since the methanogenic Archaea reside in the rumen without entering into the mucosa or affecting the mucosal surface (e.g. by inflammation), the vaccine must target the topical mucosal response via the salivary glands. However the salivary glands are not easily accessible and indeed vaccination requiring administration directly would not be practical at any scale. Evidence indicates that the main source of salivary IgA in ruminants is the sub-lingual gland. While the nasal cavity is a logical target for vaccination against respiratory diseases in ruminants, it is not the approach of choice to stimulate secretion of immunoglobulins in saliva. The preferred target is vaccination per the sub-lingual route. Considering that a keratinized oral mucosa is found in ruminants and rodents, findings in rodents should be readily transferrable to ruminants.

The proposed approach involves use of a Virus-like Particle vaccine incorporating the target antigens (VLPs are self-assembling protein complexes derived from recombinant systems expressing viral proteins; while they lack a viral genome, they have the immunogenic competence of actual viruses). Targeting of the sub-lingual system is recognized as a particular challenge.

In conclusion, the review recommends an approach that targets sub-lingual vaccination using VLPs (e.g. based on VP60, the virus capsid protein of Rabbit Hemorrhagic Disease virus, or similar). The vaccine would be based on specific target motifs common to the surface of archaeal species (but not other rumen microbial classes) and which can be expected to affect either archaeal metabolism or methane production pathways. The initial (proof of concept) experiments would be in rats targeting the sub-lingual salivary gland (not the parotid which is the likely target of a nasal vaccine) using two archaeal (model) antigens; the response criteria would include both the serum antibody response and the mucosal antibody response (per a lung lavage sample). Should the protocol be successful, it would then be evaluated in sheep in terms of both the immunogenic (antibody) response and the impact on methane production.

Table of Contents

Terms of Reference

Produce a review of the scientific, technical and patent literature to provide an overview of the options for development of a vaccination regime for the delivery of high levels of salivary antibody (in this case targeted specifically the ruminal Archaea). The specific focus will include particle vaccines, and a broad, non-specialised, overview of freedom to operate in terms of vaccine technology.

1. Benefit: The Review will provide input into decision-making by MLA re investment in research into vaccine technology targeted at the ruminal Archaea; this is part of the MLA investment in productivity improvement through methods to reduce the wastage of energy in the rumen from methane and to reduce greenhouse bases. This project provides background for decision-making
2. Impact: should a vaccine approach prove successful, producers will have an option to vaccinate to reduce methane emissions; hence impact could be expected in both the supply & risk dimensions; an improvement in productivity will impact cost of production (supply), while the risk issue relates to the risk that methane may ultimately incur a cost to producers (although it may also lead to a Carbon Farming Initiative (CFI) methodology) and other benefits.
3. Practice changes: The outcomes will be derived via the impacts above
4. Outputs from investment: A review of the literature.

1. Introduction

1.1 Methane mitigation

World-wide, methane emissions from agriculture make up for roughly two-thirds of the anthropogenic methane emission. Enteric fermentation in livestock accounts for about a third at about 80 million tons of methane per year. The rest of these agricultural methane emissions stem from flooded rice paddies and animal wastes [1].

Since methane, as opposed to carbon dioxide, has a higher global warming potential (25 times that of CO2) as well as a short (12-year) atmospheric lifetime, mitigation of methane emissions is considered as a valid approach to delay global warming [2].

Moreover, reducing methane production in the rumen could potentially enhance productivity and feed efficiency in ruminants, as methane losses account for about 6-10% of gross feed intake [3]. Mitigation strategies have included the dietary supplementation with oils, chemical modulation of the ruminal climate and/or methanogenesis itself, defaunation (elimination of protozoa), probiotics to modify the rumen environment and immunization against methanogens [1,2,3].

All of these approaches have shown limited success for a number of reasons including adaptation of the ruminal microorganisms and limited knowledge of the methanogens themselves and of ruminal ecology.

1.2 Potential of a vaccine approach

With the recent progress in the field of vaccine development, it seems reasonable to once more review the possibilities for an immunization approach to methane mitigation. There are precedents for a vaccination approach. For example, vaccination against *Streptococcus bovis* and *Lactobacillus* (per intramuscular injection) was effective in preventing lactic acidosis in cattle challenged with a 90% wheat diet [4]. Similar results were reported in sheep providing evidence of the feasibility of modulating the ruminal flora via vaccination [5,6,7].

Attempts to reduce methane emissions in sheep by inducing a systemic immune response utilizing crude cell solutions date back to the early 2000s, when methane emissions were reduced in sheep vaccinated with an Archaeal vaccine [8]. Unfortunately, these results could not be confirmed so that a careful consideration of the vaccine and the vaccination protocol is required to find a suitable approach.

A reliable vaccine to lower methane emissions will need to target a wide array of methanogens and induce a mucosal, and potentially a systemic, response.

This review of the vaccine approach assumes that appropriate target motifs are available. In fact this area is the subject of a number of projects within both AgResearch and within CSIRO.

2. Vaccine option

2.1 Targeting the rumen

The rumen is both a part of the digestive system and a rich microbial ecosystem where methanogens are one of many groups f bacteria, protozoa, Archaea and fungi present [8]. Even though the normal pH of the ruminal fluid of around 6.5 leads to a

slightly acid environment and, thus, to a lot less strain on introduced particles or proteins than further along the gastrointestinal tract, there are still a number of proteolytic enzymes present as well as innumerable challenges in microbial digestion that can quickly inactivate functional proteins such as enzymes or antibodies [9].

