



DIVA TECHNOLOGY: INDISPENSABLE TOOL FOR THE CONTROL OF JOHNE'S DISEASE

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ABSTRACT

Ruminant Paratuberculosis (Johne's disease) is categorized as List B disease by OIE. Paratuberculosis is a disease of socio-economic and public health importance and has significant effect on in the international trade of animals and animal products. Control of paratuberculosis is priority in many countries and different countries have designed their own control programs tailored to their farming practices and geographical conditions. However, the major component shared by these control programs is "Test and Cull" policy. Due to inability of detecting paratuberculosis in early stages this policy has globally failed to control the disease and hence there is global urgency in developing control measures. Vaccination has shown promise in controlling this disease. However, vaccination in present form cannot be used due to lack of DIVA (Differentiation of Infected from Vaccinated Animals) technology, because present vaccines interfere with diagnosis of naturally infected paratuberculosis animals and animals infected with tuberculosis. Therefore markers are needed to be identified for developing DIVA. This paper summarizes the findings of vaccination trials conducted in different countries and highlights the importance of vaccination in controlling paratuberculosis and also discusses strategies for developing DIVA for paratuberculosis vaccines.

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1 Introduction

Johne's disease (JD) or Paratuberculosis has emerged as wide-spread, highly prevalent and economically devastating infectious disease of domestic livestock around the world (Singh et al., 2014). JD is characterized by persistent diarrhea with progressive loss of weight. Chronic diarrhoea results in protein losing enteropathy. Disease is caused by an extremely fastidious microbe known as *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is highly resistant to environmental stress like temperature, drying and is able to persist for years in farm soil (Singh et al., 2013). In India disease has been widely reported from all the domestic ruminant species (Singh et al., 2014). In India MAP has also been reported from wild ruminants such as blue bulls, hog deer, bison (Singh et al., 2010a; Singh et al., 2011a) as well as other animals like rabbit and primates (Singh et al., 2011b; Singh et al., 2012).

Paratuberculosis is a spectral disease where it takes years for clinical signs to appear in animals. In very early stages (silent stage) typically there are no signs of disease and none of the available tests can detect infected animals at this stage, this stage progresses to sub-clinical disease, where shedding of MAP in feces can be occasionally seen without any signs of the disease (Tiwari et al., 2006). This stage often then progresses to clinical infection. At this stage animal have intermittent diarrhea and progressive weight loss without reduction of appetite. Sporadic signs at this stage generally give way to more severe infection. These animals will give positive results with fecal culture tests, because of host shedding massive numbers of organism. Animals in this stage are also positive on serological assays. Clinical signs continue for months, which may usually results in death.

Paratuberculosis is increasingly being recognized as significant problem affecting animal health, farming and the food industry due to the high prevalence of the disease across the world. Paratuberculosis can cause significant economic loss in affected herds, as a result of reduced milk yield, poor milk quality, poor feed conversion, increased susceptibility to disease in general, reduced reproductive efficiency, premature culling and reduced slaughter values. It is estimated that 68.0% of US dairy herds are infected with JD, costing between \$200 million to \$1.5 billion per year to dairy industry (Sohal et al., 2015). A study from India by Vinodhkumar et al. (2013) estimated loss of Rs 1,840 (US\$ 38.33) per infected sheep/year. Another study from India in a Holstein Frisian (H/F) dairy farm estimated loss of Rs 1,63,800.0 (US\$ 2465) in 180 days due to JD (Rawat et al., 2014). Besides costly to animal husbandry, MAP is gaining interest as a zoonotic and food-borne pathogen. Evidences suggest the involvement of MAP in human diseases like Crohn's disease and type I diabetes (Sohal et al., 2015). MAP is not killed by pasteurization and milk has been considered as main source of infection transmission to humans. MAP has frequently been

isolated from pasteurized milk and milk products (Shankar et al., 2010). Rising concern of the MAP zoonosis has generated lot of awareness among veterinarians and medicos seeking to control this disease in animals. Therefore paratuberculosis needs immediate attention for control in animals. This paper discusses the role of vaccination and DIVA (Differentiation of Vaccinated & Infected Animals) technology in efforts to control Johne's disease.

