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**Review Article**
Derleme

# DIVA (Differentiating Infected from Vaccinated Animals) vaccines and strategies

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**Abstract:** In veterinary medicine, vaccination is one of the most effective techniques for control and prevention of diseases. When other methods fail in control of animal diseases, different vaccines and strategies are developed**.** DIVA (Differentiating Infected from Vaccinated Animals) vaccines lacking one or more antigenic epitope(s)/ protein(s) in the prevailing field strain and the accompanying diagnostic tests are effective in eradication and control of diseases. While currently used vaccines can not distinguish between vaccinated and infected animals, DIVA vaccines can be very useful for the purpose. Serological tests, such as ELISA (Enzyme-Linked ImmunoSorbent Assay), used together with DIVA vaccines, can be used to determine which animals are infected and which are vaccinated. DIVA vaccines and strategies for some animal diseases have been developed and continue to be developed.

In this review, it is aimed to explain the general characteristics, importance, production, advantages of DIVA vaccines and strategies and their use in various animal diseases.

**Keywords:** DIVA, marker vaccines, vaccine, veterinary medicine.

## DIVA (Differentiating Infected from Vaccinated Animals) aşı ve stratejileri

**Özet:** Veteriner hekimlik alanında aşılama, hastalıkların önlenmesi ve kontrolünde en etkili yöntemlerden birisidir. Hayvan hastalıklarının kontrolünde mevcut yöntemler başarısız olduğunda, farklı aşı ve stratejiler geliştirilmektedir. Saha suşunda yer alan bir veya daha fazla antijenik epitop veya proteinden yoksun olan DIVA (Differentiating Infected from Vaccinated Animals) aşıları ve beraberinde kullanılan tanı testleri, hastalıkların eradikasyonu ve kontrolünde oldukça etkilidir. Mevcut aşılar, aşılı ve enfekte hayvanların ayrımını sağlayamazken, DIVA aşıları bu alanda önemli yararlar sağlar. Bunun yanında ELISA (Enzyme-Linked ImmunoSorbent Assay) gibi DIVA aşılarıyla birlikte kullanılan serolojik testler, hangi hayvanların enfekte ve hangilerinin aşılı olduğunun ayrımı için kullanılabilir. Bazı hayvan hastalıklarına yönelik DIVA aşı ve stratejileri geliştirilmiş ve diğerleri için de geliştirilmeye devam edilmektedir.

Bu derlemede, DIVA aşı ve stratejilerinin genel özelliklerini, önemini, üretimini, avantajlarını ve bunların çeşitli hayvan hastalıklarında kullanımını açıklamak amaçlanmıştır.

**Anahtar kelimeler:** aşı, DIVA, marker aşıları, veteriner hekimlik.

## Introduction

Vaccines are biological substances prepared either by using the agents or their antigenic molecules in order to protect living organisms against pathogenic microorganisms (Arda 2011). Edward Jenner initially used the "vaccine" term from the "vacca" Latin for cow and "vaccinia" Latin for cowpox to explain the protection of humans against smallpox virus. In order to provide this protection, people were inoculated with cowpox vaccine (Meeusen et al. 2007). Vaccination is one of the most important medical developments to date and many researchers played a significant role in the development of vaccines (Sareyyüpoğlu and İzgür 1999; Morgan and Parker 2007). Vaccines can be used both to control, prevent, eliminate and eradicate diseases at the population and the weakening of clinical signs in infection (Meeusen et al. 2007).

Exposure to a live pathogen followed by recovery is a way of immunization (Owen et al. 2013). The aim of the vaccination strategy is to generate a naturally acquired immunity by inoculation of a particular pathogen, or its immunojenic but non-pathogenic components (Meeusen et al. 2007). These components stimulate antigen-specific lymphocytes and allow the formation of memory cells. Vaccination is an event, whereas immunization is a potential consequence of this event (Owen et al. 2013).

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Vaccination is widely used in veterinary medicine as the cost-effective intervention tool for the control and prevention of diseases. It also plays a vital role in global eradication programs (Baron et al. 2018). The primary purpose of vaccines in veterinary medicine is to improve the welfare and health condition of domestic animals and to prevent disease spread from both domestic and wild animals to humans. These different aims have led to various veterinary vaccine approaches (Meeusen et al. 2007).

Due to antibodies formed in serum after vaccination, it is difficult to distinguish between antibody titers resulting from conventional vaccination and exposure to the actual agent. To make this distinction possible, marker vaccines and/or DIVA (Differentiating Infected from Vaccinated Animals) vaccines have been developed (Day and Schultz 2014).