Moreover, the function of the rumen as a resorptive organ dictates certain differences between the ruminal mucosa and intestinal mucosa. For example, the ruminal wall lacks lymphoid follicles and contains only a small number of solitary immune cells such as dendritic cells, lymphocytes and plasma cells [10], which effectively limits the possibilities for local stimulation of production and secretion or penetration of antibodies. Due to this, the major portion of immunoglobulins reaching the ruminal lumen is not secreted locally, but is of salivary origin.

2.2 A mucosal immune response

Even though IgG is the predominant immunoglobulin class in serum, the main immunoglobulin in bovine saliva is IgA [11] and while high levels of blood-derived IgG and IgA can be found in mucosal secretions in sheep, IgA is the effector group of the mucosal immune response [12].

Since IgA antibodies, specifically secretory IgA (S-IgA), have a relatively high tolerance to proteolytic enzymes [13], they have a better chance of surviving in the ruminal environment and hence are the preferred target for a vaccination solution. Although an intramuscular or subcutaneous injection of antigen will induce an IgG and monomeric serum IgA response, stimulation of secretory IgA production requires direct involvement of the mucosal immune system [14].

The adaptive mucosal immune response is triggered by contact between antigen and mucosal antigen presenting cells (APCs) in the mucosa-associated lymphatic tissue (MALT). However cross-presentation can also occur – this is the situation where an antigen that would normally be presented on MHC II and hence lead to an antibody response (due to recognition by antigen-specific $CD4^+$ T helper cells) is also presented on MHC I, a process normally reserved for endogenous antigen such as a replicating virus in a cell. MHC I presentation leads to a cell-mediated immune response (CMI) via a CD8+ cytotoxic cell response. Stimulation via VLP results in the generation of antibody but some can also be cross presented to generate a CMI response [15, 62].

In addition to the few B lymphocytes affected directly by the presenting antigen, CD4+ cells attract and activate B lymphocytes to develop into plasma cells capable of producing high levels of specific antibodies. Additionally, antigen-loaded APCs and sensitized immune cells leave the point of contact and enter the lymphatic system to be drained into the nearest lymphatic organ, most commonly a lymph node, where more immune cells are presented with the antigen by the APCs. Then, due to a high level of compartmentalization of the mucosal immune system regulated by surface factors (“homing factors”) on immune cells and corresponding “addressins” on cells in the mucosal regions (as well as a number of cytokines expressed by immune and epithelial cells [15]), they return to the specific region in which they were primed initially via the blood stream.

This highly sophisticated system ensures that the mucosal immune system, as the largest immune system in the organism, does not get overloaded at every antigen contact but is capable of a targeted response, even though a linkage between certain sites does exist.

The B cells recruited and activated by T helper cells at the effector sites as well as in draining lymph nodes and central lymphatic organs (such as spleen and bone

marrow) migrate to the sub-mucosa at the point of contact, guided by the information provided by the T helper cells. Here, they mature into plasma cells and start producing immunoglobulins.

Notably, in order to be able to produce anything but IgM (the "default" immunoglobulin), B cells need to undergo **class switch recombination**, which involves the deletion of parts of their genome [16]. Only after this class switch can a B cell produce IgA, which is then secreted (in oligomeric form and usually as dimer) and taken up by epithelial cells via the polymeric immunoglobulin receptor (pIgR) that is in turn internalized and transported to the apical surface. When pIgR reaches the epithelial surface, it is then partially externalized and cleaved by proteolysis in order to produce S-IgA, consisting of an IgA dimer linked to secretory component, a former part of pIgR [17]. Since pIgR is produced by mucosal epithelial cells and is essential for the production of S-IgA, (up-) regulation of the expression of the pIgR gene is key to a successful secretion of S-IgA.

Common promoters of the polymeric immunoglobulin receptor gene are pro-inflammatory cytokines such as interferon-gamma (IFNγ), tumour necrosis factor (TNF) and interleukin-1 (IL-1), but pathogen-associated molecular patterns (PAMPs) have been shown to up-regulate pIgR expression in the absence of inflammation [18] via Toll-like Receptors (TLRs) or other host pattern-recognition receptors.

This is of special interest for mucosal vaccine design, because an inflammatory response to the administration of the vaccine, as after intramuscular application, is unlikely. On the other hand, systemic immunization alone cannot induce a focused response in any particular mucosal region, since the B cells recruited in the process have not been primed by T helper cells (or the surroundings in the region itself), and therefore lack the necessary homing factors [19].

2.3 Possible approaches to mucosal vaccination

Direct stimulation of the mucosal immune system at several different sites has been shown to elicit both a mucosal S-IgA response and a systemic response, with the latter mainly involving IgG [20,21,22,23,24]. However, the immune response to topical mucosal vaccines is not regarded as a strong response so that successful mucosal vaccination requires a powerful mucosal adjuvant such as choleratoxin (CT) or cytosine-phosphate-guanosine (CpG) [25,26] in order to generate a satisfactory response.

The use of prime-boost vaccination regimes has been shown to enhance the efficacy of vaccination. Where the objective is a strong mucosal response, the key may well lie in formulation/ application, combining a mucosal priming dose with a parenteral booster. There have been several recent studies where such prime-boost regimes have been shown to enhance efficacy where a DNA vaccine provides the priming vaccination with a protein-containing vaccine as the booster [27]. The strong immune reaction of mucosally-primed animals to a boost with an injectable cholera or polio vaccine as opposed to such a challenge in naïve animals [20,28,29] supports such an approach.