2 Vaccination as tool of Paratuberculosis Control

Due to predominant subclinical nature of disease and prolonged course of infection, early diagnosis is not possible therefore control of paratuberculosis is problematic task. The most widely practiced control strategy for paratuberculosis is test and cull policy. However, due to lack of tools to detect disease in early stages; test and cull policy is not sufficient in preventing spread of MAP (Bastida & Juste, 2011). Despite test and cull policies in place disease burden has continued to increase (Singh et al., 2014). Positivity (number of positive animals vs number of tested animals) of ruminant species in India for paratuberculosis (cattle, goat, sheep and buffalo) increased from 11.6% to 23.3% over the period of 28 years (1985-2013) (Singh et al., 2014). Therefore alternate strategies are required if control of paratuberculosis is to be achieved.

Vaccination programs in past have been successfully deployed. As a result, there is increased interest in use of vaccination against paratuberculosis. Vaccination is a cost-effective strategy for paratuberculosis containment (Singh et al., 2007; Juste & Perez, 2011; Bush et al., 2008; Dhand et al., 2013). Vaccination reduces morbidity & mortality due to JD, reduces shedding of MAP in feces, improves clinical signs (reduces diarrhea & increases body weight), cures intestinal lesions and enhances flock immunity to JD (Singh et al., 2007; Singh et al., 2010b; Singh et al., 2013). Studies have confirmed that vaccination not only reduces the prevalence of JD but also has economic benefits to farmers (Groenendaal et al., 2015). Vaccination also provides revival against MAP infection i.e. therapeutic effects observed in already infected animals (Singh et al., 2010b).

Benefits of vaccination have been summarized in Table 1. Therefore vaccination is being considered an economically attractive tool for controlling Johne's disease (Sohal et al., 2015). Examples are there from countries like Iceland and Australia where compulsory vaccination programs brought about sufficient reductions in prevalence of paratuberculosis (Sohal et al., 2015). Another indirect benefit of paratuberculosis vaccination is that there is some degree of cross protection for tuberculosis (de Val et al., 2012). Both killed and live attenuated vaccines have the same efficiency in controlling paratuberculosis (Singh et al., 2007) however, killed vaccines have longer shelf life and are safer.