## General Information About DIVA Vaccines

The DIVA principle is based on the fact that antibody response against the causative agent antigens involved in DIVA vaccines is different from the antibody response to the actual microorganism in the field (Uttenthal et al. 2010). While DIVA vaccines do not contain one or more immunological protein or antigen, very same substances are naturally present in the field strain (Guo et al. 2017). Moreover, these vaccines provides an oppurtunity for serological differentiation between the vaccinated and the infected individuals. A marker / DIVA vaccines are used with accompanying tests. These tests identify antibodies against a protein not found in the vaccine strain (Van Oirschot et al. 1996). These vaccines can be deletion mutants of field pathogens or subunit / peptide vaccines (Francis 2017). Development of the DIVA vaccines also requires the development of an accompanying diagnostic test (Day and Schultz 2014). ELISA (Enzyme-Linked ImmunoSorbent Assay) is generally used to test the efficiencies of a vaccination (Akan et al. 2006; Arda 2011; Dhama et al. 2016). As a result, these serologic tests can detect antibody response to specific protein(s) after infection (Van Oirschot et al. 1996). Current vaccines often do not provide differentiating between vaccinated and infected animals, while DIVA vaccines differentiate those vaccinated from the infected ones (Selke et al. 2007; Holzer et al. 2016).

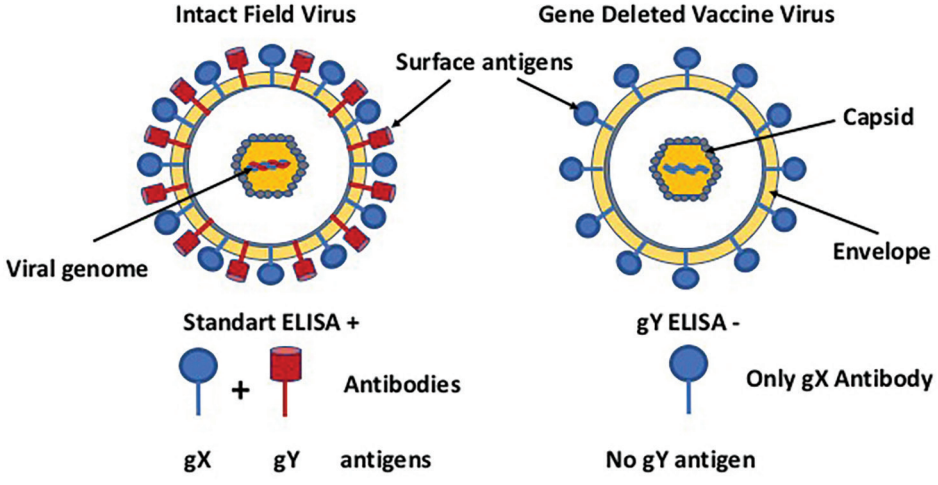


**Figure 1.** Use of ELISA in DIVA vaccines.

When creating DIVA vaccines, this is significant to detect which points are essential for the eradication or control of certain diseases. Each vaccine technology has benefits that can be used to develop the vaccine with desired properties; Thus, vaccines and accompanying diagnostic tests will have a significant effect on the eradication or control. As marker vaccines; by the help of the conventional technologies, subunit vaccines, DNA vaccines, live vector vaccines and deletion mutants have been developed. For the development of diagnostic tests; conventional methods (ELISA, neutralization tests, etc.), western blot technology, nucleic acid (PCR, real-time PCR) and biosensor technologies have been developed (Henderson 2005; Dhama et al. 2016).

The ideal marker (DIVA) vaccine should include: no long-term or short term side effects, provide genetic stability in the targeted species, should be durable, should be easy to produce and the cost of production should be low, act early and provide lifelong immunity, should be easily detectable in the body, should be effective against all virus vari-

ants, the antigenic components they carry should have good immunogenicity, should protect the host from horizantal and vertical transmission, should not have cross reactions, should permit subsequent vaccinations, should have a specific DIVA differential test (Arda 2011; Blome et al. 2017b). DIVA vaccines and accompanying diagnostic tests must meet established licensing necessities for efficacy, safety and purity, and the sensitivity and spesifity of the diagnostic test should be high (Henderson 2005).