Since the methanogenic Archaea reside in the rumen without entering into the mucosa or affecting the mucosal surface (e.g. by inflammation), there is a need for the vaccine to target the topical mucosal response [20] via the salivary glands. This is the only option as there is no potential for ruminal pIgR expression or immunoglobulin production, and in addition, as described earlier, transport (leakage) of immunoglobulins across the ruminal wall is likely to be minimal.

Unfortunately, the salivary glands are usually not easily accessible. In humans, nasal

or tonsillar application of immunogen has shown good results in terms of an increased antibody titre in saliva [15] but virtually no response in the gut. Moreover, tonsillar application in the ruminant presents a major practical challenge due to difficulties with access to the tonsils (especially when compared to that in humans) and indeed would not be practical at any scale.

A number of studies in humans and laboratory rodents [30,31] have concluded that the parotid, sub-mandibular and sub-lingual salivary glands contribute to IgA in saliva. However there has also been one study of pIgR in bovine salivary glands which suggested that the main source of salivary IgA in cattle is the sub-lingual gland, with the parotid barely expressing pIgR at all. These findings have also found support in a study where only low titres of antibodies were detected in parotid saliva in sheep [31].

With nasal application of the immunogen, it is to be expected that the greater part would be transported into the lymphatic tissue in the parotid gland after penetrating the mucosal epithelium, since the nose is part of its drainage area. While this would lead to IgA production in humans, a salivary IgA response would not be expected in the ruminant due to the limited capacity for S-IgA secretion from the parotid.

Hence, while the nasal cavity is a logical target for vaccination against respiratory diseases in ruminants, it does not appear to be worthwhile as an approach to stimulate secretion of immunoglobulins in saliva.

However recently there has been a lot of attention given to a relatively new method of vaccination per the **sub-lingual route**. This, as opposed to tonsillar vaccination, can be expected to be practical (e.g. combined with routine work such as drenching of animals). It has also been shown to evoke strong intestinal and systemic immune responses [20] as well as inducing a significant rise in specific secretory IgA in saliva [32]. This response is due to the fact that, other than in oral application, the antigen is not digested and/or processed in the liver (which would possibly lead to antigen tolerance [23]) but, at least in part, directly enters the bloodstream via the well-perfused sub-lingual mucosa [25]. Here, the immunogen comes into contact with the sub-lingual lymphatic tissue, or is transported into the lymphatic centres located inside the sub-lingual salivary glands, where an adequate immune response can be generated.

Considering the distinctive differences between the non-keratinized epithelium found in the sublingual region of humans and the keratinized oral mucosa in ruminants, the good results achieved in sub-lingual human allergy treatment [24] may not be transferrable to a sub-lingual delivery approach in cattle or sheep. Nevertheless, mice also exhibit keratinization of the oral mucosa [24,33], and thus findings in laboratory rodents should be readily transferrable to ruminants.

In this respect, there have been several studies in mice and guinea pigs (e.g. using Human Papilloma Virus (HPV) [23], *Salmonella* proteins [32], Foot-and-Mouth Disease Virus (FMDV) [34] and Ebola Virus [24]). All of these studies found vaccination to be effective in creating an immune response and/or protective against an otherwise lethal pathogen challenge. Further, in the FMDV study, a commercial injectable vaccine was administered sub-lingually without modification at the recommended dosage or at one-quarter or 1/16 of that dose and all animals were found to be protected from a challenge with FMDV. Moreover, since sub-lingual application of an immunogen induces a good mucosal response in the gut [20] and there is a linkage between gut and distant mucosal response sites such as the salivary glands and mammary glands [15,20], it might even be possible, given the right vaccination regime, to induce a mammary response strong enough to supply newborns with antibodies via the colostral route. This, however, would call for further investigation of the level of intestinal stimulation required.

In a study comparing the immune response generated by sublingual vaccination with adjuvanted killed Influenza virus, non-adjuvanted HPV16 Virus-like Particles and *Helicobacter pylori* extract with the one elicited by natural infection, the VLP formulation was found to be as effective as the whole killed influenza virus administered with an adjuvant [20]. 'Memory' is also an important component of an immune response – a memory response is important for a long lasting response (less frequent re-boost) and often requires an adjuvant.

The above discussion points towards the likely feasibility of a sub-lingual approach utilizing non-adjuvanted Virus-like Particles as carriers.

2.4 Particle vaccines and a mucosal immune response

Vaccines based on live viruses (generally attenuated or naturally apathogenic strains) are well-accepted as generating strong immune responses. Using genetic engineering approaches, there is the option to create recombinant viruses that are apathogenic to the targeted host, but still fully capable of replication, and express single antigenic proteins to induce a long-lasting immunity to the desired pathogen (an example is a recombinant Canarypox virus carrying West Nile virus antigens for immunization of horses).

In order to reach the lymphatic tissue in the sub-lingual sub-mucosa, the agent needs to be able to penetrate the surface layers of epithelial cells or be internalized to be transported below. One of the reasons that live attenuated pathogens form vaccines with superior immunogenicity is their ability to invade tissues, even if they lack characteristics necessary for infection [31].

For an immunogen to mimic a live pathogen in terms of tissue penetration, it would need to be very small and possibly of a particulate nature, because M-cells and dendritic cells (the two main cell types involved in pathogen phagocytosis and processing in mucosal regions), more readily internalize particles with size and density characteristics that mimic pathogens [35]. They should also present antigens in a way that resembles that of pathogens.

