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**Comparison of antibody titres between intradermal
and intramuscular rabies vaccination using inactivated
vaccine in cattle in Bhutan**

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Abstract

In developing countries, the cost of vaccination limits the use of prophylactic rabies vaccination, especially in cattle. Intradermal vaccination delivers antigen directly to an area with higher number of antigen-presenting cells. Therefore, it can produce equivalent or higher antibody titres than conventional intramuscular vaccination even when a lower dose is given.

This study aimed to compare the antibody response in cattle vaccinated intramuscularly with 1mL of inactivated rabies vaccine (Raksharab, Indian Immunologicals) against intradermally vaccinated cattle with 0.2mL of the same vaccine. The study was conducted in Haa province of Bhutan where rabies is not endemic. One hundred cattle from 27 farms were selected for the study. Virus neutralising antibody (VNA) response was measured using the fluorescent antibody virus neutralisation test on the day of vaccination (day 0) and 14, 30, 60 and 90 days later.

Overall, 71% of intradermally vaccinated cattle and 89% of the intramuscularly vaccinated cattle produced a protective response (≥ 0.5 IU/mL). This difference was significant ($P < 0.02$) on days 14 and 30 post vaccination with 36 and 56% in the intradermal group having titres ≥ 0.5 IU/mL respectively compared to the equivalent figures of 78 and 76% in the intramuscular group. The mean VNA titres were lower for intradermal group than intramuscular group ($p < 0.001$) with the mean difference being greater than 0.6 IU/mL. Although low dose intradermal vaccination did produce a detectable antibody response, it was inferior to intramuscular vaccination. Thus, although, intradermal vaccination has the potential to reduce the cost of vaccination by reducing the dose required, this study showed that a single dose of 0.2mL intradermally was inferior to an intramuscular dose of 1mL. Further research evaluating dose and dose regimen is needed before intradermal vaccination using the Raksharab rabies vaccine can be recommended in cattle.

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Introduction

Rabies is a fatal zoonotic viral disease caused by a non-segmented single-stranded negative-sense RNA (Ribonucleic acid) virus of the genus *Lyssavirus* under the family *Rhabdoviridae* (Gluska *et al.*, 2014). The virus causes an acute disease of the central nervous system in almost all mammals including humans. The infection is fatal as death is inevitable once clinical signs develop. Globally rabies accounts for 59 000 human deaths per year and an economic impact of US \$ 8.6 billion per year, with 6% of those losses being due to rabies in livestock (WHO, 2013; Hampson *et al.*, 2015).

The rabies virus appears as bullet-shaped particles under the electron microscope (Hummeler *et al.*, 1967). The inner core of the virus consists of RNA, nucleoprotein (N), phosphoprotein (P) and large protein (L, RNA-dependent RNA polymerase). This forms the infectious unit that is surrounded by matrix protein (M). Two layers of lipid membrane cover the infectious unit and the matrix protein complex. The outermost layer is made up of glycoprotein (G) spikes (Hummeler *et al.*, 1967; Dietzgen *et al.*, 2017). The glycoprotein layer is the main antigenic component of the virus and has a critical role in induction of host immune response. It is also responsible for virus uptake by the host cells and neuronal spread of the virus (Cox *et al.*, 1977).

The rabies virus is highly neurotropic (Baloul & Lafon, 2003; Schnell *et al.*, 2010). After inoculation of the virus, either through natural (usually a bite) or experimental exposure, the virus multiplies in the muscle cells at the inoculation site (Murphy & Bauer, 1974; Charlton *et al.*, 1997). The virus then accumulates around sensory or motor nerve endings and travels centripetally through neurotendinous or neuromuscular spindles to the peripheral nerves and spinal cord before it enters the brain (Baer, 1975; McKay & Wallis, 2005). Once in the peripheral nerves, the virus spreads rapidly at a rate of 50-100 mm per day along the axons to the central nervous system (Baer, 1975; Tsiang *et al.*, 1991). Using this axonal transport mechanism, the virus can be distributed to different parts of the body including glandular tissue, particularly the salivary glands (which play an important role in transmission of rabies virus via biting) (Dean *et al.*, 1963; Dierks *et al.*, 1969). The virus can be detected in the saliva of infected animals between 3- 14 days before the onset of clinical signs (Vaughn *et al.*, 1965; Fekadu, 1988).

Clinical signs depend on the parts of the central nervous system in which the virus replicates. For instance, greater replication and accumulation of virus in the limbic system impairs motivational, emotional, behavioural and long term memory functions (Murphy, 1977). The typical incubation period of the virus is one to three months but it can vary between few days to many years. The incubation period is shortened when the virus is inoculated into the head or neck regions (Fekadu & Shaddock, 1984; Leung *et al.*, 2007).

Rabies is manifested in two forms: furious and dumb. Furious or encephalitic rabies is more common than the dumb form and is observed in 70-80% of the cases (Fekadu & Shaddock, 1984; Hudson *et al.*, 1996; Leung *et al.*, 2007). The clinical signs include excessive vocalisation and salivation, change in behaviour, tremors, hyperesthesia, trismus and pharyngeal paralysis, lameness and recumbency (Fekadu & Shaddock, 1984; Hudson *et al.*, 1996; Leung *et al.*, 2007; Thiptara *et al.*, 2011). Death usually occurs between 3 to 7 days after the development of clinical signs (Hudson *et al.*, 1996; Leung *et al.*, 2007). Rabies is widely distributed across the world with the exception of Antarctica. Mammals of the order Carnivora and Chiroptera are the primary reservoir hosts of rabies virus. Domestic canines are the major reservoir hosts and vectors of rabies globally, especially in developing countries in South East Asia, Africa, the Middle East and Central America. In North America, raccoons and skunks are the major reservoir hosts whereas in Europe foxes and bats maintain the virus (Krebs *et al.*, 2001). In South America vampire bats are the main source of rabies virus (Chomel, 1993). Different species of mongoose are also important reservoir hosts in some parts of Africa, Asia and the Caribbean (Niezgoda *et al.*, 2002; Rupprecht *et al.*, 2002). Reservoir hosts of rabies other than carnivores and bats include kudu antelopes in Namibia (Scott *et al.*, 2013) and non-human primates (marmosets) in Brazil (Favoretto *et al.*, 2013). Both domestic and wild cats are vectors of rabies but they do not serve as a reservoir hosts (Rupprecht *et al.*, 2002).

The virus is typically maintained in two different cycles, urban and sylvatic. In the urban cycle, domestic dogs maintain the virus while in the sylvatic cycle, the virus is maintained by wild carnivores and bats. The virus is transmitted within and between species in both cycles (Wallace *et al.*, 2014). The disease occurs as epizootic outbreaks in both reservoir and non-reservoir hosts. These epizootics have been explained using population density models. Increases in the density of the reservoir population lead to

an increase in the proportion of that population that is susceptible to rabies infection. Consequently, high-risk individuals develop rabies and spread disease to other members of the population. The rate of infection is high due to the high proportion of susceptible animals. The disease wipes out a large proportion of the reservoir population and subsequently disease incidence reduces. The next epizootic occurs when the reservoir population increases to its initial size and has a low proportion of resistant individuals (Mayen, 2003; Sterner & Smith, 2006; Wallace *et al.*, 2014).

Non-reservoir or dead end hosts of rabies such as humans and livestock animals acquire disease as spill-over infection during outbreaks in reservoir populations (Wallace *et al.*, 2014). Thus, the epidemiology of disease in the non-reservoir hosts is associated with the ecology, density and dynamics of the reservoir hosts (Carey & McLean, 1983; Hampson *et al.*, 2009). For example, in areas where vampire-bat-rabies is prevalent, rabies outbreaks in cattle have been reported as lasting for 18 months with 4-year gaps between outbreaks. These outbreaks in cattle were closely related to large outbreaks of disease in the bat population (Mayen, 2003).

A bite from an infected animals is the most common route of rabies transmission (Knobel *et al.*, 2005). Other much rarer routes of infection include ingestion, aerosol, transplacental and via organ transplants (Martell *et al.*, 1973; Barnard *et al.*, 1982; Gibbons, 2002). Transmission via aerosol has been reported in laboratory personnel dealing with rabies vaccine production, humans and animals exposed to rabies virus in bat cages (Constantine, 1962; Winkler *et al.*, 1973). There are documented evidences of human-to-human transmission of rabies through transplantation of organs such as liver, kidney, cornea and arteries from a patient that died of unknown encephalitis (Houff *et al.*, 1979; Srinivasan *et al.*, 2005; Bronnert *et al.*, 2007). Although rabies virus is rarely detected in blood and contact from tissue fluids are limited, cases of rabies in babies from a rabid human mother and calves from rabid cows have been recorded (Martell *et al.*, 1973; Aguemon *et al.*, 2016).

Rabies is a disease of significant public health importance. People contract the disease from infected dogs, cats, bats or other wild animals (Yakobson *et al.*, 2015). Rabid dogs are the major source of human infection and account for almost 99% of human deaths (mainly in Asia and Africa) (Knobel *et al.*, 2005). Although wildlife rabies is common in many parts of Europe and Americas, the rate of human cases is much lower than in

countries where dogs are the principal source of rabies. In the US, only 3 human cases of rabies linked to wildlife were reported in 2014 (Monroe *et al.*, 2016) while in Europe about 10 such cases were reported per year (Cliquet *et al.*, 2014).

Infected livestock such as cattle are of low risk to the general public. However, they are of potential risk to animal handlers and veterinarians especially when the disease is confused with other neurological diseases. Risk from bovines include infections from saliva and infected wounds (Simani *et al.*, 2012). There are documented cases of human mortality from cattle rabies contracted after an oral examination and wound treatment of infected cattle (de Brito *et al.*, 2011; Simani *et al.*, 2012). Although infection from cattle to humans is rare, the furious form of rabies in cattle is a significant zoonotic risk (Liu *et al.*, 2016).

Bovine rabies causes significant economic losses to livestock farmers (Mayen, 2003; Anderson *et al.*, 2014; Feng *et al.*, 2016; Jibat *et al.*, 2016). The losses incurred are in the form of cattle mortality, decrease in weight and milk production and damage to hide in case of vampire bat feeding (Anderson *et al.*, 2014). The added loss due to rabies in livestock include costs of euthanasia, diagnosis, replacement stock and vaccination of at risk herds (Thiptara *et al.*, 2011; Vos *et al.*, 2014). The impact of rabies on livestock is higher in developing countries as livestock animals, mainly cattle, are used as a source of food, manure and draft power and therefore serve as the main livelihood of farmers (Vos *et al.*, 2014). In areas where canine rabies is endemic, stray dogs are the principle source of rabies for livestock especially where livestock are allowed to range freely. Thus, canine rabies is a major risk to livestock in countries where free range grazing is common, especially in developing countries in Asia and Africa (Feng *et al.*, 2016; Jibat *et al.*, 2016).

The global economic loss due to rabies in livestock was estimated at US\$ 512 million in 2015 (Hampson *et al.*, 2015). In Asia and Africa, the losses amounted to US\$ 12.3 million per year (Knobel *et al.*, 2005). In Africa, Jemberu *et al.* (2013) reported that the incidence of cattle rabies was 19.89 cases per 100 000 cattle, while Jibat *et al.* (2016) observed an incidence rate of 11-21% in Ethiopia. These livestock mortality figures are likely to be a gross underestimate of the actual figures (Knobel *et al.*, 2005; Anderson & Shwiff, 2015), as rabies outbreaks in cattle are commonly underreported in many countries due to lack of proper surveillance and reporting systems. Furthermore, these

mortality estimates were derived from samples submitted for diagnosis and did not include animals that were not tested. The losses due to rabies are often calculated based on probable costs and are thus unlikely to reflect the true economic impact (Knobel *et al.*, 2005).