The various advantages of these vaccines are: they can differentiate vaccinated animals from infected ones, they do not reproduce, spread in/out of the body, and free of complete microorganisms and their nucleic acids (except mutant strains), they are easily tolerated, prevalence and incidence of infection in vaccinated populations can be studied under the field conditions, their efficacies can be measured well. Thus they can be applied in vaccination eradication programs (Van Oirschot et al. 1996; Arda 2011).

**Development of DIVA Vaccines**

Despite the regulations on imports of animal and animal products, the risk of emergence of animal diseases still continues in some countries. Particularly in areas with crowded animal populations, eradication efforts based on quarantine measures may be insufficient in preventing diseases. These conditions, combined with increasing economic and ethical concerns, have provoked the development of different vaccines and strategies to control outbreaks (Pasick 2004).

Vaccine efficacy in a population is a function of the ability of the vaccine to decrease the virus contamination. If the antibody response to the vaccine antigens is indistinguishable from the infection, it may complicate serological follow-up studies and subsequent eradication strategies. Thanks to the DIVA vaccines and the accompanying diagnostic tests, this disadvantage can be easily overcomed (Pasick 2004).

The first licensed DIVA vaccine was produced to identify wild type pseudorabies virus with the ELISA kit that blocks protein A (Liu et al. 2013). The term "DIVA" was initially offered by J. T. van Oirschot in the Netherlands in 1999. This term is used as an abbreviation for 'Differentiation of Infected from Vaccinated Animals' (Pasick 2004). DIVA vaccines are originally deletion mutants of wild microorganism strains (Uttenthal et al. 2010). Diagnostic tests should be used with these vaccines to identify antibodies formed against epitopes (Ganguly et al. 2015). DIVA vaccines are generally based upon the lack of minimum one immunogenic protein in the vaccine strain. In the DIVA strategy, after vaccination with DIVA vaccines, diagnostic tests are used to determine antibodies against antigens that are not in the vaccine strain (Liu et al. 2013). This system makes it possible to mass vaccinate as well as to allow serological follow-up of a sensitive animal population for effective disease control (Pasick 2004).

**Use of DIVA Strategy Against Some of the Significant Diseases in Veterinary Medicine**

DIVA strategies are being developed for the control of significant diseases such as bovine rhinotracheitis, avian influenza, pseudorabies, bovine tuberculosis, classical swine fever and PPR in veterinary medicine (Dhama et al. 2016; Singh 2021).

**Infectious Bovine Rhinotracheitis (IBR)**

DIVA vaccines containing the glycoprotein E (gE) deleted strain of BoHV-1 (Bovine Herpesvirus) the agent of IBR, has been reported to be an effective and safe strategy to IBR, which can be effectively used in countries with high prevalence of diseases (Muratore et al. 2017). Accordingly, the IBR marker vaccine contains a genetically modified virus that does not produce glycoprotein E. A suitable serological test (ELISA) is developed to be used with the vaccine. A vaccinated cow with serum antibody against glycoprotein E is interpreted as being exposed to the field virus (Arda and Sareyyüpoğlu 2004; Day and Schultz 2014; Petrini et al. 2020).

**Avian Influenza (AI)**

One deficiency of inactivated vaccines is the difficulty of controlling avian influenza virus (the agent of avian influenza) because it cannot distinguish between vaccinated and naturally infected poultry by serologically widely used methods. Until recently, some DIVA strategies have been developed for avian influenza (AI) (Suarez 2005). Current research results show that the DIVA control strategy can be a tool for the control of AI infections (Capua et al. 2003; Sun et al. 2021). These strategies include observation, subunit vaccine use (have included just hemagglutinin), non-structural protein 1 (NS1) strategies (this protein is produced in infected cells, so naturally infected poultry can develop antibodies to the NS1), and heterologous neuraminidase strategy (Suarez 2005). The heterologous neuraminidase strategy is based upon the use of an inactivated oil emulsion vaccine including the same hemaggluti-

nin (HA) subtype but different neuraminidase (NA) subtype. (Capua et al. 2003). Vaccinated poultry will be protected on the basis of HA antibodies, but if the poultry are infected with the virus that causes the outbreak, different NA antibodies will be produced which can be separated from the NA antibodies produced after the use of the vaccine (Lee et al. 2004). For example, if the wild strain is H7N2, the animals can be vaccinated with H7N3 or the remaining seven appropriate combinations. Serological monitoring against N3 protein confirms that the flock is vaccinated, whereas, serological monitoring against N2 protein can be observed with wild type strain. That is, it can be determined that the animals are vaccinated or infected. Commercially, this strategy was first implemented in 2010, when the H7N1 outbreak occurred in Italy, by vaccinating with the H7N3 vaccine (Suarez 2005).