Table 1 Beneficial effects of vaccination against MAP

S. No.	Name/ kind of vaccine	Country	Species	Major Observation	Reference	
1.	Laboratory Scale (Live)	USA	Cattle	Vaccination reduces fecal shedding of MAP	Larsen et al., 1974	
2.	Fromm (Killed)	USA	Cattle		Hurley & Ewing 1983	
3.	Laboratory Scale (Live)	Denmark	Cattle		Jorgensen, 1983	
4.	Laboratory Scale (Live)	France	Cattle		Argente, 1992	
5.	Phylaxia (Killed)	Hungary	Cattle		Kormendy, 1994	
6.	Neoparasec (Live)	Germany	Cattle		Klawonn et al., 2002	
7.	Lio-Johne (Live)	Spain	Sheep		Aduriz, 1993	
8.	Laboratory Scale (Live)	Greece	Sheep		Dimareli-Malli et al., 2013	
9.	Live Vaccine	New Zealand	Sheep		Gwodz et al., 2000	
10.	Killed vaccine	-	Goat		Kalis et al., 2001	
11.	Weybridge (Live)	UK	Cattle	Vaccination improves production	Wilesmith, 1982	
12.	Lelystad (Killed)	Netherlands	Cattle		Kalis et al., 1992	
13.	Lio-Johne (Live)	Spain	Sheep		Aduriz, 1993	
14.	Gudair (Killed)	Australia	Sheep		Windsor et al., 2003	
15.	Neoparasec (Live)	New Zealand	Sheep		Gwozd et al., 2000	
16.	Laboratory Scale (Killed)	Netherlands	Cattle		Histological improvement after vaccination	van Schaik et al., 1996
17.	Silirum (Killed)	Spain	Cattle	Garcia-Pariente et al., 2005		
18.	Laboratory Scale (Killed)	Iceland	Sheep	Sigurdsson, 1960		
19.	Lio-Johne (Live)	Spain	Sheep	Aduriz, 1993		
20.	Mycopar (Killed)	USA	Sheep	Thonney & Smith, 2005		
21.	Laboratory Scale (Live)	Norway	Goat	Saxegaard & Fodstad, 1985		
22.	Laboratory Scale (Killed)	USA	Goat	Kathaperumal et al., 2009		
23.	Laboratory Scale (Killed)	India	Goat	Histological improvements, reduction fecal shedding, improves production and therapeutic effects		Singh et al., 2007; Singh et al., 2010b
24.	Gudair (killed)	Australia, New Zealand and Spain	Goat and Sheep			Griffin et al., 2009; Reddacliff et al., 2006; Eppleston et al., 2004; Corpa et al., 2000
25.	Killed Vaccine	Iceland	Sheep	Reduction in mortality, improves clinical signs and reduction in fecal shedding of MAP		Sigurdsson & Gunnarson, 1983
26.	Live Vaccine	Cyprus	Sheep		Crowther et al., 1976	

3 Issues of Paratuberculosis Vaccination

Vaccination efforts against paratuberculosis have succeeded numerous times (Table 1); however, there are several issues in implementing vaccination programs. First, vaccination against paratuberculosis may interfere with routine diagnosis of paratuberculosis. ELISA is the most widely used test for diagnosing paratuberculosis because of low cost, rapid turnaround time and high sensitivity. Other tests are costly, time consuming, have poor sensitivity and requires sophisticated facilities so are of limited utility when incorporated into paratuberculosis control programs. However, presently available ELISAs cannot discriminate vaccinated individuals from naturally infected individuals. ELISA results can be a problematic in certifying herds for disease (paratuberculosis) free status where a compulsory vaccination has either lowered the prevalence or eradicated the disease from herd due to the fact that these tests can't discriminate between pathogen and vaccine-induced immunological responses. Since positive ELISA diagnostic test results for MAP are often sufficient in triggering herd cull responses, false positive results can be economically disastrous for cattle farms. Secondly, vaccination against paratuberculosis will interfere with diagnosis with tuberculosis and there are evidences on this (Juste & Perez, 2011).

MAP and *M. bovis* share antigenic structures; therefore immune responses generated by vaccination against these two can interfere with diagnosis of paratuberculosis as well as tuberculosis. Hence, implementing vaccination against MAP will not only affect the diagnosis of paratuberculosis but will also affect the tuberculosis control programs. Considering these issues most of the countries are hesitant to vaccinate against MAP. This problem is not just restricted to paratuberculosis, there are number of animal diseases where vaccines are available but cannot be used. One example is FMD; vaccines are available and are quite effective in controlling clinical disease but are not used in disease free countries, as it interferes with diagnostic test results. Positive immunological test results could ruin the disease free status even of vaccinated healthy livestock populations, which, in turn, can lead to serious economic losses in a region's agronomy (Meeusen et al., 2007).

4 DIVA Technology

The primary goal of vaccination is to help in elimination of disease. However, vaccinations elicit immune responses similar to those found infected animals, thereby rendering traditional diagnostic screening protocols useless as a means of determining true herd disease status. Therefore it is essential to differentiate immune responses due to vaccination compared to natural infection. The term Differentiation of Infected from Vaccinated Animals (DIVA) was coined in 1999 by Jan T. van Oirschot (van Oirschot, 1999). It is now generally used in place of older term 'marker vaccines'. The DIVA principle has

now also been extended to include killed whole organism vaccines (Pasick, 2004). The general DIVA principle is that antibody response produced by vaccination can be differentiated from the antibody response elicited by natural infection.