While micro-particles are candidates for delivery in terms of modifiability and antigen presentation (size ranging up from 100nm, 0.1µm), they still have problems being taken up readily by M-cells [19]. Moreover, "true" nanoparticles, which in theory are smaller than 1µm, are a difficult target in drug or antigen delivery systems, as they are regarded as too small to incorporate a reasonable amount of the substrate [36].

Thus, the potential uptake by APCs of particulate formulations is considered to be limited. In some micro-particle compositions, biodegradation is expected or may even be desirable (e.g. using muco-adhesive qualities of alginate capsules [21] or APC-activating characteristics of poly(lactide-co-glucolide) [22]), this issue also presents a problem in respect of the immunogenicity of such preparations. However this problem can be overcome by the utilization of **Virus-like Particles** (VLPs).

VLPs are self-assembling protein complexes derived from recombinant systems expressing viral proteins. As such, the key feature is that they lack a viral genome (whether that be a DNA or RNA-based) although they still have the immunogenic competence of actual viruses [37]. Some of them contain only one protein, while others can resemble the original virus (e.g. influenza virosomes) [38]. Delivering antigen by way of VLPs can be done in two ways:

1) inserting the antigen into the VLP shortly before or at the time of self-assembly, or
2) tethering the antigen to the surface of the already assembled and purified VLP.

In any case, the structure of the VLP leads to a certain measure of protection of the

immunogen as well as facilitated delivery to the desired surface/cell type.

Most VLPs are small (and are no smaller than their virus of origin ranging from only 20 to 100nm, a standard size for many viruses), which means that, in addition to efficient phagocytosis by APCs, they might even reach the draining lymph nodes without being intercepted by the immune system [39].

In addition to their superiority over other particle formulations in terms of size, all VLPs also share one crucial characteristic: immunogenicity. The evolutionary impact of millions of years of viral challenge means that the physiological characteristics of VLPs generally lead to both a cellular and a humoral immune response [40]. With their size, shape and density resembling those of pathogens and the organized, highly repetitive fashion in which antigens can be presented, VLPs reliably stimulate APCs [39], especially dendritic cells, because they are recognized as foreign [41]. Due to the optimal spacing of antigens on their surface they can also have the ability to cross-link the antibodies that constitute the B cells receptors and stimulate B cells directly [42].

These properties of the vaccine could make the use of an adjuvant obsolete, especially if the virus used to construct the VLPs actually expressed tropism towards the very same tissue that is targeted for response [43] as long as the VLP retains the ability of the virus to penetrate cells (e.g. by incorporating certain surface proteins).

While some VLPs have been shown to elicit a natural killer T cell (NKT)/CD8+ T cell weighed response [44], the primary goal of an efficient vaccine against Archaea should be induction of a B cell response leading to antibody production; in fact this is the primary response of some VLPs [45]. These findings call for careful consideration of the type of VLP used in a vaccination study. In this respect, the ideal VLP vector should be:

1) inexpensive to produce and purify;
2) easy to engineer in order to add in the desired antigen;
3) of small size and a dense particulate nature; and
4) be one where the target organism is unlikely to have been challenged by the native virus as this is known to interfere with VLP survival and antigen presentation due to pre-existing immunity (PEI) [24]

VLPs (based on VP60, the virus capsid protein) of Rabbit Hemorrhagic Disease virus (RHDV) have been successfully used as a vaccine against RHD in rabbits. They are also easy to produce in modified baculovirus/insect cell systems with a high yield of purified VLPs after a comparatively simple purification process. In addition, different techniques for the incorporation of protein antigens have been investigated, and the researchers have found that linkage of small peptides to the scaffold of the RHD VLP is possible at a high level [46].

The RHD VLP is typical of many VLPs; it is only about 40nm in size and constitutes a single protein assembled in a highly repetitive manner, so the VLP meets the structural requirements for a VLP to be used in sub-lingual vaccination. Even though these VLPs should generate the desired immune response on their own, incorporation of an adjuvant into the particle might be necessary in order to facilitate a sufficient response (and may also be important in ensuring long-term memory development) and should be evaluated in any study. Several different approaches to this have been investigated including loading VLPs with CpG [49], α-galactosylceramide [44] and T-cell epitopes [49]. Since CpG is a potent mucosal adjuvant, further investigation is indicated; in contrast, α-galactosylceramide is an activator of natural killer T-cells so that this is contra-indicated as such a response is not that desired.

A vaccine targeted at the sub-lingual mucosa appears to offer an opportunity. However in order to prolong the exposure time of the sub-lingual mucosa to the immunogen, there are possibilities to create a vaccine formulation with muco-adhesive properties. Several muco-adhesive polymers are well-known in pharmaceutical development and some have been investigated for their ability to adhere to bovine sub-lingual mucosa. These include Carbopol 974P and Pharmacoat 606 [60], while there is evidence that poly-acethylene polymer adheres well to bovine buccal mucosa [61].

2.5 The ruminal methanogenic Archaea as a vaccination target

In vaccination against pathogens, vaccine target identification is relatively straight-forward, as it is generally limited to one species. However in this case the target is a wide group of microbes.

The general consensus appears to be that the Archaeal population of the rumen constitutes around 3 to 3.5% of ruminal microbes [50]; most, if not all of the Archaea are methanogens. Around 120 species of ruminal Archaea within 33 genera have been described; their distribution differs greatly with diet and environment as well as genetic makeup, health, age and geographical location of the host. However it seems that on a roughage based diet, *Methanobrevibacter* species make up about 60% of the Archaeal population [8,50].