**Pseudorabies (Aujeszky's Disease)**

The DIVA strategy for control of pseudorabies has been used successfully in many countries (Freuling et al. 2017). With the deletion of the gE gene, it is aimed to enable the DIVA approach for the control of this disease (Meeusen et al. 2007). Pseudorabies are the main examples of DIVA in combination with ELISA (Freuling et al. 2017). Thanks to the ELISA, it can distinguish between animals producing antibodies against gE after wild type virus infection and animals those do not have antibodies against gE after vaccination, though they can carry antibodies against other PrV (Pseudorabies virus) glycoproteins (Freuling et al. 2017; Mettenleiter 2020). In prV eradication, gB / gE ELISA showed high specificity and sensitivity; could determine antibody from serum, blood and colostrum. Recently, high-precision techniques have been developed to differentiate vaccine virus genetic material from the wild strain genome (Freuling et al. 2017).

**Classical Swine Fever**

Classical swine fever virus (CSFV) is an RNA virus from Pestivirus genus (Dong and Chen 2007; Blome et al. 2017a). The glycoprotein E2 in the envelope is the most important immunogen of pestiviruses. Antibodies of E2 and antibodies against glycoprotein $E^{rns}$ and the non-structural NS3 protein is present in infected host. In the first generation marker / DIVA vaccines (E2 subunit vaccines), $E^{rns}$-specific antibodies in the ELISA respond positively in the animals infected with the wild type strain, whereas only CSFV E2 specific antibody responses develop in the vaccinated animals. As an alternative, detection of NS3 antibodies may be used as an option. E2 subunit marker vaccines safety has been confirmed but may have disadvantages over live vaccines (Blome et al. 2017a).

Some studies have been conducted on the basis of epitope vaccines (EVs), and also combined CSFV-E2 and NS3 vaccines have been produced to contribute to the development of the immunogenicity of E2 marker vaccines (Uttenthal et al. 2010).

In 2014, the vaccine "CP7_E2alf" was licensed as the first live attenuated marker vaccine against Clasical Swine Fever (Blome et al. 2017b; Wei et al. 2021). This vaccine is based upon pestivirus chimera "CP7_E2alf" that carries the basic immunogen of CSF virus "Alfort/187"and glycoprotein E2, in a type 1 bovine viral diarrhea backbone ("CP7") (Blome et al. 2017b).

**Foot and Mouth Disease**

In this disease, DIVA strategies are needed using serological tests (ELISA) to differentiate vaccinated animals (Uttenthal et al. 2016; Diaz-San Segundo et al. 2017). In contrast to vaccination, foot and mouth disease virus (FMD) infection reveals a powerful antibody response to NSP (nonstructural proteins) and viral proteins. Various tests based on different NSPs (2A, 2B, 2C, 3A, 3B, 3ABC and 3AB) have been developed to differentiate vaccinated and non-infected animals from other vaccinated, which are afterwards clinically or subclinically infected with foot and mouth disease virus (Uttenthal et al. 2016; Bhatt et al. 2018).

**Rift Valley Fever**

The RNA genome of Rift Valley Fever Virus (RVFV) encodes 4 structural proteins and two non-structural proteins NSs and NSm, that play a significant role in viral pathogenesis. In addition, information on the molecular biology of RVFV was used to develop DIVA-compatible vaccines (Faburay et al. 2017). Vaccination policies and types of vaccines used may vary in non-endemic and endemic countries. In non-endemic countries, eradication and control programs are a major objective against outbreaks, and the use of vaccines that provide oppurtunity for DIVA is the best choice (Faburay et al. 2017; Wilson et al. 2021). In endemic countries, vaccine candidates that can be used to control rift valley fever are highly flexible. In this case, the use of DIVA vaccines may be a better choice if the goal is eradication. Vaccines such as VLPs, DNA vaccines and recombinant protein based vaccines are included in the DIVA-compatible vac-

cines group with no universal risk and high safety profile (Faburay et al. 2017).

In some studies, RVFV vaccines include deletion of NSs and NSm genes. These may be useful in controlling RVFV in endemic areas as well as for DIVA. However the accompanying diagnostic tests are not yet commercially present (Alhaj 2016).