Because the rabies virus is highly neurotropic and has the ability to evade the host immune system, the disease is invariably fatal once clinical signs develop (Baloul & Lafon, 2003; Dietzschold *et al.*, 2008). However, it is a preventable disease both in humans and animals provided strict control measures are put in place (Fu, 1997; Dodet, 2006). Vaccination is one of the most effective measures for preventing rabies in both humans and animals (Rupprecht *et al.*, 2006). The normally long incubation period of the virus (provided that entry of the virus has not occurred in the head or neck) means that post exposure prophylaxis is very effective, especially in humans (Fu, 1997; Jackson, 2003). Thus, pre-exposure prophylaxis is only recommended in high risk individuals such as veterinarians, laboratory workers and animal handlers (WHO, 2005; Nigg & Walker, 2009). Post-exposure prophylaxis is much more difficult to achieve in wild and stray domestic animals, so mass prophylactic vaccination of reservoirs and vectors of rabies is crucial for eradication and control of rabies (Fu, 1997). For livestock such as cattle, vaccination is essential to prevent economic losses to farmers.

Literature review

1.0 Types of rabies vaccine

Several types of rabies vaccine are available for veterinary use. Based on the substrate for vaccine production, they can be broadly classified as first generation and second generation vaccines. These generations of vaccines are further categorised as attenuated (live) and inactivated (killed) vaccines (Meslin *et al.*, 1996). With the help of newer technologies, recombinant or subunit vaccines are being developed. These are termed as third generation vaccines (Nandi & Kumar, 2010).

1.1 First generation rabies vaccines

The first generation of rabies vaccines were produced from animal substrates such as brain tissue, and embryonated eggs. Use of adult animal nerve tissue for vaccine production was based on techniques developed by Louis Pasteur. Nerve tissues from sheep, goats, rats, rabbits and mice have all been used for vaccine production, as have duck and chicken embryos (Umeno, 1921; Sikes, 1969). Both live and inactivated first generation vaccines have been used widely in animals (Perez & Paolazzi, 1997).

Encephalomyelitis and polyneuritis were the most common complications following administration of adult animal brain tissue vaccines in humans (Meslin *et al.*, 1996). Similar signs have also been observed in sheep, dogs, primates and laboratory animals (Jervis, 1954; WHO, 2013). Studies demonstrated that the complications were due to allergic reactions to myelin basic protein (Eylar *et al.*, 1969; Hemachudha *et al.*, 1987). To overcome neurological and other side effects related to adult animal brain tissue vaccines, brain tissues from suckling neonates were used, as they were considered free of myelin. Vaccine production using birds embryos were advocated as an alternative to reducing neurological complications (Peck *et al.*, 1955), but these vaccines still resulted in neurological complications, although to a lower degree than nervous-tissue vaccines (Trejos *et al.*, 1974).

Rabies vaccine prepared using nerve tissues or embryonated eggs, inevitably contained large amounts of tissue proteins. These proteins caused allergic reactions and also competitively inhibited the expression of host antibodies to viral proteins thereby decreasing the vaccine potency (Fenje, 1960). Nevertheless, nervous tissue vaccines are

still used to vaccinate animals in parts of Africa, Latin America and Caribbean as they are cheaper to produce than the alternatives (Cliquet & Picard-Meyer, 2004).

1.2 Second generation rabies vaccines

In 1958, Kissling successfully propagated rabies virus in hamster kidney cells (Kissling, 1958). Subsequently, the rabies virus was also propagated in porcine and canine kidney cells (Abelseth, 1964; Cleaveland *et al.*, 2006). This led to the development of second-generation cell-culture-derived vaccines (Fenje, 1960; Roumiantzeff *et al.*, 1985). Both attenuated and inactivated vaccines can be produced using cell culture (Abelseth, 1964; Cleaveland *et al.*, 2006).

Baby hamster kidney (BHK-21) cell or chick embryo cell lines (NIL2) are commonly used for rabies vaccine production for veterinary use (Kallel *et al.*, 2002). BHK-21 cell cultures have the advantage of rapid virus replication that yields large volumes of virus and allows for large-scale production of vaccines (Sureau, 1987; Perez & Paolazzi, 1997).

One of the main issue with production of vaccines from cell culture was the requirement for the addition of animal sera to enhance cell proliferation. This was a significant drawback as high quality animal sera is expensive and therefore significantly increased the cost of cell culture vaccine production compared to tissue-derived vaccines. Further, issues with cell culture vaccine production include significant variation between culture lots and the risk of contamination by microorganisms such as bacteria, fungi and viruses (Pay *et al.*, 1985).

1.2.1 Attenuated or live vaccines

Live vaccines can be produced either by serial attenuation in vitro using cell cultures (BHK-21) or in vivo passages in mice (Meslin *et al.*, 1996). The harvested viral suspension is then diluted to produce a viral dose which is sufficient to immunise the target species. Potency is measured as unit of infectivity or median lethal dose for mice (LD50). The vaccine is stored in freeze-dried form with suitable excipients (Meslin *et al.*, 1996).

Two types of live vaccine are available: parenteral-modified and oral-modified (Nandi & Kumar, 2010). The strains of virus used to prepare modified live vaccines for parenteral use include Flury and Kelev, Street Alabama Dufferin (SAD) and Evelyn

Rokitnicki Abelseth (ERA). Flury and Kelev strains were adapted in chick embryo whereas SAD and ERA were propagated in hamster and porcine kidney cells respectively (Fenje, 1960; Abelseth, 1964; Lawson & Crawley, 1972).

Modified live oral vaccines were developed to control rabies in wild and free ranging animals, and have been successfully used to control fox-propagated rabies in Europe. Apathogenic mutants of SAD Bern strains such as SAG-1 and SAG2 (SAD avirulent Gif) were widely used for preparation of these oral rabies vaccines (Artois *et al.*, 1997). The SAG 2 oral rabies vaccine widely used for wildlife vaccination was also licensed to use in dogs (Cliquet *et al.*, 2007; Pradhan *et al.*, 2008). Efficacy trials were conducted using SAG2 in India, Mexico, Indonesia and South Africa. Although vaccinated dogs were protected against a lethal challenge, antibody titres and seroconversion rates were low (Cliquet *et al.*, 2007; Lucas *et al.*, 2008). Furthermore, the use of oral vaccination in stray and free-roaming dogs posed a risk to humans and other domestic animals as the bait had to be distributed in the vicinity of human settlements (Rupprecht *et al.*, 2005).

Live vaccines are simpler and cheaper to produce as the requirements for viral cultures and cells are much lower. In addition, since the virus is live, it can infect and replicate in host cells which induces a better immune response (both in terms of titre and length of protection). Vaccination is simple as vaccines can be given orally, intranasally or intraocularly as well as intramuscularly (Meeusen *et al.*, 2007). It is especially useful for rabies control in wild animals where oral administration via baits is the most practical method (Brochier *et al.*, 1991).

The principal problem with live vaccine is the risk of residual pathogenicity. There have been reported cases of vaccine-induced rabies in wild carnivores and bovines after vaccination with ERA live vaccine (Fehlner-Gardiner *et al.*, 2008). Residual pathogenicity also poses a potential risk to handlers and environmental contamination if the virus reverts back to its pathogenic wild type (Meeusen *et al.*, 2007). In addition, live vaccines were sensitive to slight variation in temperatures and resulted in loss of immunogenicity (Rodrigues da Silva *et al.*, 2000).

1.2.2 Inactivated or killed vaccines

Currently, these are the most widely used vaccines for rabies prevention (Sugiyama & Ito, 2007). The whole virus is killed or inactivated either with heat or chemical agents.

Beta-propiolactone, ethylene-imine and thimerosal are commonly employed agents for virus inactivation (Minke *et al.*, 2004). They kill the virus without affecting its antigenicity. After inactivation, the virus is concentrated by ultrafiltration, and adjuvants such as aluminium hydroxide and aluminium phosphate are added to enhance the immune response. The vaccine can be stored either as freeze dried plug or in liquid suspension (Meslin *et al.*, 1996). The strains of virus used for inactivated vaccines production included standard challenge virus 11 (CVS), Pittman-Moore, Nishigahara and Pasteur virus. Most of the currently used inactivated vaccines are produced using cell culture such as the BHK-21 cell lines (Yang *et al.*, 2013).

Inactivated vaccines are more stable and safer to use than live vaccines (Meslin *et al.*, 1996), as if prepared properly there should be no risk of reversion to its pathogenic form (Meeusen *et al.*, 2007). After the completion of vaccination protocols, inactivated vaccines can produce long-term immunity. Trials in humans have shown the protective antibody titres to last for as long as 14 years. Further, challenge experiments in dogs have demonstrated protective immunity even at low detectable antibody titres (Meslin *et al.*, 1996). Another advantage is that inactivated vaccines can be used in combination with other vaccines. Successful trials were conducted using combined vaccine containing rabies and foot-and-mouth disease in calves (Palanisamy *et al.*, 1992).

However, inactivated vaccines are not as immunogenic as live vaccines. In particular, the immune response is slower and large amounts of antigen, strong adjuvants and booster doses are required to induce adequate immunity. As host cells are not infected by inactivated vaccines, they are weaker inducer of cell mediated immune system than live vaccines (Minke *et al.*, 2004). Although adjuvants such as aluminium hydroxide and aluminium phosphate enhance antibody mediated immune response (Eldred *et al.*, 2006), they can cause allergic reactions at the site of injection and as well as systemic sarcomas and autoimmune disorders (Day, 2006). Finally incomplete inactivation could be hazardous to personnel and environment (Minke *et al.*, 2004).

The need for higher antigenic mass, strong adjuvants and an effective process of inactivation means that even if animal sera are not required, the cost of production of inactivated vaccines is higher than that of live vaccines (Meeusen *et al.*, 2007).

1.3 Third generation rabies vaccines

Third generation rabies vaccines include deoxyribonucleic acid (DNA) and recombinant vaccines. These vaccines were developed to reduce the pathogenicity of live rabies vaccines and at the same time to increase their potency (Yang *et al.*, 2013). DNA-based vaccines are constructed on a plasmid vector that expresses rabies virus glycoprotein. These types of vaccines are easy to produce and in addition, the bacterial plasmid has an adjuvant effect that enhanced the magnitude and duration of immune response (Wang *et al.*, 1998).

Recombinant rabies vaccines have been developed using viruses such as poxvirus, adenovirus and rhabdoviruses as vectors (Yang *et al.*, 2013). Avirulent viral phenotypes were obtained by replacing the arginine codon at position 333 in the glycoprotein gene sequence with other amino acids such as glycine, leucine or cysteine (Tuffereau *et al.*, 1989). This avirulent gene sequence was then inserted into vector viruses to produce a recombinant rabies vaccine (Brochier *et al.*, 1991). Recombinant vaccines are effective when given orally and are thus mostly used in wild animals. The vaccine is safer as there is no live rabies virus used in its production (OIE, 2013). However, the efficacy and side effects of recombinant rabies vaccine need further exploration. The vectors may cause disease in inoculated animals and humans. Mutations could occur in the inserted genes and thereby restore rabies virulence (Sugiyama & Ito, 2007).