DIVA tests work by detection of immune response against specific antigens which are present in the infectious agent but in the absence of vaccine. Successful DIVA technologies has been developed for animal vaccines like bovine rhinotracheitis (IBR), pseudorabies, classical swine fever (CSF) etc (Meeusen et al., 2007). Strategies for developing DIVA based vaccines for other diseases like bovine tuberculosis (Vordermeier et al., 2001), avian influenza (Rahn et al., 2015), PPR (Liu et al., 2014) and bluetongue virus (Calvo-Pinilla et al., 2014) are also under development. Though there has been great demand to develop DIVA strategies for paratuberculosis, so far no progress has been made and to our knowledge there is no laboratory working on it. Till date, vaccination is the only practical method for controlling paratuberculosis.

Since popular commercially available paratuberculosis vaccines are whole cell killed preparations, simple strategies can be designed to develop DIVA technology to differentiate infected and vaccinated animals. Killed whole cell vaccines will generate immune response only against cellular antigens i.e. vaccinated animals will only have antibodies against cellular antigens. However, in naturally infected animals will have antibodies against both cellular and secreted (culture filtrate antigens) antigens. Immune response against secreted antigens can be selectively used to differentiate vaccinated and naturally infected animals (Fig. 1). Therefore secreted antigens of MAP can serve as markers of differentiation between vaccinated and naturally infected animals and can be used to develop DIVA. There have been reports that secreted antigens are released early during the infection process and elicit antibody responses (Ahmad, 2010), hence these can be used as markers for early sero-diagnosis of paratuberculosis in early or subclinical stages (Facciuolo et al., 2013). Presently available commercial ELISAs contain a crude antigen mixture termed PPA, which is prepared by thorough physical disruption of mycobacterial bacilli followed by removal of cell debris.

The low sensitivity of available conventional ELISA tests can be attributed to the lack of secreted antigens. Hence, a simple ELISA based test can be developed using secreted antigens to diagnose paratuberculosis as well as the same secreted antigens based ELISA can be used to differentiate vaccinated and infected animals if used in conjunction with conventional ELISA protocols (Table 2). If MAP specific secreted antigens are incorporated in this system then same ELISA regimen can be used for diagnosis, DIVA marker detection and for differentiating paratuberculosis & tuberculosis infection. Table 3 summarizes the MAP secreted proteins that can be used to develop DIVA for killed whole cell vaccines and for differentiating paratuberculosis and tuberculosis.