A parenteral vaccine targeted against *Streptococcus bovis* and/or *Lactobacillus* provided proof-of-concept for a vaccine targeted at rumen microbes [4,5,6,7]. However such an approach has not proved reliable with a methanogen vaccine with several studies in sheep in Australia and New Zealand and even though one of the groups found an 8% reduction in emissions [51], these results could not be confirmed by others [52,53]. Nevertheless, all of these vaccination studies utilized whole-cell solutions or relatively crude protein preparations, in which case the antibodies generated are likely to be specific for the species used in the vaccine. Even if a number of methanogens are used, the response will still target only a fraction of the reported 120 species of Archaea and as these are knocked out by specific antibodies, others will almost certainly migrate into the vacant niche.

A much broader approach targeting a range of methanogens is likely to be necessary. Therefore common (small) protein motifs on the archaeal surfaces are probably preferred as a target; surface proteins potentially provide ready access to the antibodies [54]. Ideally, the protein motifs would be essential to the processes of either archaeal metabolism or methane production and thus present in most, if not all methanogens.

The sequence of the entire genome of *Methanobrevibacter ruminantium* offers a number of possible targets, some of which have been further specified and selected as likely suitable immunogens [55]. Other approaches such as phage display are also being pursued. However approaches utilising comparative genomics and reverse vaccinology, a computational procedure in which the entire genome is scanned for possibly antigenic genes, should help identify common antigens shared by a number of methanogens. There are also studies examining purified proteins generated in heterologous systems as possible vaccines [56]. Unfortunately, there are distinct differences between three fractions of methanogens, *Methanobrevibacter*, *Methanomicrobium* and the largely unspecified *Rumen Cluster C (RCC)* [27]. As these fractions also differ greatly in the structure of their cell wall [57], identification of an overall common antigen might prove very complex.

Nevertheless, there have been some efforts to classify the surface proteins of *Methanosarcina* species and identify possible targets [58], so that a combined

vaccine targeting *Methanobrevibacter* as well as *Methanosarcina* could prove more appropriate.

2.6 Scale of antibody response required

An estimate of the scale of antibody response required to effect a change in ruminal methane production through targeting ruminal Archaea is a formidable task for which no obvious data are available in the literature. However Wright *et al* found an 8% reduction in methane output in sheep (serum (salivary) titres of IgG and IgA of 450,000 (1,750) and 90,000 (300) respectively) [51]. Williams *et al* found similar antibody titres (serum IgG of 550,000 and salivary IgG of 900) but actually detected an increase in methane output in the vaccinated animals [53]. This unexpected result might be due to a rise in a previously disadvantaged methanogen species.

The dose of immunogen is an issue to consider. For example, in a study on oral vaccination of human volunteers with a Norwalk virus VLP, there was no difference between the immune response to 250, 500 or 2000 µg VLPs given orally. This could suggest that there are only a certain number of antigen processing sites in a certain area of mucosa that can be saturated with antigen [59]. This result, however, helps highlight the need to carefully consider the actual formulation and application of the vaccine, as a longer exposure period to the mucosa could be expected to yield better results due to receptor recycling; also a parental boost might be necessary to achieve the desired antibody titres.

3. Conclusion and recommendation

3.1 Overview

In this review we argue for an approach that targets sub-lingual vaccination using VLPs (e.g. based on VP60, the virus capsid protein of Rabbit Hemorrhagic Disease virus, or similar). The vaccine would be based on specific target motifs common to the surface of archaeal species (but not other rumen microbial classes) and which can be expected to affect either archaeal metabolism or methane production pathways[1].

3.2 Recommendation

That MLA working with the PGgRC bring together a meeting of key researchers (Drs Chris McSweeney, Neil Wedlock, Peter Jannsen, Professor Vernon Ward (University of Otago)), an immunologist (possibly ex CSIRO) with MLA and PGgRC nominees in early 2014 to discuss the next steps. Assuming the decision is to progress assessment of the concept, the group would then take the first steps around making decisions as to seeking investment, the development of protocol (with timelines), and engagement of a project team.

[1] Note that this review of the vaccine approach assumes that appropriate target motifs are available, an area of research within both AgResearch and CSIRO.

4. Indicative project: Outline

Establish a protocol for induction of a mucosal secretory IgA and IgG response, via the sub-lingual salivary gland (not the parotid which is the likely target of a nasal vaccine) in rats using two archaeal (model) antigens. An indicative series of initial steps is outlined below.

Stage 1

1. Call meeting as per Recommendation
2. Establish target protein motifs

   The objective is two archaeal (model) antigens. A project to identify potential candidates is currently underway at CSIRO.
3. Commission the development of the concept for a VLP-based vaccine where the key facets to be considered include:
   a. *purpose:* the vaccine is to target the mucosal secretory IgA and IgG responses, via the sub-lingual salivary gland;
   b. *species:* rodents (rats) are an appropriate model for vaccination per the sub-lingual route;
   c. *vaccine conjugate:* simple conjugation would be the most appropriate starting point as the focus is on the immune response and not the technology;
   d. *sampling:* serum by venipuncture at 3 week intervals, plus lung lavage in sacrificed rats
   e. *assay:* the possibilities include an assay of methanogens on a plate or a simple conjugate of epitopes to a carrier such as BSA;
   f. *vaccination protocol*: this should include treatments as below
      - without/with adjuvant (to further stimulate IgA production) and be formulated to target the 'right' response (i.e. a B cell rather than an NK cell response),
      - a combination of a mucosal primer targeted at the sub-lingual gland with a mucosal or a sub-cutaneous booster[2], with the vaccine administered at 3-week intervals;