2.0 Rabies vaccination of reservoir hosts and vectors

Rabies control measures vary significantly depending on the main reservoir species. In most developing nations, particularly those in Africa and Asia, stray and free-roaming domestic dogs remain the major source of rabies for humans and other domestic animals (Knobel *et al.*, 2005; Rinzin *et al.*, 2016). Rabies is prevalent in countries with large number of stray and free roaming dogs, poor dog ownership regulations and poor garbage management (Wandeler *et al.*, 1993).

In countries which have eliminated endemic rabies in the domestic dog (such as many European countries, USA and Canada), wildlife rabies is the major source of rabies in humans and domestic animals (Belotto *et al.*, 2005). Rabies in wildlife emerged as a significant spill over infection from domestic animals in the 1940s; with the disease eventually becoming established in wild canids (Wandeler *et al.*, 2004; Velasco-Villa *et al.*, 2008). Over 6000 rabies cases were detected in Europe in 2012 with almost equal

number of cases in wild and domestic animals. These figures indicated that there had been an increase in spill-over infection to domestic animals (Miyamoto & Matsumoto, 1967; Cliquet *et al.*, 2014). In the US, wildlife rabies represents over 90% of the 6000 rabies cases reported every year, with the majority of cases observed in raccoons followed by bats, skunks and foxes (Dyer *et al.*, 2014; Monroe *et al.*, 2016). In 2014, spill-over infection to domestic animals affected 78 cattle (1.29% of all rabies cases), 59 dogs (0.98%) and 272 cats (4.5%) (Monroe *et al.*, 2016).

2.1 Rabies control of free ranging wildlife

Large scale oral vaccination has been the most effective tool for elimination of rabies in wildlife (Cliquet & Aubert, 2003; Blancou, 2008; Freuling *et al.*, 2013). Baits containing rabies vaccines were distributed with the help of aeroplanes, a technique that was developed in the 1950s to poison ground rodents (Marsh, 1967). Currently bait-distributing planes are fitted with automated systems that record the date, location, number and time of each bait released (Müller *et al.*, 2012). In addition to dropping bait over a wide area, trap–vaccinate–release (TVR) programmes and hand placement of vaccine baits have been used to vaccinate small fox and raccoon populations within cities (Rosatte *et al.*, 1992; MacInnes *et al.*, 2001).

In Europe, the success of the first oral rabies vaccination trial in Switzerland in 1978 drew the attention of many other European countries, and initiated a co-operative vaccination programme across many European states (Steck *et al.*, 1982). By the mid-1990s, 17 European countries were using oral vaccines to control rabies in foxes, with, approximately, 15 million baits distributed annually. This coordinated effort brought down the rabies cases from over 18000 in 1980 to less than 7000 in 1999 (Vitasek, 2004). By 2010, 22 oral vaccination programs covering 24 states and 1.9 million km² area had been implemented in Western and Central Europe (Freuling *et al.*, 2013). Currently, 12 European countries are free from rabies (Cliquet *et al.*, 2014). These programs have been strongly supported by the European Union (EU). In addition to national programs, the EU has also co-financed rabies vaccination in neighbouring states to maintain a 100 km wide vaccination zone along the borders (Demetriou & Moynagh, 2011). However, the disease still remains a threat in eastern and southern Europe with red foxes, bats and raccoon dogs being the primary reservoirs (Cliquet & Aubert, 2003; Singer *et al.*, 2008). Illegal importation of pets from rabies endemic areas

and introduction of rabies to new reservoir hosts pose challenges in rabies eradication programs and maintaining rabies-free status (Cliquet *et al.*, 2014; Muller *et al.*, 2015).

In Ontario, Canada, oral rabies vaccination was initiated in 1989 to eliminate rabies in the arctic fox. Approximately 20 baits/km² were distributed to cover 8850 to 29590 km². The strategy was termed as ‘*progressive elimination*’ and started at the foci of the outbreak and expanded outwards every year (MacInnes *et al.*, 2001). By 1996, the number of rabies cases in foxes had dropped to 4 cases per year from the initial 203 and by 1997, the spill-over infection to striped skunks and livestock had dropped to zero (Rosatte *et al.*, 1992; MacInnes *et al.*, 2001).

In the USA, oral rabies vaccination programs began in the mid-1990s and by 2003 included 16 states. In 2003, ten million vaccine-laden baits were distributed to about 1.8 million km² area to cover rabies virus in raccoons, grey fox and coyotes (Slate *et al.*, 2005). By 2004, rabies had been eliminated from coyotes and substantially reduced in grey foxes (Sidwa *et al.*, 2005; Blanton *et al.*, 2007). Vaccination coverage of 40-50% was sufficient to break the rabies cycle and 50-60% to provide herd immunity (Sidwa *et al.*, 2005). Nevertheless rabies virus harboured by wild species such as skunks, raccoons, foxes and mongoose still remains a significant source of rabies in domestic animals and humans in the USA (Blanton *et al.*, 2007).

In South America, vampire bats are a significant source of rabies for humans and domestic species. As yet no feasible method of vaccination has been found for controlling disease in these species (Johnson *et al.*, 2014).

2.2 Rabies control in domestic dog reservoirs

2.2.1 Canine rabies free regions of the world

Restricted dog movement and muzzling were the main rabies control measures implemented in Europe in the 18th and 19th centuries (Müller *et al.*, 2004). In the later part of the 19th century, additional measures such as elimination and tracing of stray dogs, quarantine, disease notification, and regulation of sanitary and strict import policies were implemented. These measures were successful in controlling urban rabies and eliminating dog-mediated rabies from several European states including Denmark, Austria, Germany, Netherlands and UK (Muir & Roome, 1997; King *et al.*, 2004).

Large scale dog vaccination began in the early 20th century, and alongside the regulation of ownership and restrictions of dog movements formed the backbone of rabies control

in Europe (King *et al.*, 2004; Müller *et al.*, 2012). As a result, the burden of canine rabies was reduced in several European states within a few decades. By 1990s, many European countries including Greece, France and Yugoslavia were free of canine rabies (King *et al.*, 2004).

Rabies was eliminated as an endemic disease in the domestic dog population from most of North America including Canada in the 1960s (Held *et al.*, 1967). In rest of the Americas, coordinated efforts by the Pan American Health Organisation (PAHO) reduced canine rabies by 90% by 2005 (Belotto *et al.*, 2005) and by 98% in 2015 (Velasco-Villa *et al.*, 2017).

2.2.2 Canine rabies control in Asia and Africa

Only a few Asian countries including Japan, Taiwan, and Singapore have achieved canine rabies free status through strict quarantine measures and compulsory dog vaccination programmes (Acha & Arambulo, 1985; Takayama, 2000; Briggs & Schweitzer, 2001). Several countries in Asia and Africa have now implemented mass vaccination and sterilisation programmes along with improved quarantine and surveillance systems. Through these programmes rabies incidences have been reduced in some of these countries including Philippines (Lapiz *et al.*, 2012), Sri Lanka, Indonesia (Putra *et al.*, 2013) and Tanzania (Cleaveland *et al.*, 2003).

In spite of having efficient measures for canine rabies control and the success stories observed in many countries, rabies is still endemic in many developing countries particularly in Asia and Africa (Knobel *et al.*, 2005; Davlin & VonVille, 2012). Dog vaccination coverage is very low compared to other countries (OIE, 2014). For instance, Latin America vaccinated 2.8 million dogs for every human life lost whereas in Africa and Asia the numbers of dogs vaccinated remain extremely low at 1000 and 200 respectively for every human life lost. Similarly, the number of dogs vaccinated divided by the number of cattle was 1.7 million in Latin America, 1900 for Asia and 600 for Africa (OIE, 2014). Lack of technical expertise, political commitment and sustainable financial sources have been the major impediments to successful rabies control programmes (Knobel *et al.*, 2005; Wilde *et al.*, 2005). In many of the highly rabies endemic countries including India, China and Pakistan, mass vaccination of dogs against rabies is not a priority (Davlin & VonVille, 2012). Thus, rabies remains a neglected disease in many countries with a high disease burden (Bourhy *et al.*, 2010;

Davlin & VonVille, 2012). These countries remain a threat to neighbouring countries where good rabies control programs exist. Elimination of canine rabies is near impossible from a country or an area unless every country makes an effort in controlling canine rabies through national, regional and international cooperation.

3.0 Rabies vaccination in cattle

Attempts to vaccinate cattle against rabies dates back to as early as 1881. In the initial trials cows and calves were inoculated intravenously with 10- 20 drops of infected rabbit brain emulsion. The inoculated animals did not survive when challenged, probably because the dose of brain emulsion was very low. Of four sheep given 3 mL of the same emulsion, three survived when challenged intraocularly with pathogenic virus (Roux and Nocard, 1888 cited in Koprowski, 1955). Later treatment trials were carried out in cows bitten by a rabid dog using dog brain tissue suspension and all cows survived. However, because the trials lacked controls, it could not be confirmed whether it was the vaccine that prevented rabies infection in these cattle (Moncet, 1898 cited in Koprowski, 1955)

Moving away from crude preparation of vaccine with street virus, laboratory-fixed rabies virus was used to prepare rabies vaccine. In the early 20th century, several European countries vaccinated cattle using these vaccines. The expected mortality in untreated animals was 60-70% and in vaccinated animals the mortality varied between 1.1% (n=851) to 7.8% (n=228) (Koprowski, 1955).

Most of these early attempts were focused on inoculating cattle either known or assumed to be exposed to rabies. These attempts led to the prevention of rabies in cattle by vaccination with the main motive of preventing economic losses incurred to farmers (Koprowski *et al.*, 1955). Initially, mass vaccination of cattle took place alongside the vaccination of vector population such as dogs and wild canids; so by the mid-20th century millions of cattle had been vaccinated against rabies in endemic areas (King *et al.*, 2004). However, in the later part of the 20th century vaccination campaigns were focused more on reservoir hosts and vectors rather than spill-over species such as cattle (King *et al.*, 2004; Müller *et al.*, 2012).

Despite this change in focus, vaccination is still an effective method of preventing rabies in cattle (Yang *et al.*, 2014; Yakobson *et al.*, 2015). In Russia, mass vaccination of cattle against rabies began in the late 1970s, with around one to eight million cattle

being vaccinated annually. By 1985, the morbidity rate due to rabies in cattle was <12% of what it had been before vaccination, while in France a vaccination program that targeted both reservoir hosts and cattle decreased the incidence of rabies in cattle by 99.7% (Aubert, 1996; King *et al.*, 2004).

The World Organisation for Animal Health recommends the prophylactic vaccination of cattle against rabies in endemic regions (OIE, 2014). However, few endemically infected countries routinely vaccinate cattle against rabies. In Kazakhstan where dog-mediated rabies is a major problem, livestock animals are regularly vaccinated. In 2015 around 1.5 million cattle and 2.5 million sheep were vaccinated (Sultanov *et al.*, 2016). Korea is one of the few Asian countries where cattle vaccination against rabies is enforced by law (Cheong *et al.*, 2014; Yang *et al.*, 2017). In some countries, such as Israel and Turkey, vaccination of cattle against rabies is voluntary (Vos *et al.*, 2014; Yakobson *et al.*, 2015) except for milking cows and for those animals that will be in close contact with humans (Yakobson *et al.*, 2015). In other endemically infected countries with a high rate of canine rabies such as India, China and Bangladesh, cattle and other livestock are sometimes vaccinated during an outbreak or after being bitten by rabid dogs (post exposure prophylaxis) (Islam *et al.*, 2016; Liu *et al.*, 2016). A few studies have shown that post exposure prophylaxis is successful in cattle (Basheer *et al.*, 1997; Wilson *et al.*, 2010). However, there are issues beyond effectiveness under controlled conditions. Firstly, administering vaccine to rabies-exposed cattle is risky to the personnel involved, and, secondly, under field conditions, it can often be difficult to confirm whether animals have been exposed let alone the timing of that exposure. Thus, despite its huge economic impact especially on smallholder dairy farmers, rabies prevention in cattle remains neglected (Lembo *et al.*, 2010; OIE, 2014; Liu *et al.*, 2016). The key reasons for this neglect are lack of resources, lack of appropriate vaccines, diversion of the resource to control rabies in vector population, low perceived risk of rabies from cattle and high cost of vaccination (Gilbert *et al.*, 2015; Yakobson *et al.*, 2015).