**Peste des petits Ruminants (PPR)**

Conventional live vaccines used for PPR do not differentiate between vaccinated and infected animals. Therefore, it is important to develop vaccines suitable for the DIVA concept (Fakri et al. 2021). This concept may play a significant role in reduction of disease in endemic regions (Parida et al. 2015). Thus, adenovirus-based DIVA vaccines have been developed in East African goats against the peste des petits virus (PPRV) challenge. H and F are surface glycoproteins of PPRV. As a result the vaccine consisting of (AdF +AdH) appears to be successful and provides a DIVA vaccine potential when used in conjunction with the ELISA (Holzer et al. 2016).

**Newcastle Disease**

VLP (virus-like particle) vaccines have been indicated high levels of protection against different viral agents. In a study, Newcastle disease virus (NDV) VLP was developed and poultry immunized with NDV VLP vaccines. It has been strongly shown that the use of this vaccine in poultry may be a suitable strategy for control of NDV. Accordingly, the VLP vaccine and accompanying HI test may give opportunity to use the DIVA strategy (Park et al. 2014).

**Infectious Laryngotracheitis**

Distinction between ILTV (infectious laryngotracheitis virus) wild type strain and vaccine strains is essential for control of disease. ILTV DIVA vaccines are developed to overcome this problem (Shil et al. 2012). In one study, it was shown that the TaqMan real-time PCR test together with the ΔgG ILTV vaccine has the possible to be used in the DIVA strategy for control and eradication of ILT (Shil et al. 2014).

**Bovine Tuberculosis**

Bovine tuberculosis, caused by *Mycobacterium bovis*, is an important economic and global animal welfare issue. The development of the DIVA test is required for slaughter control strategies in addition to traditional bovine vaccines in countries (Vordermeier et al. 2016a; García et al. 2020).

The DIVA test, based upon the interferon gamma blood test platform, is similar to the tests used to diagnose human tuberculosis. The interferon gamma DIVA test is based on a response combination of 3 antigens, Rv3615c, CFP-10 and ESAT-6 (Conlan et al. 2015). Antigens used in blood tests were additionally evaluated for their use in skin tests (Vordermeier et al. 2016b). The DIVA skin test approach is effective in detecting infected animals without the use of immunomodulators, while giving negative results in non-infected or BCG-vaccinated cattle. There are also two skin tests that are potential for DIVA. Of these, APHA-1; is based on mixture of Rv3615c CFP-10, ESAT-6 proteins, while APHA-2; it is based on the Rv3020 protein in addition to the three proteins (Vordermeier et al. 2016a ).

**Paratuberculosis (Johne's Disease)**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) cause paratuberculosis in ruminants. Popular commercial paratuberculosis vaccines are prepared from whole cell killed MAP which will generate antibodies to cellular antigens. Nevertheless, field infected animals will have antibodies to both cellular and secreted antigens. A simple ELISA-based test, when used in conjunction with conventional ELISA protocols, can be used to differentiate between vaccinated and infected animals using MAP secreted antigens to diagnose paratuberculosis (Dhama et al. 2016).

**Salmonella Infections**

In *Salmonella* Choleraesuis DIVA vaccines, the *ompA* gene was deleted from live attenuated ΔrpoS and ΔphoP vaccine strains. The *ompA* is found in whole *Salmonella enterica* serovars. The results show that *Salmonella* Choleraesuis ΔrpoS ΔompA and ΔphoP ΔompA may be useful as DIVA strains carriying foreign antigens, thus generating new probability for the production of invaluable live vaccines for live animals. Also ELISA was used as differential test. Furthermore, these information show that *OmpA* may be proper negative marker for DIVA vaccines (Herrero-Gil et al. 2016).

For Pullorum disease, the *S.* Pullorum ΔspiC ΔwaaL mutant strain was developed. In vaccine strain the truncated Salmonella lipopolysaccharides have a differentiating use as a serological marker. The efficacy, safety, and DIVA characteristics of this vaccine candidate were evaluated in broilers. The results show that the double mutant strain may be a effective, cross-protective and safe vaccine against Salmonella infection in a poultry in accordance with the requirements of the DIVA plan (Guo et al. 2017).