      nothing that at this stage, the focus must be on the immune response/ outputs and not the technology; therefore the simplest approach will likely be to anaesthetise the rats so that the dose can be placed under the tongue and to then hold them under anaesthesia for say 30 minutes; once the basic protocol with anaesthetised rats is developed, there is a very strong case to include a muco-adhesive agent (e.g. poly-acethylene) to enhance uptake of the VLP by the sub-lingual gland (and possibly a PAMP to stimulate TLRs to up-regulate the sub-lingual cell polymeric immunoglobulin receptor activity);
   g. *animal ethics approval*: this is a relatively straight-forward protocol, so no particular issues are anticipated.
4. Commission the project, sign the contract and initiate the project

5. Initiate the project

[2] The rationale is to target the mucosal immune system by priming so that the (subcutaneous) boost then targets the cells that already been primed (memory cells which already harbour the appropriate information).

6. Proof of concept - first (rat) experiment: The initial project will involve a simple experiment and would constitute the first *go/no-go point*.
   a. *Indicative design* (a 'best-bet' protocol) –
      - RHDV VLPs with two antigens,
      - *2 adjuvant treatments:*
        - without adjuvant
        - with adjuvant (an adjuvant that is already approved or likely to be readily approved for use in ruminants[3])
      - *3 prime-boost protocols:*
        - subcutaneous primer/ mucosal boost)
        - mucosal primer/ subcutaneous boost
        - mucosal primer/ mucosal boost
      - *2 ages at slaughter*
        - 3 weeks after the first booster
        - 3 weeks after the third booster
        - hence the experiment would include a control plus 6 treatment groups (2 x 3 x 2) with 5 rats per treatment sacrificed at 3 weeks after the first booster, and a further 5 sacrificed 3 weeks after the third booster; this requires a total of 72 rats.
   b. *Response:* serum response (samples at 3-week intervals) & a lung lavage at slaughter (to define the mucosal response) at 3 weeks after the first booster (first group) and 3 weeks after the third booster (second group).
7. Second (rat) experiment: The second experiment with rats would include an evaluation of the preferred treatment with/without a muco-adhesive agent compared with the anaesthesia-based approach; this would constitute the second *go/no-go point*.
8. Preliminary evaluation in sheep: The next experiment would seek to extend the work to an evaluation in sheep. It would involve both assessment of the antibody response and the effect on ruminal methane production. There sheep can be sampled as live animals.

An indicative timeline for Stage 1 would be:

1. Call meeting as per Recommendation — March 2014
2. Establish target protein motifs — March 2014
3. Commission the concept development for a VLP-based vaccine — May 2014
4. Develop the project & sign the contract — August 2014
5. Initiate project — September 2014
   - Project development, production of VLP-antigen conjugate, assay development

[3] Seppic with their Montanide range are an excellent potential source of such adjuvants (http://www.seppic.com)

- Ethics approval

6. First (rat) experiment: February 2015
   - February to May 2015; rat experiment & ongoing assay development will be concurrent
   - Review meeting June 2015 (first *go/no go*)
7. Second (rat) experiment: August 2015
   - August to November 2015
   - Review meeting December 2015 (second *go/no go*)
8. First phase evaluation in sheep: March 2016
   - March to August 2016
   - Final Report of Stage 1 and Review meeting October 2016 (third *go/no go*)

5. Bibliography

1. Moss AR, Jouany JP, Newbold J (2000) Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49, 231–253
2. Hook SE, Wright ADG, McBride BW (2010) Methanogens: Methane Producers of the Rumen and Mitigation Strategies. Archaea Volume 2010, Article ID 945785, 11 p
3. Cottle DJ, Nolan JV, Wiedemann, SG (2011) Ruminant enteric methane mitigation: a review. Animal Production Science 51, 491–514
4. Shu Q, Gill HS, Hennessy DW, Leng RA, Bird SH, Rowe JB (1999) Immunisation against lactic acidosis in cattle. Research in Veterinary Science 67, 65–71
5. Gill HS, Shu Q, Leng RA (2000) Immunization with *Streptococcus bovis* protects against lactic acidosis in sheep. Vaccine 18, 2541-2548
6. Shu Q, Gill HS, Leng RA, Rowe JB (2000) Immunization with a *Streptococcus bovis* Vaccine Administered by Different Routes Against Lactic Acidosis in Sheep. The Veterinary Journal 159, 262–269
7. Shu Q, Bird SH, Gill HS, Duan E, Xu Y, Hillard MA, Rowe JB (2001) Antibody Response in Sheep Following Immunization with *Streptococcus bovis* in Different Adjuvants. Veterinary Research Communications 25, 43-54
8. Wright ADG, Klieve AV (2011) Does the complexity of the rumen microbial ecology preclude methane mitigation? Animal Feed Science and Technology 166-167, 248-253
9. Russell JB (2009) Rumen. Encyclopedia of Microbiology (3rd Edition), Oxford, 2009, 163–174
10. Eurell JA, Frappier BL (2013) Dellmann's Textbook of Veterinary Histology (6th Edition), 190-192
11. Butler JE, Kiddy CA, Pierce CS, Rock CA (1972) Quantitative Changes Associated with Calving in the Levels of Bovine Immunoglobulins in Selected Body Fluids. Can J Comp Med. 36(3), 234–242
12. McNeilly TN, McClure SJ, Huntley JF (2008) Mucosal immunity in sheep and implications for mucosal vaccine development. Small Ruminant Research 76, 83–91
13. Underdown BJ, Dorrington KJ (1974) Studies on the Structural and Conformational Basis for the Relative Resistance of Serum and Secretory Immunoglobulin A to Proteolysis. The Journal of Immunology 112(3), 949-959
14. Premier RR, Jacobs HJ, Lofthouse SA, Sedgmen BJ, Meeusen ENT (2003) Antibody isotype profiles in serum and circulating antibody-secreting cells following mucosal and peripheral immunisations of sheep. Veterinary Immunology and Immunopathology 98, 77–84
15. Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nature Medicine Supplement 11(4), S45-S53
16. Chen K, Cerutti A (2010) Vaccination Strategies to Promote Mucosal Antibody Responses. Immunity 33(4), 479–491
17. Johansen FE, Kaetzel C (2011) Regulation of the polymeric immunoglobulin receptor and IgA transport: New advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. Mucosal Immunol. 4(6), 598–602
18. Asano M, Komiyama K (2011) Polymeric Immunoglobulin Receptor. Journal of Oral Science 53(2), 147–156
19. Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nature reviews. Immunology, 6(2), 148 - 158
20. Czerkinsky C, Holmgren J (2010) Mucosal Delivery Routes for Optimal Immunization: Targeting Immunity to the Right Tissues. Current Topics in Microbiology and Immunology 354, 1–18