4.0 Immune response to rabies virus and rabies vaccination

The response of the host immune system to the rabies virus has been principally studied under experimental conditions in laboratory animals, with fewer studies in wild animals, vaccinated dogs and humans (Baer, 1991; Srithayakumar *et al.*, 2014). The mechanism of immune response that occurs during natural infection in animals such as dogs and

cattle is limited (Johnson *et al.*, 2010; Srithayakumar *et al.*, 2014). It is still unclear as to how the virus can remain at the site of entry or inoculation and yet not stimulate the host immune system (Wang *et al.*, 2005), as although rabies virus is highly neurotropic, it does not immediately enter the neurons to escape recognition by immune system (Charlton *et al.*, 1997). In addition, immune response is influenced by rabies virus strain, dose and the inoculation route (Wiktor *et al.*, 1977; Hirai *et al.*, 1992). The immune mechanisms involved after infection or vaccination with rabies virus are described under are innate, cell mediated and humoral immunity.

4.1 Innate immunity

The innate immune response is rapid but nonspecific. It is an important defence mechanism during the early stages of infection. The incoming antigens are recognised as non-self and are phagocytised for elimination. The antigens also stimulate the sensors of innate immune cells (RIG-1 and MDA-5), triggering inflammation and release of cytokines and chemokines. This mechanism creates an antiviral environment to kill the virus and/or virus-infected cells either directly or through recruitment of inflammatory cells (Chelbi-Alix *et al.*, 2006; Faul *et al.*, 2010; Choppy *et al.*, 2011). The phagocytised antigens are further processed and presented to the professional antigen presenting cells (dendritic cells) to be recognised by naïve B or T cells. Thus, innate immune cells have crucial role in the initiation of the specific adaptive immune response (Hoebe *et al.*, 2004; Pashine *et al.*, 2005).

However, because pathogenic rabies virus has immunoevasive strategies ('T cell destruction and B7-H1 overexpression'), the induction of the host innate immune system, as just described, occurs only with attenuated rabies virus strains or after vaccination (Choppy *et al.*, 2011). In mice, infection by attenuated rabies virus produced marked inflammatory responses with increased expression of innate immune protein genes, interferons and chemokines compared to infection by pathogenic virus (Wang *et al.*, 2005). The response of the innate immune system was quicker when virus was inoculated intracerebrally as opposed to peripherally. The gamma and beta interferons and antiviral proteins were observed within 2 days of infection after intracerebral inoculation compared to 4 days after peripheral inoculation (Johnson *et al.*, 2006).

Vaccination and revaccination with inactivated rabies virus have both been shown to induce natural killer (NK) cells as the major cell type (Horowitz *et al.*, 2010). The NK

cells as markers of vaccine efficacy have been demonstrated in humans (Panpanich *et al.*, 1992) and mice (Megid & Kaneno, 2000). The incorporation of dendritic cell activating genes into rabies virus has been shown to enhance the activation and recruitment of dendritic cells and B cells. (Wen *et al.*, 2011).

Thus, the innate immune system is crucial in the induction and stimulation of the adaptive immune response. However, long term immunity to rabies infection is dependent on adequate stimulation of the adaptive immune system.

4.2 Adaptive immune response

The adaptive immune response is slow to develop irrespective of whether an animal is infected or vaccinated. Pathogenic rabies virus is highly neurotropic and immunoevasive, which means by the time adaptive immunity has developed sufficiently to provide some protection, the infected naïve host has already succumbed to rabies. Thus, to prevent the development of rabies, vaccination whether prior to or immediately after exposure needs to stimulate the adaptive immune response (Wunderli *et al.*, 1991; Solomon *et al.*, 2005; Franka *et al.*, 2009). This protection is conferred via cell-mediated and humoral immune responses (Wiktor *et al.*, 1977).

4.2.1 Cell mediated immune response

Cellular immunity is mediated by T lymphocytes and initiated upon recognition of antigens. There are two types of T lymphocytes, T helper and cytotoxic T cells. The major role of cellular immunity in rabies vaccination is conferred by T helper cells which induce B cells to produce virus neutralising antibodies (VNA) (Baer, 1991). Turner (1976) demonstrated the essential role of T lymphocytes in immunity to rabies. Thymectomized mice that were devoid of T cells could not produce VNA in response to inactivated rabies vaccination and succumbed to infection upon challenge, whereas all normal mice were unaffected by challenge. Similarly, Zanetti *et al.* (1998) also showed the importance of cellular immunity in protection against rabies virus infection, as mice that resisted challenge to pathogenic rabies virus demonstrated high levels of gamma interferon (produced by T cells). Several other studies have demonstrated increase in the levels of lymphocytes and T cell mediated lymphokines, gamma interferon and interleukins in vaccinated individuals, including cattle (Celis *et al.*, 1986; Thraenhart *et al.*, 1994; Benisek *et al.*, 2006; Bouet-Cararo *et al.*, 2011).

Studies in mice have demonstrated that T cells help to reduce virus replication in the CNS of rabies-infected animals, increase the permeability of the blood-brain barrier and facilitate the entry of antibody-secreting B cells that can clear the virus (Dietzschold *et al.*, 1992). T-cell activity, like the innate response is enhanced in animals infected with the attenuated rabies virus compared to those infected by pathogenic virus (Hooper *et al.*, 2009). Although cell mediated immunity can control rabies virus replication, the complete clearance of the virus from infected host is dependent on the production of VNA antibodies.

4.2.2 Humoral immune response

Stimulation of humoral immunity is critical for protection against rabies (Wunderli *et al.*, 1991). The development of the humoral immune response to rabies virus depends on priming the T helper cells with antigen-presenting cells and loading them with antigens. The antigen-laden T cells migrate to the T and B cell junctions in the secondary lymphoid organs (Okada *et al.*, 2005). Once the follicular B cells are exposed to the antigen, they differentiate to form short-lived plasma cells and early B memory cells or rapidly proliferate to form germinal centres. Within the germinal centres, the B cells further differentiate to form long-lived plasma cells or B memory cells (Nutt & Tarlinton, 2011). These cells secrete large amounts of antibodies upon exposure and re-exposure to pathogens. The fully matured memory B cells and long-lived plasma cells are developed a few days to weeks after vaccination. Thus, the neutralising antibodies appear a few weeks after vaccination (Dorfmeier *et al.*, 2012). The mechanisms by which VNA antibodies remove virus or virus-infected cells include direct antigen binding, inhibition of RNA transcription; restriction of cell-to-cell spread of virus and antibody-induced 'cell-mediated cytotoxicity or complement-dependent lysis' (Davies & Metzger, 1983; Levine *et al.*, 1991; Dietzschold *et al.*, 1992).

Many studies have observed a clear correlation between VNA titres and survival after challenge with virulent rabies virus strains. Thus, measurement of VNA titres has been used as an indicator of effective immune response to vaccination (Abelseth, 1964; Larghi & Nebel, 1985; Wunderli *et al.*, 1991). Although different immune responses complement each other in their functions, VNA have a major role in early and rapid clearance of rabies virus from infected host. Pre-existing rabies-specific VNA can counter rabies virus before it crosses the neuromuscular junctions and prevent its entry

into the CNS. This is crucial as once in the CNS, the virus is shielded from the immune system (Xiang *et al.*, 1995).

5.0 Tests for detection and quantification of rabies virus antibody titres

Several tests are available for detection and quantification of rabies virus neutralising antibodies (VNA) (Chauhan *et al.*, 1991; Shiota *et al.*, 2009). The mouse neutralisation test (MNT) was the first standard test in, which live mice were used to detect and quantify antibodies (Smith *et al.*, 1973). A constant amount of virus was mixed with serial dilutions of sera and was injected intracerebrally to weaning mice. Death of mice 6-14 days after inoculation was considered to be due to rabies infection. Antibody titre was calculated based on the greatest dilution which prevented infection in the mice (Webster & Dawson, 1935).

The requirement for live mice means that the MNT has been replaced by the rapid fluorescent focus inhibition test (RFFIT) and fluorescence antibody virus neutralisation test (FAVNT) as the standard tests for the quantification of VNA titres (Servat & Cliquet, 2006; OIE, 2013). These two tests are based on the principle of in vitro neutralisation of serial dilutions of the serum to be tested with a constant amount of ‘challenge virus standard’ (CVS) followed by inoculation of the cells that are susceptible to rabies (usually BHK-21 cell lines) (OIE, 2013). Antibody titre is determined by counting the proportion of fluorescing cells for each dilution and calculating the dilution which would result in a 50% infection rate (Zalan *et al.*, 1979). Recently rabies-based enzyme-linked immunosorbent assays (ELISA) have been developed as alternatives to RFFIT and FAVNT (Cliquet *et al.*, 2004). Serum is added to antigen-coated wells, and the amount of bound antibody is measured by using anti-IgG linked to colour or fluorescence development (Moore & Hanlon, 2010). The titre levels, calculated from the optical density readings are expressed in equivalent units (EU/mL) based on comparison with a reference serum sample (Knoop *et al.*, 2010).

While choosing a standard test for VNA quantification, the test properties such as sensitivity, specificity, repeatability, rapidity and cost should be considered (Briggs *et al.*, 1998).

5.1 Sensitivity and specificity

Sensitivity and specificity are the common measures used to evaluate the quality of test methods. Use of these terms requires careful distinction as to whether it is for analytical

or diagnostic purposes (Saah & Hoover, 1997). “Analytical sensitivity” is the ability of a test to identify a low concentration of substance or organism in a sample. “Analytical specificity” is the ability of a test to detect a certain organism or substance in the presence of other organisms or substances (Saah & Hoover, 1997). “Diagnostic sensitivity” is the ability of a test to detect the presence of a condition in a population that truly has the condition. “Diagnostic specificity” is the ability of a test to detect the absence of a condition in a population that does not have the condition (Lalkhen & McCluskey, 2008).

In quantitative assays the sensitivity and specificity are dependent on the cut-off values (Lalkhen & McCluskey, 2008). Taking 0.5 IU/mL as the cut-off point and using MNT and RFFIT as reference tests, the specificity of FAVN was found to be 100%. The VNA titres for negative samples obtained by the FAVN test were significantly lower than those obtained using RFFIT and thus represented a clear difference between positive and negative samples (Cliquet *et al.*, 1998). Sensitivity was highly variable between tests, with most of these variations observed with VNA titres close to the cut-off point or below 1 IU/mL (Briggs *et al.*, 1998; Cliquet *et al.*, 1998). Of the three tests, RFFIT was considered to be the most sensitive test (Fitzgerald *et al.*, 1979; Cliquet *et al.*, 1998), but the higher sensitivity of RFFIT was often due to false positives as RFFIT is prone to cytotoxicity from contaminated samples (Cliquet *et al.*, 1998).