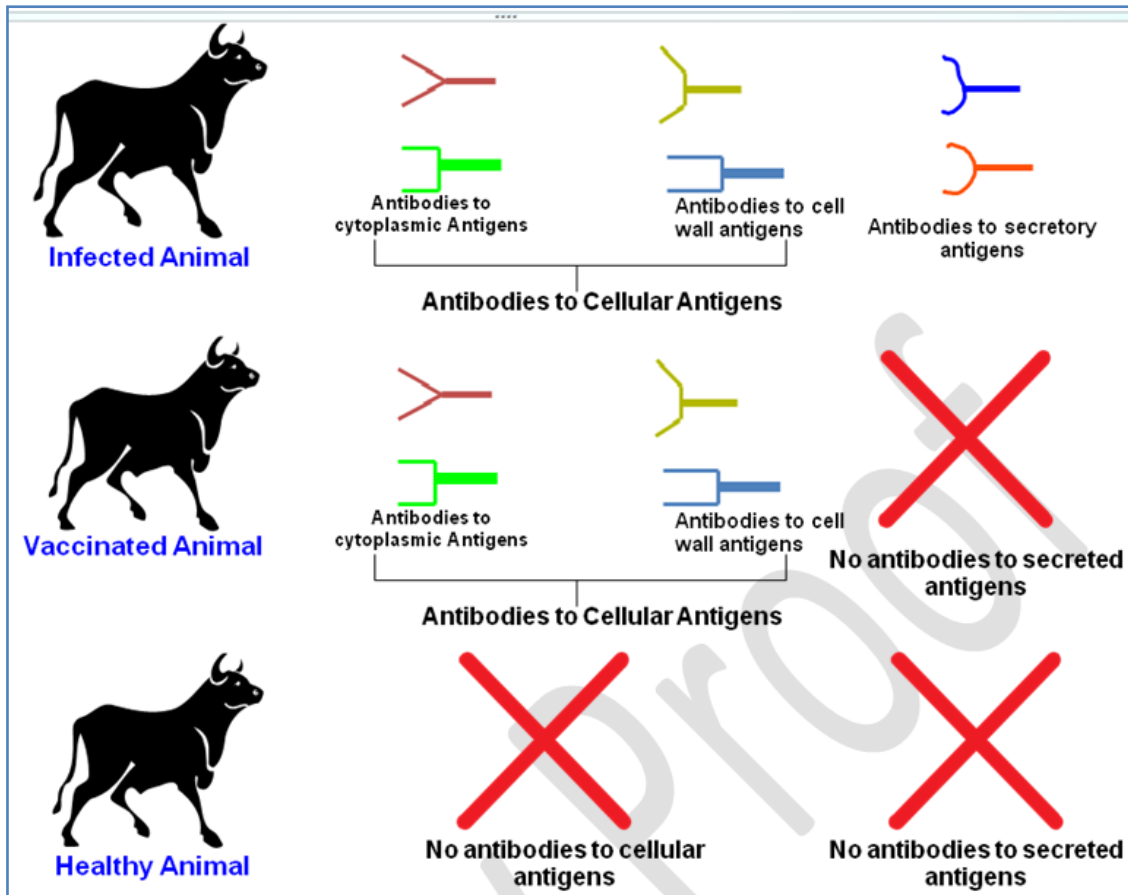


Figure 1 Scheme of the immune response that can be used to differentiate vaccinated and naturally infected animals.

Table 2 Scheme of the differentiation of healthy, infected and vaccinated animals

Kind of Animal	Status of Animal in Conventional ELSIA	Status of Animal in Secreted Antigens based ELISA
Healthy	Negative	Negative
Naturally Infected	Positive	Positive
Vaccinated	Positive	Negative

Concluding Remarks

Paratuberculosis is a devastating disease, negatively affecting the livestock agronomy throughout the world, its presence triggers trade restrictions and raises serious public health concerns. Therefore control of paratuberculosis is of the utmost urgency. Most extensively accepted “Test and Cull” policy is very costly to farmers and governments; moreover it is not particularly effective in stemming the physical spread of MAP

from one region to the next. Through the scientific, technical and farming experiences it is becoming clear that vaccination is the only practical solution for controlling this disease. However, in the absence of DIVA technology, vaccination programs cannot be implemented at national scale, as vaccinations often elicit immune responses indistinguishable from those generated by pathogens (using standard test regimens).

Table 2 List of secreted MAP proteins that can be used to develop DIVA (in killed whole cell vaccine system) and for differentiating paratuberculosis and tuberculosis.