21. Rebelatto MC, Guimond P, Bowersock TL, HogenEsch H (2001) Induction of systemic and mucosal immune response in cattle by intranasal administration of pig serum albumin in alginate microparticles. Veterinary Immunology and Immunopathology 83, 93-105

22. Kavanagh OV, Adair BM, Welsh M, Earley B (2013) Immunogenetic responses in calves to intranasal delivery of bovine respiratory syncytial virus (BRSV) epitopes encapsulated in poly(DL-lactide-co-glycolide) microparticles. Research in Veterinary Science 95, 786–793

23. Kweon MN (2011) Sublingual mucosa: A new vaccination route for systemic and mucosal immunity. Cytokine 54, 1–5

24. Choi JH, Schafer SC, Zhang L, Kobinger GP, Juelich T, Freiberg AN, Croyle MA (2012) A Single Sublingual Dose of an Adenovirus-Based Vaccine Protects against Lethal Ebola Challenge in Mice and Guinea Pigs. Mol Pharm. 9(1), 156–167

25. Pavot V, Rochereau N, Genin C, Verrier B, Paul S (2012) New insights in mucosal vaccine development. Vaccine 30, 142-154

26. Eriksson K, Holmgren J (2002) Recent Advances in Mucosal Immunization Using Virus-like Particles. Mol. Pharmaceutics 10, 1596−1609

27. Fournillier A et al. (2013) A heterologous prime/boost vaccination-strategy enhances the immunogenicity of therapeutic vaccines for hepatitis C virus. Journal of Infectious Diseases, Advance Access published 17/06/13, http://jid.oxfordjournals.org

28. Holmgren J, Svennerholm AM (2012) Vaccines against mucosal infections. Current Opinion in Immunology 24, 343–353

29. Meeusen ENT (2011) Exploiting mucosal surfaces for the development of mucosal vaccines. Vaccine 29, 8506– 8511

30. Brandtzaeg P (2007) Do Salivary Antibodies Reliably Reflect both Mucosal and Systemic Immunity? Annals of the New York Academy of Sciences 1098, 288–311

31. Sakaguchi K, Yokota H, Miyasho H, Maeda N, Nakamura K, Onaga T, Koiwa M, Matsuda K, Okamoto M, Hirayama K, Taniyama H (2012) Polymeric immunoglobulin receptor expression and local immunoglobulin A production in bovine sublingual, submandibular and parotid salivary glands. The Veterinary Journal 197, 291–296

32. Huang CF, Wang CC, Wu TC, Wu KG, Lee CC, Peng HJ (2008) Neonatal Sublingual Vaccination with *Salmonella* Proteins and Adjuvant Cholera Toxin or CpG Oligodeoxynucleotides Induces Mucosal and Systemic Immunity in Mice. Journal of Pediatric Gastroenterology and Nutrition 46(3), 262–271

33. Treuting PM, Morton, Jr. TH (2012) Oral Cavity and Teeth. Comparative Anatomy and Histology: A Mouse and Human Atlas, 95-110

34. Chen HT, Liu YS (2013) Immunity of Foot-and-Mouth Disease Serotype Asia 1 by Sublingual Vaccination. PLoS ONE 8(5), e63839

35. Jabbal-Gill I (2010) Nasal vaccine innovation. Journal of Drug Targeting, 18(10), 771–786

36. Kunda NK, Somavarapu S, Gordon SB, Hutcheon GA, Saleem IY (2013) Nanocarriers Targeting Dendritic Cells for Pulmonary Vaccine Delivery. Pharm Res 30, 325–341

37. Roldão A, Silva AC, Mellado MCM, Alves PM, Carrondo MJT (2011) Viruses and Virus-Like Particles in Biotechnology: Fundamentals and Applications. Comprehensive Biotechnology (2nd Edition), 625-649