ELISA tests have lowered sensitivity and specificity compared to the standard tests (Servat & Cliquet, 2006; Knoop *et al.*, 2010; De Benedictis *et al.*, 2012). The sensitivity and specificity of ELISA kits ranged from 37-82% and 68-100% respectively (Knoop *et al.*, 2010). The collaborative study among 16 European Union reference laboratories that tested 1675 samples found ELISA to be highly heterogeneous. The rate of false positives ranged from zero to as high as 85. The coefficient of concordance ranged between 50 to 90% and only three laboratories could produce the expected coefficient of 90%. The higher agreement could be achieved only with FAVN titres above 5 IU/mL (Servat & Cliquet, 2006).

The main disadvantage with ELISA is that it detects both neutralising and non-neutralising antibodies resulting in increased false positives. Insufficient concentration of antigens coating the ELISA microplates decreases the sensitivity and increases false negatives (Moore & Hanlon, 2010). Lack of a standard calculation to derive the titre

values from the optical density results and sensitivity of ELISA to sample quality also affected the performance of ELISA (Knoop *et al.*, 2010). In addition, the performance of the ELISA is dependent on the type of antigens used. For example, crude glycoprotein G is highly immunosorbent and better at detecting neutralising antibodies than other antigens (Servat *et al.*, 2007).

Therefore, ELISA tests require significant improvements before they can replace the standard FAVN test. In particular, samples detected as negative by ELISA still needs to be tested by standard tests such as the FAVN. Thus, ELISA tests are recommended as a screening tests only (Cliquet *et al.*, 2004).

5.1.1 Sample quality

Samples for the analysis of rabies antibody titres included sera from blood, muscle extract and fluids from body cavities. The presence of haemolysed blood cells and tissues reduced the sensitivity and specificity of tests especially ELISA (Cliquet *et al.*, 2004; Bedekovic *et al.*, 2013). RFFIT is prone to cytotoxicity from contaminated samples with low dilutions and thus increases the risk of false positives (Kurz *et al.*, 1986). Although poor quality samples does not appear to affect the performance of the FAVN test, correct storage temperatures are recommended for better test results (Cliquet *et al.*, 1998, 2004). Serum samples chilled at 4°C are recommended to be tested within 4-5 days; if long term storage is required the samples should be stored at –20°C (Cliquet *et al.*, 2004).

5.2 Reproducibility

A test should be reproducible and consistent when performed by different laboratory personnel. Repeated analysis of a sample should produce homogenous results with a small coefficient of variation (Louie *et al.*, 1975; Fitzgerald *et al.*, 1979). The reproducibility of MNT was affected by variation in susceptibility of the mice used in the test to the rabies virus (Louie *et al.*, 1975; Bedekovic *et al.*, 2013). In RFFIT, reading is done by randomly selecting 20 microscopic fields per microplate and looking for fluorescing cells. This process is subjective which can lead to different endpoint titres with the same samples. Thus, repeatability across laboratories can be poor as the results can vary due to differences between microscopes such as eyepiece, objective and magnification. Further, there is a high risk of re-reading the same field by mistake (Zalan *et al.*, 1979; Ma *et al.*, 2012).

The FAVN test is more objective and reproducible than RFFIT (Briggs *et al.*, 1998) as the test uses an ‘all-or-nothing method’ of reading, with wells being positive if any cells fluoresce (Cliquet *et al.*, 1998). However, there are few studies comparing the reproducibility of RFFIT and FAVN. One collaborative study found FAVN to be less variable than RFFIT (Briggs *et al.*, 1998). Cliquet *et al.* (1998) established a correlation coefficient of more than 99% for the FAVN test with repeated titrations using standard and diluted serum samples, confirming that the test was highly reproducible.

5.3 Rapidity

ELISA is the most rapid test and can be completed in 4 hours (Cliquet *et al.*, 2004) whereas MNT is the most time consuming test taking 14 days to complete (Haase *et al.*, 1985). Although RFFIT can be completed in 24 hours as opposed to 48 hours for the FAVN test, reading 20 microscopic fields per test for the RFFIT is tedious and time consuming.

The FAVN test has been automated in an attempt to decrease the time needed for reading (Briggs *et al.*, 1998); however, computerised automated reading was found to be less sensitive than the visual reading method. Additionally, the automated reading equipment was expensive and needed careful calibration before each test. Thus, automated reading of FAVN was not recommended (Peharpre *et al.*, 1999).

5.4 Cost

Use of live animals and time required for testing made MNT a very expensive test (Zalan *et al.*, 1979; Haase *et al.*, 1985). FAVN and RFFIT are both cheaper than MNT and if facilities are available, it can be scaled up easily to test large numbers of samples (Fitzgerald *et al.*, 1979; Cliquet *et al.*, 1998). ELISA tests do not require sophisticated laboratory equipment or highly trained personnel so are cheaper and easier to use (Cliquet *et al.*, 2004).

5.5 Summary

Table 1 represents the summary of different tests used to measure rabies antibody titres. The FAVN test is the best in terms of overall test quality. Although ELISA testing is cheaper and faster, the test quality is poor and thus ELISAs cannot be used as a standard test for VNA titre determination especially when evaluating vaccine efficacy or the immune status of animals being imported from rabies endemic areas to rabies free areas and vice versa.

Test parameters	MNT	RFFIT	FAVNT	ELISA
Cost	Most expensive	Expensive	Expensive	Cheap
Time	14 days	24 hours	48 hours	4 hours
Test method	In vivo	In vitro	In vitro	In vitro
Reading method	Death of mice	20 microscopic fields	All or none	Change in colour
Test automation	No	No	Yes	yes
Repeatability	MNT < RFFIT	MNT < RFFIT < FAVNT	FAVNT > ELISA, RFFIT	ELISA < FAVNT
Specificity	100%	95%,	100%	68-100%
Sensitivity	83.3%	100%	89.8%	37-82%
Citations	(Louie <i>et al.</i> , 1975; Haase <i>et al.</i> , 1985; Cliquet <i>et al.</i> , 1998)	(Louie <i>et al.</i> , 1975; Zalan <i>et al.</i> , 1979; Haase <i>et al.</i> , 1985)	(Briggs <i>et al.</i> , 1998; Cliquet <i>et al.</i> , 1998)	(Servat & Cliquet, 2006; Knoop <i>et al.</i> , 2010; De Benedictis <i>et al.</i> , 2012)

Table 1 Summary of different tests used to measure rabies antibody titres

5.6 Limitations of virus neutralisation tests

The relatedness of the challenge virus used for the antibody assay and the seed virus of the vaccine is a major factor that can affect the measurement of antibody titres. Studies have shown that when the challenge virus is closely related to the seed virus in the vaccine, higher antibody titres are obtained compared to when they are not related (Zanetti *et al.*, 1998; Rodrigues da Silva *et al.*, 2000). Thus, taking account of this association is essential in assessing protection after vaccination and when evaluating the potency of a new vaccine (Lafon *et al.*, 1990; Moore *et al.*, 2005).

Additionally, the challenge virus standard is a product of several passages in an animal host or in cell culture (Wiktor & Koprowski, 1980). Antigenic variation occurs due to selective pressures such as adaptation to host species (Benmansour *et al.*, 1992). Failure

of post exposure prophylaxis has been documented in humans that could be due to antigenic variation of the rabies virus in natural infection. Failure to cross protect between variants has been demonstrated in the murine model. Vaccination of mice with a variant obtained by single point mutation did not protect against challenge with a different variant or to the parent virus (Wiktor & Koprowski, 1980).

6.0 Efficacy of rabies vaccination in cattle

Effective vaccines reduce the incidence and prevalence of disease. They protect animals from both morbidity and mortality, as well as prevent the spread of infection and the replication of infectious agents (McVey & Shi, 2010). However, most vaccines do not provide complete protection in which under some circumstances vaccinated animals will develop disease. The likelihood of this happening (i.e. the degree of protection) is usually determined by the manufacturers during the development of the vaccine, based on the results of efficacy studies (McVey & Shi, 2010). Efficacy testing involves measuring the virus neutralising antibody titres and duration of immunity in the target species in an experimental environment set up to mimic, as much as possible, the field situation. For rabies vaccinations, efficacy studies generally involve the measurement of VNA titres in vaccinated animals followed by challenge with a virulent strain of virus (OIE, 2013). However, although these studies accurately measure vaccine potency they are not always successful in predicting vaccine efficacy in the field (McVey & Shi, 2010).

6.1 Indication of vaccine efficacy

Animal welfare issues limit the use of challenge studies (Broom, 1991; Ibrahim, 2006), so in the field, the standard test for the efficacy of rabies vaccination is the measurement of VNA titres in vaccinated animals (de Oliveira *et al.*, 2000). This is justified because a clear positive correlation has been established between the rabies VNA titres and survival when challenged with a virulent strain of rabies virus (Aubert, 1992; Côrtes *et al.*, 1993).

When vaccine efficacy is based on VNA titres alone, a cut-off point is needed to distinguish between animals protected if they are exposed to rabies virus and those that will not (Moore & Hanlon, 2010). Different studies have used different cut-off points to compare VNA titres and resistance to challenge. Abelseth (1964) used the reciprocal of the undiluted serum that protected 50% of the mice as cut-off point. Hundred percent

survivability was observed in animals with a titre above this cut-off point. Survivability was reduced to < 25% in animals that had lower serum titres less. Netto *et al.* (1973) assessed the impact of VNA titres on survivability in cattle using three different vaccines: an inactivated sheep brain vaccine, an inactivated tissue culture and a live tissue culture vaccine. All vaccinated cattle were challenged with bovine rabies virus strain M95/60. Similar to Abelseth (1964), Netto *et al.* (1973) used the reciprocal of the serum dilution that protected 50% of the mice as the cut-off point. Based on this, a titre of '10' was chosen. Eighty-four percent of the animals with a VNA titre >10 survived (n=19), whereas only 50% with VNA titres <10 survived (n=20). Similarly, Larghi and Nebel (1985) reported hundred percent survivability of animals (n=28) with VNA titres >48 IU/mL when challenged. The proportion of surviving animals decreased with decreasing VNA titres. When the VNA titre was 1.9 IU/mL the proportion of cattle that survived the challenge was 12/14.

Currently, a VNA titre ≥ 0.5 IU/mL is used as cut-off point to indicate a protective VNA titre. The World Health Organisation (WHO) and the World Organisation for Animal Health (OIE) recommends that all humans and animals vaccinated against rabies should have VNA titre of ≥ 0.5 IU/mL (OIE, 2013; WHO, 2013). As per the European standard, vaccine efficacy should be tested on a minimum of 20 animals of the target species and during the vaccine's protection period the mean VNA titre should not fall below 0.5 IU/mL and, at all times more than 10% of the animals should have a titre of 0.1 IU/mL (European pharmacopoeia, 2013).

In cattle, when vaccinated animals with VNA titre ≥ 0.5 IU/mL were challenged against virulent strain of rabies virus, the survival rate ranged from 85% (n=10) (Côrtes *et al.*, 1993) to 100% (n=10) (Larghi & Nebel, 1985). The survival rate reduced with decreasing VNA titre and at a titre ≥ 0.24 IU/mL, 80% (n=10) of the cattle survived (Côrtes *et al.*, 1993). However, in dogs and cats VNA titres as low as 0.1 IU/mL have been shown to protect 95-100% of animals challenged with virulent rabies virus (n=288) (Aubert, 1992). Darkaoui *et al.* (2016) also found 100% (n=8) survivability in dogs with VNA titre ≥ 0.24 IU/mL.