### Brucella Infections

In a study, the modified *B. abortus* S19 strain called S19Δper was described. Deletion of the *perosamine synthetase gene* caused a significant attenuation of the mutant S19Δper without affecting the immunogenic properties. Then S19Δper DIVA capability was assessed by using Rose Bengal Plate Test. As a result the mutant S19Δper with a moderately similar phenotype showed a significant similarity to the S19 vaccine strain with improved immunogenicity, safety and DIVA properties for the bovine brucellosis control, and can thus be used as the DIVA vaccine (Lalsiamthara et al. 2015; Chaudhuri et al. 2021).

### Mycoplasma Infections

The control strategy of the contagious bovine pleuropneumonia is to vaccinate with live attenuated strains. In addition, an lppQ−mutant of the current vaccine strain T1 / 44 has been developed. T1lppQ-mutant strain lacks lipoprotein LppQ, which provides a possible virulence of *M. mycoides* subsp. *mycoides*. TaqMan real-time PCR based upon the lppQ gene has been developed for the detection of mycoplasma and for the discrimination of wild-type strains from the lppQ− mutant vaccine strain. Consequently this has been described as a live DIVA vaccine strain. (Vilei and Frey 2010).

**Table 1.** DIVA (Marker) vaccines used in veterinary medicine (Capua et al. 2003; Arda and Sareyyüpoğlu 2004; Suarez 2005; Dong and Chen 2007; Meeusen et al. 2007; Uttenthal et al. 2010; Vilei and Frey 2010; Day and Schultz 2014; Park et al. 2014; Shil et al. 2014; Conlan et al. 2015; Lalsiamthara et al. 2015; Dhama et al. 2016; Herrero-Gil et al. 2016; Holzer et al. 2016; Vordermeier et al. 2016a; Vordermeier et al. 2016b; Blome et al. 2017a; Freuling et al. 2017; Muratore et al. 2017).

| Disease | Causative Agent | DIVA/Marker Vaccine Strategy | Deleted Gene | Accompanying Test |
|---|---|---|---|---|
| Infectious Bovine Rhinotracheitis (IBR) | Bovine herpesvirus | Gene deletion mutant virus | Glycoprotein E | ELISA for gE analysis |
| Avian Influenza (AI) | Avian Influenza virus | Subunit vaccine use | - | ELISA tests |
| | | Non-structural protein 1 (NS1) | - | ELISA tests |
| | | Heterologous neuraminidase (H7N2 (wild)/H7N3 (vaccine)) | - | N2/N3 monitoring |
| Pseudorabies (Aujeszky's Disease) | Pseudorabies virus (PrV) | Gene deletion mutant virus | Glycoprotein E | ELISA for gE analysis |
| Classical Swine Fever (CSF) | CSF virus (CSFV) | E2 subunit vaccine use | - | ELISA tests |
| Foot and Mouth Disease (FMD) | FMD virus (FMDV) | Comparison of serologic response against nonstructural proteins (NSPs) with serologic response against vaccine antigens | - | ELISA tests |
| Peste des petits Ruminants (PPR) | PPR virus (PPRV) | Adenovirus-based vaccine use | - | ELISA tests |
| Newcastle Disease (ND) | ND virus (NDV) | Virus like particles | - | HI test |
| Infectious Laryngotracheitis (ILT) | ILT virus (ILTV) | Gene deletion mutant virus | Glycoprotein G | TaqMan real-time PCR |
| Bovine Tuberculosis | *Mycobacterium bovis* | APHA-1 or APHA-2 use | - | DIVA skin test approach |
| Paratuberculosis (Johne's Disease) | *M. avium* subsp. *paratuberculosis* (MAP) | Evaluation of MAP secreted antigens and cellular antigens | - | Classic and MAP secreted ag ELISA |
| Salmonella Infections | *Salmonella* Choleraesuis | Gene deletion | *OmpA gene* | ELISA tests |
| Brucella Infections | *Brucella abortus* | Gene deletion | Per gene | Rose Bengal Plate Test |
| Contagious Bovine Pleuropneumonia | *M. mycoides* subsp. *mycoides* | Gene deletion | Lipoprotein LppQ | TaqMan real-time PCR |

## Conclusion

DIVA strategies as a differentiation method between infected and vaccinated animals are significant tools for disease control and eradication. There is an important need to develop DIVA vaccines and accompanying tests, as they are predicted to be more effective for disease eradication than the standard vaccines used. These vaccines will also be an important approach for the control of infectious diseases that may emerge in the future. With the development of technologies in the field of veterinary medicine, the use of new DIVA strategies against various disease seems to be inevitable. These vaccine strategies have been tried to be developed for many animal disease agents and are still being studied today.

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