38. Vacher G, Kaeser MD, Moser C, Gurny R, Borchard G (2013) Recent Advances in Mucosal Immunization Using Virus-like Particles. Mol. Pharmaceutics 10, 1596−1609

39. Buonaguro FM, Buonaguro L, Tagliamonte M, Tornesello ML (2011) Developments in virus-like particle-based vaccines for infectious diseases and cancer. Expert Reviews of Vaccines, 10(11), 1569-1583

40. Peek LJ, Middaugh CR, Berkland C (2008) Nanotechnology in vaccine delivery. Advanced Drug Delivery Reviews 60, 915–928

41. Kushnir N, Streatfield SJ, Yusibov V (2012) Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development. Vaccine 31, 58– 83

42. Liu F, Ge S, Li L, Wua X, Liu Z, Wang Z (2011) Virus-like particles: potential veterinary vaccine immunogens. Research in Veterinary Science 93, 553–559

43. McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H (1992) The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 10 (2), 75-88

44. McKee SJ, Young VL, Clow F, Hayman CM, Baird MA, Hermans IF, Young SL, Ward VK (2012) Virus-like particles and α-galactosylceramide form a self-adjuvanting composite particle that elicits anti-tumor responses. Journal of Controlled Release 159, 338–345

45. Quan FS, Huang C, Compans RW, Kang SM (2007) Virus-Like Particle Vaccine Induces Protective Immunity against Homologous and Heterologous Strains of Influenza Virus. Journal Virol. 81 (7), 3514–3524

46. Peacey M, Wilson S, Baird MA, Ward VK (2007) Versatile RHDV Virus-Like Particles: Incorporation of Antigens by Genetic Modification and Chemical Conjugation. Biotechnology and Bioengineering 98 (5), 968-977

47. Win SJ, Ward VK, Dunbar PR, Young SL, Baird MA (2011) Cross-presentation of epitopes on virus-like particles via the MHC I receptor recycling pathway. Immunology and Cell Biology 89, 681–688

48. Schwarz K, Meijerink E, Speiser DE, Tissot AC, Cielens I, Renhof R, Dishlers A, Pumpens P, Bachmann MF (2005) Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles. Eur. J. Immunol. 35, 816–821

49. Jemon K, Young V, Wilson M, McKee S, Ward V, et al. (2013) An Enhanced Heterologous Virus-Like Particle for Human Papillomavirus Type 16 Tumour Immunotherapy. PLoS ONE 8(6): e66866. doi:10.1371/journal.pone.0066866

50. Janssen PH, Kirs M (2008) Structure of the Archaeal Community of the Rumen. Applied and Environmental Microbiology 74 (12), 3619-3625

51. Wright ADG, Kennedy P, O'Neill CJ, Toovey AF, Popovski S, Rea SM, Pimm CL, Klein L (2004) Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine 22, 3976–3985

52. Wedlock DN, Pedersen G, Denis M, Dey D, Janssen PH, Buddle BM (2011) Development of a vaccine to mitigate greenhouse gas emissions in agriculture: Vaccination of sheep with methanogen fractions induces antibodies that block methane production in vitro. New Zealand Veterinary Journal 58 (1), 29-36

53. Williams YJ, Popovski S, Rea SM, Skillman LC, Toovey AF, Northwood KS, Wright ADG (2009) A Vaccine against Rumen Methanogens Can Alter the Composition of Archaeal Populations. Applied and Environmental Microbiology 75, 1860–1866

54. Wedlock DN, Janssen PH, Leahy SC, Shu D, Buddle BM (2013) Progress in the development of vaccines against rumen methanogens. Animal 7 (2), 244–252

55. Leahy SC, Kelly WJ, Altermann E, Ronimus RS, Yeoman CJ, et al. (2010) The Genome Sequence of the Rumen Methanogen Methanobrevibacter ruminantium Reveals New Possibilities for Controlling Ruminant Methane Emissions. PLoS ONE 5(1): e8926. doi:10.1371/journal.pone.0008926

56. Buddle BM, Denis M, Attwood GT, Altermann E, Janssen PH, Ronimus RS, Pinares-Patiño CS, Muetzel S, Wedlock DN (2011) Strategies to reduce methane emissions from farmed ruminants grazing on pasture. The Veterinary Journal 188, 11–17

57. Kandler O (1994) Cell Wall Biology in Archaea and its Phylogenetic Implications. Journal of Biological Physics 20, 165-16

58. Rohlin L, Leon DR, Kim U, Loo JA, Ogorzalek Loo RR, Gunsalus RP (2012) Identification of the Major Expressed S-Layer and Cell Surface-Layer-Related Proteins in the Model

Methanogenic Archaea: Methanosarcina barkeri Fusaro and Methanosarcina acetivorans C2A. Archaea Volume 2012, Article ID 873589, 10p

59. Tacket CO, Sztein MB, Losonsky GA, Wasserman SS, Estes MK (2003) Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers. Clinical Immunology 108, 241–247

60. Accili D, Menghia G, Bonacucina G, Di Martino P, Palmieri GF (2004) Mucoadhesion dependence of pharmaceutical polymers on mucosa characteristics. European Journal of Pharmaceutical Sciences 22, 225–234

61. Tiwari D, Sause R, Madan PL (1999) Evaluation of Polyoxyethylene Homopolymers for Buccal Bioadhesive Drug Delivery Device Formulations. AAPS Pharmsci 1 (3) 13

62. Schuette V, Burgdorf S (2014) Current Opinion in Immunology 26, 63–68