6.2 Factors influencing the efficacy of rabies vaccination

Multiple factors influence the efficacy of rabies vaccination. Extrinsic factors such as vaccine potency, vaccine type, and the strain of virus used in the vaccine as well as dose

and route of vaccine administration can all be manipulated in order to increase vaccine efficacy (Piza *et al.*, 2002; McVey & Shi, 2010). However, intrinsic factors such as animal health status, animal age and physiological state as well as the innate response of each animal to those extrinsic factors is much more difficult to control. Few studies have recorded the individual animal variation in response to rabies vaccination under same study conditions (Albas *et al.*, 1998). In a study in Brazil that evaluated the efficacy of high egg passaged (HEP) Flury strain of rabies vaccine in cattle, two animals had identical antibody titres 30 days post vaccination. One of the animals succumbed to rabies while the other survived when challenged (Carneiro *et al.*, 1955). Similarly, Albas *et al.* (1998) reported a wide range of VNA titres in cattle vaccinated against rabies (n=21). The mean VNA titre at 90 days after vaccination was approximately 8 IU/mL but the titre ranged from a minimum of 0.5 IU/mL to a maximum of 47 IU/mL. In some animals (n=3), a VNA titre of 0.5- 1 IU/mL was detected on day 90 with zero titre on subsequent days (i.e. 180, 270 and 360 days after vaccination). Although individual variation may not affect the detection of VNA titres, the presence of outliers with higher values than the mean value may significantly influence that mean, and affect the interpretation of results (Zuur *et al.*, 2010).

6.2.1 Effect of vaccine potency

The potency of a vaccine is a measure of the antigen content (OIE, 2013) or the relative strength of that vaccine as defined by USDA. Potency testing is crucial to control vaccine quality before it is released into the market for use. The potency of rabies vaccine can be tested and validated using a variety of methods such as the National Institute of Health (NIH) potency test, the Habel test, rapid fluorescent focus inhibition test, the single radial immunodiffusion test and ELISA (Meslin *et al.*, 1996; Fournier-Caruana *et al.*, 2003). The NIH potency test in live mice is currently the standard test for potency testing of vaccine (Schiffelers *et al.*, 2014).

A desired level of antigen content is required for the vaccine to be potent (McVey *et al.*, 2003). WHO (2005) states that rabies vaccines for use in animals should have a minimum potency of 1 IU/ml when administered via the standard intramuscular route, and that at this potency the vaccine should provide protection for at least one year. This means up to one year following vaccination, the vaccinated animals should survive when challenged with virulent rabies virus in an experimental setting or an outbreak of

rabies in the field. However, a potency of 0.5 IU/ml was sufficient to produce adequate VN titres in cattle when administered intradermally (Benisek *et al.*, 2006).

Although the vaccine potency is essential in the success of vaccination, a potent vaccine is not always efficacious. Factors such as variation in disease pathogenesis and individual immune response determine whether a potent vaccine is protective. This variation between potency and efficacy is more commonly observed with inactivated adjuvanted vaccines than with attenuated vaccines (McVey *et al.*, 2003).

The efficacy of a potent vaccine is measured in terms of its ability to elicit adequate antibody titres in the target species (Heldens *et al.*, 2008). In general, there is a positive correlation between antigen content of a vaccine and VNA production (Wunderli *et al.*, 1991; Fournier-Caruana *et al.*, 2003; McVey *et al.*, 2003). However, as discussed earlier, the actual VNA titre is strongly associated with the type of antigen (live vs inactivated) present in the vaccine. However, even when the type of antigen is consistent there are still issues with the NIH potency test (Schiffelers *et al.*, 2014). One key area that is often neglected is the use of mice in potency tests when the vaccine is to be used in a different species. Piza *et al.* (2002) studied the correlation between NIH potency values obtained from mice to the antibody titres obtained from cattle after vaccination with inactivated adjuvanted vaccines. The study found no correlation between NIH potency values and antibody titres in vaccinated cattle. Instead, they identified a positive correlation between the concentration of virion-attached glycoproteins and antibody titres after vaccination.

Thus, while estimated vaccine potency and efficacy are related, it is not only important to take into consideration antigen content but also the type of antigen present, species of animals and the tests used to assess the potency.

6.2.2 Effect of vaccine storage

Vaccine quality is crucial in determining efficacy. The manufacture of rabies vaccine involves an intricate process of seed virus management, as well as the production of quality substrate for viral growth. At all stages there are essential control processes which include sterility, potency and safety tests. Maintenance of high quality standards at all manufacturing steps is vital if good quality vaccine is to be produced (OIE, 2013).

However, biological products such as vaccines are sensitive to changes in temperature and storage conditions in the field. This is of particular importance to tropical countries

where cold chain facilities are limited. One solution for this issue is to develop rabies vaccines, which are stable at ambient temperatures. Preservation by vaporisation has shown promising results. Smith *et al.* (2015) reported that using that technique the ERAG333 strain of attenuated rabies vaccine could be stored for 23 months at 22°C and 15 months at 37°C, with the vaccine producing protective titres and resistance to challenge in mice. However, these techniques have not yet been tested using currently available inactivated rabies vaccine or applied in the field. Until such testing is done all rabies vaccines must be stored and maintained at 4-8°C to retain their efficacy.

6.2.3 Effect of different types of vaccine

The type of vaccine is a major factor in determining rabies vaccine efficacy in cattle. In calves, vaccination with live vaccine produced rapid and rising antibody titres with protective immunity lasting for 3 to 4 years. The mean antibody titre with these vaccines remained similar irrespective of the dose and booster (Atanasiu *et al.*, 1972; Lawson & Crawley, 1972).

In Colombia, the efficacy of live vaccine was compared with chloroform-inactivated nervous tissue vaccine in cattle. Cattle were injected intramuscularly with 10 to 20 mL of live vaccine and 30 mL of inactivated vaccine. All the animals were challenged after one year with a dog-originated street rabies virus strain. Based on survivability to challenge and antibody titres, live virus vaccine provided better protection than the inactivated vaccine (Gomez *et al.*, 1955), probably because of secondary multiplication of virus in the vaccinated cattle (Abelseth, 1964; Atanasiu *et al.*, 1972).

In contrast, Rodrigues da Silva *et al.* (2000) reported that a currently available commercial live (ERA strain) vaccine was less efficacious than an inactivated vaccine (PV strain). Cattle (n=29) vaccinated with the inactivated vaccines had protective titres that lasted for the duration of the study (180 days), whereas the animals vaccinated with live vaccine had protective titres for <90 days post vaccination. One key difference was that animals given the inactivated vaccine were revaccinated 30 days after the first vaccination whereas animals vaccinated with live vaccine were not. Furthermore, the vaccine strain and strain used for the measurement of VNA titres were homologous for the inactivated vaccine and heterologous for the live vaccine.

Among inactivated rabies vaccine, vero and BHK-21 cell culture vaccines were better in eliciting immune response compared to nervous tissue vaccines in calves. The antibody

titres with tissue culture vaccine were highest until 95 days after vaccination; in contrast peak antibody titres with nervous tissue vaccine were seen up to 40 days post vaccination. Calves vaccinated with tissue culture vaccine were protected when infected with street rabies virus (Basheer *et al.*, 1997).

6.2.4 Efficacy based on virus strains

A large number of rabies virus strains have been used for vaccine production including Pasteur's original 1885 strain and its derivatives such as Pasteur Virus, Pitman-Moore, and Challenge Virus Standard, and more recently isolated strains such as Street-Alabama-Dufferin (SAD), Flury, Vnukovo and Kelev (OIE, 2013).

Significant differences were noted in antibody production after vaccinations with commercially available vaccines based on different strains of rabies virus. Yang *et al.* (2014) compared vaccines prepared from different rabies virus strains such as Pasteur RIV, VP12, SVR-289, G52, PV and ERA strains. All the vaccines were inactivated except for ERA. All the cattle (n=32 per strain) vaccinated with vaccines containing Pasteur RIV, VP12, SVR-289 and the ERA strains had seroconverted when analysed 28 days post vaccination. In addition, the Pasteur RIV, SVR-289 and ERA-based vaccines produced protective VNA titres (≥ 0.5 IU /mL) in all cattle. However, only 24/32 cattle vaccinated with the G52 strain seroconverted and only 16/32 of those vaccinated with the PV strain. Gilbert *et al.* (2015) reported that an ERA-based live virus vaccine was marginally superior to another live vaccine prepared with Street Alabama Dufferin (SAD) strain. Adequate VNA titre was established at 50% or greater neutralisation of virus with 1.25 serum dilution, corresponding to a VNA titre of ≥ 0.2 IU/mL. Using this threshold level, 63% of cattle vaccinated with the ERA strain developed adequate VNA titres whereas only 36% of those vaccinated with the SAD strain did.

6.2.5 Effect of booster vaccination

The revaccination of cattle is emphasised in rabies endemic areas as protection from primary vaccination declines after six months to one year. Albas *et al.* (1998) reported that cattle which received a booster vaccination had protective VNA titres for a longer period (270 days) than those with only a primary vaccination (180 days). At the end of 270 days after vaccination, 71% (15/21) of the cattle that received the booster vaccination had protective VNA titres compared to 7% (1/14) in cattle with only a primary vaccination. Furthermore, the duration of circulating protective VNA titre has

been shown to be dependent on the number of boosters administered to the animals. Yakobson *et al.* (2015) found that all cows (n=10) that received two booster vaccinations 36 and 54 months later had protective VNA titres when assessed 36 months after the last booster vaccination. In comparison, only 59% of the cows that received one booster vaccination had protective VNA titres at that time.

6.2.6 Effect of age and colostral antibodies

Both age at first vaccination and vaccination history of dam have been shown to affect rabies vaccine response (Arnold *et al.*, 1973; Filho *et al.*, 2012; Yakobson *et al.*, 2015). Yakobson *et al.* (2015) reported that calves vaccinated with inactivated adjuvanted vaccine at 6.5 months of age produced significantly higher VNA titres than vaccinating at 3 and 4 months of age. Sixty percent of the calves vaccinated at 6.5 months had protective antibody titres (≥ 0.5 IU/mL) when tested 21-36 months after vaccination. On contrary none of the calves (n=9) vaccinated at 3 months of age had protective VNA titres and only one out of 27 calves vaccinated at 4 months of age had VNA titres at the end of 36 month post vaccination. Similarly, Filho *et al.* (2012) demonstrated positive correlation between age at first vaccination and protective titres. The titres were evaluated 12 months after primary vaccination with inactivated rabies vaccine. The proportion of calves with protective titres were higher in those vaccinated at 6 months of age (1.1 IU/mL) compared to those vaccinated at 2 (0.5 IU/mL) and 4 months (0.8 IU/mL) of age. However, the differences were statistically significant for the comparison between calves vaccinated at 2 and 6 months of age only. Arnold *et al.* (1973) also showed that delaying first vaccination increased VNA titres and linked this to a decrease in colostrum-derived antibodies.

7.0 Effect of route on rabies vaccination response in cattle

In cattle, intramuscular vaccination remains the standard route of vaccination, although some vaccines (e.g. Aluarbiffa, Merial Brazil) are authorised for subcutaneous use only and others (e.g. Imrab 3, Merial USA) are licensed for both subcutaneous and intramuscular use. Vaccine response is dependent on antigen-presenting cells taking antigens from the site of entry and presenting them to the lymphoid tissues and organs for subsequent stimulation of the immune response (Sadat *et al.*, 2017). Both the subcutaneous tissue (principally fat and connective tissue) and muscle have relatively few antigen presenting cells (Fathallah *et al.*, 2013; Liang & Lore, 2016) so the immune response is dependent on transport of the antigen to local lymph nodes and migration of

antigen-presenting cells into the inoculated area (Fathallah *et al.*, 2013). This migration can be enhanced by the inflammatory response produced by adjuvants (Liang & Lore, 2016).