S. No.	Secreted Protein	Function	Remark
1.	MAP 2609	-	Tested for antigenicity by Willemsem et al. (2006)
2.	MAP 2942c	-	Tested for antigenicity by Willemsem et al. (2006)
3.	MAP 0210c	-	Tested for antigenicity by Willemsem et al. (2006); Mon et al. (2012)
4.	MAP 0209	-	Tested for antigenicity by Mon et al. (2012)
5.	MAP 0187c	-	Tested for antigenicity by Mon et al. (2012)
6.	MAP 1272	Putative invasin, NlpC/P60 superfamily	Tested for antigenicity by Mon et al. (2012)
7.	MAP 1569/ ModD	-	Tested for antigenicity by Souza et al. (2011)
8.	MAP 0471	-	Tested for antigenicity by Facciuolo et al. (2013)
9.	MAP 1981c	-	Tested for antigenicity by Facciuolo et al. (2013)
10.	MAP 0196c	-	Tested for antigenicity by Facciuolo et al. (2013); Mon et al. (2012)
11.	MAP 1693c	Peptidyl-prolyl cis-trans isomerase	Tested for antigenicity by Mon et al. (2012); Roupie et al. (2012)
12.	MAP 0853	-	Tested for antigenicity by Bannantine et al. (2008)
13.	MAP 4308c	-	Tested for immunogenicity by Roupie et al. (2008)
14.	CobT	Phosphoribosyl transferase	Tested for immunogenicity by Byun et al. (2012)
15.	MAP 2168c	-	Tested for antigenicity by Cho et al. (2007)
16.	MAP 1022c	-	Cho et al. (2006)
17.	Antigen 85C	Mycolyl transferase	Tested for antigenicity by Shin et al. (2005)
18.	PepA (N-terminal)	Serine proteinase	Tested for antigenicity by Cho et al. (2007)
19.	PepA (C-terminal)	Serine proteinase	Tested for antigenicity by Cho et al. (2007)
20.	MAP 3273c	-	Gurung et al. (2014)
21.	AhpC	Alkyl hydroperoxide reductase C	Tested for antigenicity by Olsen et al. (2001)
22.	AhpD	Alkyl hydroperoxide reductase D	Tested for antigenicity by Olsen et al. (2001)
23.	MAP 3680c	-	Tested for immunogenicity by Carlos et al. (2015)
24.	Superoxide dismutase (Sod)	-	Tested for antigenicity by Shin et al. (2005)
25.	MAP 0586c	Possible transglycosylase SLT domain	Tested for immunogenicity by Roupie et al. (2008)
26.	MAP 2677c	Glyoxylase	Tested for antigenicity by Roupie et al. (2012)
27.	MAP 3199	-	Tested for antigenicity by Leroy et al. (2007)
28.	MAP 1272c	Putative invasin, NlpC/P60 superfamily	Tested for antigenicity by Mon et al. (2012)
29.	MAP 2942c	-	Tested for antigenicity by Gumber et al. (2009)
30.	GreA	Transcription elongation factor GreA	Tested for antigenicity by Mon et al. (2012)
31.	MAP 0593c	-	Tested for antigenicity by Gumber et al. (2009)
32.	MAP 2411	-	Tested for antigenicity by Kawaji et al. (2012)
33.	MAP2168c	-	Tested for antigenicity by Cho et al. (2007)
34.	Ppa	Inorganic pyrophosphatase	Tested for antigenicity by Gumber et al. (2009)
35.	ClpP	ATP-dependent Clp protease proteolytic subunit	Tested for antigenicity by Gumber et al. (2009)
36.	Ag85A	-	Tested for antigenicity by Rosseels et al. (2006)
37.	Ag85B	-	Tested for antigenicity by Rosseels et al. (2006)

This, in turn, greatly impairs determination of livestock herd infectious disease status- critical for the entire livestock agronomy. Since killed vaccines have good protective efficacy against paratuberculosis, the strategy proposed in the paper can be used to develop DIVA ELISA using the above comprehensive list of antigens. A careful selection and screening of secretory antigens is performed, we can develop an ELISA based test that can be used as routine diagnostic tool, as a DIVA tool and one that will be able to differentiate paratuberculosis and tuberculosis. We suggest that the development and validation of such a test be carried out on global scale, with many laboratories working in conjunction with one another, so that an effective strategy can be developed for combating the worldwide spread of Johne's disease and animal tuberculosis.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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