In contrast, although the dermis is a cell-poor layer which principally consists of fibroblasts alongside collagen and elastin fibres, it also contains a dense network of antigen-presenting cells both macrophages and specific dermal dendritic cells. Thus, intradermal vaccination result in the direct stimulation immediately after vaccination of a large population of active antigen-presenting cells (Malissen *et al.*, 2014), potentially increasing the magnitude of immune response. This potential benefit of intradermal vaccination has long been recognised, but its use, especially in humans has been limited by an increased risk of vaccine reactions (Herzog, 2014). However, in cattle, such reactions are less of an issue. Furthermore, in cattle intradermal vaccination prevents muscle damage in food animals and helps to retain meat quality (Sadat *et al.*, 2017), and their relatively thicker skin means that it is easier to administer larger quantities of vaccine compared to humans and laboratory animals (Itzchak *et al.*, 1992).

7.1 Methods of intradermal vaccination

The standard technique used for intradermal vaccination (the Mantoux injection technique; (Reichman, 1979)) uses a 27G, 3/8 “needle attached to a disposable syringe. The surface of the skin is stretched and the tip of the needle is inserted upwards and parallel to the stretched skin. The vaccine is then delivered to uppermost layer of the skin. Resistance to vaccine injection and appearance of raised papule over the skin surface indicates accuracy of the technique. The technique is inherently more difficult to execute than subcutaneous or intramuscular injection with an increased risk of leakage from the injection site (Lambert & Laurent, 2008). This led to the development of alternative methods of intradermal vaccine delivery including needle-free jet injectors and microneedle patches, as well as bifurcated and multipuncture needles (Lambert & Laurent, 2008; Arya *et al.*, 2016). In cattle however, intradermal vaccination with conventional needle and syringe was better at eliciting immune response compared to compared needle-free jet injector (Benisek *et al.*, 2006).

7.2 Efficacy of vaccination via intradermal and intramuscular routes in cattle

In cattle, there are relatively few studies, which have compared intradermal and intramuscular vaccination. Dean *et al.* (2014) reported that intradermal vaccination

against tuberculosis was as effective as intramuscular vaccination (with the same dose of vaccine) in stimulating the immune response. Gupta *et al.* (2001), using a bovine herpes virus-1 vaccine also reported that intradermal vaccination was effective at stimulating the immune response with intradermally vaccinated calves tending to have a greater immune response than intramuscularly vaccinated calves despite only being given half the dose of vaccine. However, there was no evidence that the increased immune response was reflected in better protection against bovine herpes virus 1 infection. Sadat *et al.* (2017) assessed the efficacy of bovine viral diarrhoea virus E2 protein vaccine in 15 bovine calves. The calves were injected with 50 µg of the vaccine intramuscularly and intradermally. There was no significant difference in virus neutralising titre between the two routes compared to placebo. Calves were challenged with bovine viral diarrhoea virus 56 days after primary vaccination. No difference was observed in weight loss, temperature fluctuation and virus shedding between the two groups. Itzchak *et al.*, (1992) compared the antibody response to vaccination with an enterotoxigenic vaccine after intradermal, subcutaneous and intramuscular vaccination (all used the same dose) and reported that the intradermal route produced significantly higher antibody titres than either subcutaneous or intramuscular vaccination, but that increasing the number of vaccination sites (without altering total dose) had no effect on response.

7.3 Intradermal rabies vaccination in species other than cattle

Studies with rabies vaccine in laboratory animals and dogs have shown that intradermal vaccination can be potent. Ray *et al.* (1997) reported that mice (n=6) vaccinated intradermally with one tenth of the rabies vaccine (0.1 µg) produced protective VNA titres comparable to those vaccinated intramuscularly. Challenge with rabies virus 21 days post vaccination protected 100% of the mice in the intramuscular group and 83% of those in intradermal group. In a challenge study in dogs, Lodmell *et al.* (2006) found that all dogs (n=5) vaccinated intradermally once with 50 µg of DNA rabies vaccine survived the challenge with a virulent rabies virus after one year of vaccination. This result was similar to that produced by 1 mL of inactivated rabies vaccine administered intramuscularly; all five dogs in that vaccine group had protective VNA titre (>0.5 IU/mL) compared to 3/5 in the intradermal DNA group. In contrast, none of the dogs vaccinated intramuscularly with 100 µg) of DNA vaccine or the untreated controls were protected against the challenge. In contrast, Osorio *et al.* (1999) found no benefit of the

intra-dermal route in dogs. They reported that all dogs vaccinated intramuscularly with vaccine dilution >1:1400 produced protective VNA titres but a vaccine dilution of 1:30-1:900 was required in the intra-dermal route. Dogs were not challenged in this study so it was not ascertained if VNA titres produced by intra-dermal vaccination was protective.

Intra-dermal rabies vaccination has been studied in in-depth in humans (Madhusudana & Mani, 2014). Its efficacy was first demonstrated by Nicholson *et al.* (1981), who found that 0.1mL of cell culture rabies vaccine administered intra-dermally at eight different sites produced adequate immune response. Since then many trials have been carried out to further evaluate intra-dermal vaccination (Warrell *et al.*, 1985; Madhusudana *et al.*, 2006; Khawplod *et al.*, 2012). These studies have shown that the cost of rabies vaccination can be substantially reduced by using intra-dermal vaccine as the volume of vaccine required can be reduced substantially. For example, in India the total cost of five doses of post exposure prophylaxis using a cell culture vaccine and standard intramuscular regimen of 1 mL per dose is US\$ 30. Using the intra-dermal route, the total volume of vaccine required is reduced from 5mL to 0.7mL and the cost from US\$ 30 to US\$ 4.2 (Madhusudana *et al.*, 2006). Currently, the WHO recommended intra-dermal rabies vaccination regimen for pre-exposure prophylaxis is 0.1mL on 0, 7 and 21 or 28 days. For post-exposure the regimen is 0.1mL on two-sites at 0, 3, 7 and 28 days (WHO, 2005). In addition, intra-dermal vaccination can produce protective VNA titres with lower potency vaccines than those used for intramuscular route. For example, Beran *et al.* (2005) detected protective VNA titres in all subjects (n=30) 14 days after intra-dermal vaccination using vaccines with potencies of 0.5, 0.25 and 0.032 IU/mL. Thus, intra-dermal rabies vaccination is widely practiced for both pre and post-exposure prophylaxis in humans in rabies endemic countries including Thailand and India (Verma *et al.*, 2011).

7.4 Comparison of rabies vaccination via intra-dermal and intramuscular routes in cattle

The high cost of vaccination is one of the key reasons why prophylactic vaccination of rabies is uncommon in cattle in or near endemically infected areas, especially in developing countries (Gilbert *et al.*, 2015). Intra-dermal vaccination could significantly reduce costs and thereby increase vaccination coverage in cattle. However, there are currently no rabies vaccines that are authorised for intra-dermal vaccination of cattle. This is probably because very few studies have evaluated the efficacy of intra-dermal

rabies vaccination in cattle, even though the first study to do so Koprowski *et al.* (1955) was undertaken over 60 years ago.

That study used a high egg passaged live Flury vaccines to compare the effect of route, site and dose on the response to vaccination (presence of rabies VNA as tested using the MNT). The efficacy of different routes, doses and injection sites were compared among seventy adult cattle with nine to eleven cattle in each group. Table 2 summarises the results of testing 30 days after vaccination).

Route	Site	Vaccine dose (mL)	Responders/ Total vaccinated
Intramuscular	Masseter	1.5	4/10
Intramuscular	Masseter	3	6/10
Intramuscular	Thigh	1.5	10/10
Intramuscular	Thigh	3	8/10
Intramuscular	Thigh	6	6/9
Intramuscular	Triceps	6	7/10
Intradermal	Neck region	1	11/11

Table 2: Effect of dose, route and injection site on rabies vaccine response. Adapted from Koprowski et al. (1955)

The study design, with a lack of replicates for each of the sites/routes and the small numbers in each group makes statistical analysis difficult. Re-analysis of the data from intramuscularly vaccinated cattle only using a generalised linear mixed model with a logit link showed no effect of dose or interaction between dose and site on the odds of a response to vaccination ($p > 0.1$), although there was an effect of site ($p = 0.046$). Compared to injection in the thigh, injection in the masseter muscle was less likely to produce a response (odds ratio 0.09; $p = 0.027$). Amalgamating all the data for cattle vaccinated intramuscularly in the thigh and comparing that to the data for cattle vaccinated intradermally showed that, intradermally vaccinated cattle had a higher odds of responding (odds ratio = 5.2, after 0.5 correction for a zero cell). However, small numbers in the intradermal group meant that the 95% CI of the odds ratio were too wide to make any conclusions; i.e. lower 95%CI was 0.23, which is consistent with a biologically important *reduction* in the odds of a response to vaccination if given intradermally rather than intramuscularly into the thigh. Therefore, the results of

Koprowski *et al.* (1955) support intradermal vaccination but on their own do not provide sufficient proof to recommend intradermal vaccination.

In a more recent study, Asokkumar *et al.* (2016) compared the efficacy of intradermal post exposure vaccination against rabies against intramuscular and subcutaneous vaccination. A total of 24 cattle were assigned to three groups (n=8 per group). The cattle were vaccinated on days 0, 3, 7, 14, and 28 with an inactivated cell-culture vaccine with aluminium hydroxide as the adjuvant (Raksharab, Indian Immunologicals). On day 0, the intradermal dose was 0.2 mL, while the cattle vaccinated via the intramuscular and subcutaneous routes were given 2 mL. Subsequently the equivalent doses were 0.1 mL and 1 mL respectively. Serum samples were collected on 0, 14 and 28 days post vaccination. Antibodies to rabies vaccine were quantified using RFFIT. The results are summarised in Table 3.

Day	Mean (SEM) antibody titres (IU/ml)		
	Intradermal	Intramuscular	Subcutaneous
0	0.07±00	0.07±00	0.07±00
14	0.6±0.08	0.89±0.07	0.78±0.08
28	2.54±0.32	2.81±0.40	2.19±0.27

Table 3: Effect of route of vaccination on antibody response. Adapted from Asokkumar *et al.* (2016)

All three routes produced protective antibody titres above the internationally accepted minimum threshold of 0.5 IU/mL in all animals, and no significant difference was seen in antibody titre between groups. As this study was post-exposure, there is the possibility that some of the antibody response was related to exposure to non-vaccine virus. This combined with the small numbers in each group mean that, as with Koprowski *et al.*, (1955), the results support the use of intradermal vaccination but do not provide sufficient proof to recommend it.

Benisek *et al.* (2006) undertook a similar study in animals with no potential exposure to rabies. Their study compared the efficacy of three-vaccine delivery methods- intramuscular injection, intradermal vaccination using needle and syringe, and needleless intradermal vaccination using a jet-injector. The study used 30 healthy bulls, with 10 bulls in each treatment group. A commercial inactivated aluminium hydroxide adjuvanted rabies vaccine (Rabicecell, Mevak Nitra) was used at the dose rate of 0.2 mL

for intradermal vaccination and 1mL for intramuscular vaccination. All animals were vaccinated only once. Serum samples were collected on day 0 (day of vaccination), 14, 35, 90 and 180. Antibody titres were analysed using RFFIT. The results are summarised in Table 4.

Day	Antibody titres (IU/ml)		
	Intradermal	Intramuscular	Jet injector
0	0	0	0
14	0.73 ±0.14	0.51 ±0.11	0.66 ±0.19
35	1.64 ±0.63	1.07 ±0.15	1.49 ±0.57
90	1.40 ±0.45	0.94± 0.20	1.16 ±0.44
180	1.03 ±0.34	0.81 ±0.21	0.80 ±0.27

Table 4: Effect of route of vaccination on antibody response. Adapted from Benisek *et al.* (2006)

The kinetics of the antibody titres were similar in all three groups with peak titres being observed at 35 days post vaccination followed by a continued decrease thereafter.

Intradermal vaccination with a needle and syringe produced a significantly higher antibody response than intramuscular vaccination and needleless jet-injector. However the statistical analysis used by Benisek *et al.* (2006) (unpaired t-tests) does not take into account either the repeated measures nature of the data or correct for the multiple analyses per time point, increasing the risk of type 1 error (incorrect rejection of the null hypothesis). In addition, the data were not tested for normality, which is important as one animal with a high titre can significantly skew the mean, and produce apparently significant differences when there is actually no difference in titres when that animal is excluded.

One apparent difference between the treatment groups is their variability. For example, on day 35 the standard deviation of the intradermal group (0.63 IU/mL) is significantly greater than that for the intramuscular group (0.15 IU/mL) (f-value = 17; p<0.001). Benisek *et al.* (2006) do not discuss the possible causes of this increased variation, but the technique of administration could have affected the results as intradermal injection is subject to more technical error than intramuscular injection (Tarnow & King, 2004).

These issues, combined with the small group size means that the results of Benisek *et al.* (2006) support the use of intradermal vaccination but do not provide sufficient proof

to recommend it. Larger scale studies in cattle selected randomly from a cattle population free of rabies are warranted to properly assess the efficacy of intradermal vaccination.

8.0 Cattle rabies in Bhutan

In Bhutan, rabies in cattle has a significant impact on the economics of farming, on animal welfare and to a lesser extent on human health (Tenzin & Ward, 2012). Sporadic outbreaks in areas of Bhutan which are endemically-infected with rabies resulted in the loss of livestock every year; Tenzin & Ward (2012) estimated that the economic loss due to rabies in livestock from 2001 to 2008 was approximately US\$ 0.08 million with over 80% of the loss due to cattle deaths.

8.1 Cattle farming in Bhutan

Bhutan is a small Himalayan country located in South East Asia between China and India. The country is divided into 20 districts and 205 sub districts. It is characterised as an agriculture-based country with more than 60% of its people depending on agriculture and livestock for their livelihood (MoAF, 2014).

Livestock farming in Bhutan is based on subsistence-oriented mixed smallholder farming system with cattle being the predominant species (Phanchung *et al.*, 2002). The herd size is very small with an average of 8.5 cattle per household. Cattle are reared principally for dairy production. The secondary purposes include production of manure, draught and as assets at times of need (Dukpa *et al.*, 2011). Cattle farming in Bhutan can be broadly classified into intensive and extensive rearing systems. In intensive systems cows are housed and stall fed throughout the year, whereas in extensive systems, cattle graze in the forest during the day and are housed at night. The latter system is by far the most commonly practiced cattle rearing system (Roder *et al.*, 2002; Samdup *et al.*, 2010; Dukpa *et al.*, 2011), with approximately two thirds of the cattle grazing in the forest (MoAF, 2016). When not grazing cattle can be kept either loose within the compound (73%) or tethered (23%). Both rearing systems thus allow close and frequent contact with other animals including dogs. On intensive farms, cattle sheds vary depending on the region. In the colder regions, the floor is mud with solid side walls made of mud or wooden planks. In the southern foothills, shed floors are made up of concrete without side walls that allows accessibility of other animals such as stray

dogs. The latter system is predominant in rabies-endemic regions, increasing the risk of rabies in cattle (Dukpa *et al.*, 2011).

8.2 Rabies in cattle

Rabies is a major notifiable disease in Bhutan and it is mandatory to report every suspected case (MoAF, 2001). A single confirmed case of rabies in any species of animals is considered an outbreak and requires the activation of rapid response team to control and prevent the spread of disease (MoAF, 2001; Tenzin *et al.*, 2017) The disease is common in four southern districts (59 sub districts) covering 29% of the country. The highest proportion of rabies cases are reported in Sarpang and Chukha districts (36% each) followed by Samdrup Jongkhar (16%) and then Samtse (13%). Sporadic outbreaks of rabies are reported throughout the year in these endemic areas with higher incidences in summer and spring (Rinzin K *et al.*, 2006). Rabies incidences varied between areas, with annual incidences being stable in some areas over time whereas in others it either increased or decreased (Tenzin *et al.*, 2011b).

In 2005-2007 rabies was reported in three districts of eastern Bhutan that had been previously free of rabies (Tenzin *et al.*, 2011a). The outbreak affected one human and 256 domestic animals. Cattle were the most commonly affected species (141/256; 55%) followed by dogs (106/256; 41%). The cumulative incidence in cattle ranged from 14 to 23 cases per 10,000 cattle at risk (Tenzin *et al.*, 2011a). Rabies returned to these regions in 2016 killing 6 dogs, one cat and one yak. During this outbreak, the rabid dogs bit four cattle and five people (Tenzin1 *et al.*, 2016).

Dogs are the main reservoir host and vector of rabies in Bhutan, with outbreaks in cattle and other domestic animals always being preceded by cases in dogs. Nevertheless, cattle are usually the most commonly affected species. Of the 814 rabies cases recorded in animals between 1996 and 2009, 55% were in cattle and 39% were in dogs (Tenzin *et al* 2011a). Since 2009, rabies control programmes in Bhutan have significantly reduced the number of reported cases of rabies in all species, but cattle still account for a significant proportion of all cases. Of the 285 animals cases of rabies cases reported between January 2011 and August 2017, 42% were in cattle (TAD info, 2017).

8.2.1 Risk factors

The key risk factors for rabies in animals include being in areas of Bhutan that share a border with India, that have major road networks, that have high human and cattle densities and that have a high proportion of agricultural lands used for arable purposes (Tenzin *et al.*, 2010; Tenzin & Ward, 2012). Of those factors, having a border with India is the most important as these borders are open allowing the free movement of dogs and other animals (Tenzin & Ward, 2012). The importance of these Indian connections is shown by strain typing of the viruses responsible for the outbreaks, as the strains seen in Bhutan are closely related to those circulating in India (Tenzin *et al.*, 2011c).

As discussed above, the extensive grazing system and the limited restrictions on access even when they are housed increases the risk of cattle contracting rabies from rabid dogs. This risk is exacerbated by the ecology of the dog population in Bhutan. There are ~120 000 dogs in Bhutan (1 dog for every 2.5 cattle) of which 48 000 are stray dogs. Of the remaining 72 000 owned dogs, 31% (~22 000) are free roaming (Rinzin *et al.*, 2016). These stray and free roaming dogs feed on waste food and garbage found in human settlements and have extensive uncontrolled contact with cattle (Tenzin *et al.*, 2017). Thus, a high population and density of free roaming dogs further increases the risk of rabies transmission to cattle.

8.2.2 Prevention

Considerable work has been done since 1992 to reduce the occurrence of rabies in Bhutan. As dogs are the main reservoir host and vectors of rabies (Tenzin *et al.*, 2011b; Lavan *et al.*, 2017), most of the resources spent on rabies control in Bhutan have been spent on dogs. Mass vaccination and sterilization of dogs have been carried out throughout the country on an annual basis. However, preventive measures in cattle, especially vaccination, have been a lower priority, principally because cattle are less of a zoonotic risk (Tenzin *et al.*, 2011a). This means that the government is reluctant to support routine vaccination of cattle. Therefore, since economic constraints mean that vaccination by smallholders is not feasible. In Bhutan cattle vaccination is only commonly used as part of the management of a rabies outbreak, with cattle vaccination post exposure being carried out by the government. Nevertheless, in endemic areas of Bhutan, rabies remains common in cattle and causes considerable economic losses to smallholder cattle farmers. Until rabies is eradicated in the reservoir hosts (principally

dogs), free movement of reservoir hosts across the India/Bhutan border and from rabies endemic to non-endemic areas combined with limited and accessible housing of cattle means that rabies will continue to be a problem in Bhutanese cattle and a significant economic issue for the individual affected smallholders.

As economic constraints at both government and smallholder level are a key driver for the lack of prophylactic vaccination in Bhutanese cattle, finding a way to reduce the cost of vaccination could be a useful means of increasing protection of cattle against rabies without markedly increasing the overall cost of rabies control in Bhutan.

Currently, the rabies vaccine used in Bhutan for cattle (Raksharab, Indian Immunologicals) is the same as that used in dogs. It is licensed for both species via the intramuscular or subcutaneous routes. However, as discussed earlier, Asokkumar *et al.* (2016) suggested that in a post-exposure programme, intradermal vaccination of cattle with 1/5 of the recommended intramuscular dose was as effective at stimulating the antibody response as the standard dose given intramuscularly. Meanwhile, Benisek *et al.* (2006) reported that antibody titres after a single intradermal vaccination, with a different inactivated vaccine, were similar or higher than those with intramuscular injection even though the intradermal dose was only 1/5 of the intramuscular dose. The aim of this project was therefore to test whether the vaccine currently used in cattle in Bhutan could be used intradermally at a dose of 20% of the recommended intramuscular dose, by comparing the antibody response to the lower dose intradermal vaccination against the response to the standard dose given intramuscularly. If the intradermal regime proves effective, the significant reduction in cost could stimulate increased private use of vaccines in cattle as well as increased government support of vaccination, thereby significantly reducing the impact of rabies on cattle and cattle farmers in Bhutan.

9.0 Research objectives

The objective of this research was to compare the VNA produced by intradermal and intramuscular administration of inactive rabies vaccine in cattle in Bhutan.

The hypothesis of the study was that the VNA response to intradermal vaccination (at 1/5 recommended dose) would be non-inferior to the response to vaccination using the standard intramuscular route.

10.0 Materials and Methods

10.1 Study design

The study was a multi-site non-inferiority trial with animals randomly allocated to conduct either intradermal rabies vaccination using 1/5 of the recommended dose or standard intramuscular rabies vaccination. The outcome variable tested was rabies virus neutralising titres on days 0, 14, 30, 60 and 90 after vaccination. The live handling of cattle for this study was approved by Research and Extension Division, Department of Livestock, Ministry of Agriculture and Forests, Royal Government of Bhutan.

10.2 Study area

The study was conducted in Haa district, which is located in north-western part of Bhutan. As of 2016, the district had approximately 9119 cattle and 1031 farms or household with cattle. Herd size ranged from one to hundred cattle (MoAF, 2016). There had been no record of rabies outbreak in either dogs or cattle in this district for the past five years (personal communication, Veterinary Officer, Haa). However, there are risks of future outbreaks as this district shares borders with other rabies endemic districts.

10.3 Sample size calculation

Sample size was calculated using the power calculation for a continuous outcome non-inferiority trial, from Sealed envelope Ltd., 2012. Using an α of 0.025 and a β of 0.95 and a standard deviation of 0.63 (from Benisek *et al.* (2006)) and a non-inferiority limit of 0.5, 45 animals were required in each group to detect if there was truly no difference between the intramuscular and intradermal route of vaccination in eliciting protective rabies virus neutralising antibody titres in the vaccinated cattle. The inferiority limit of 0.5 was chosen considering the threshold limit for protective rabies antibody titres of 0.5 IU/mL, and an expectation that peak titres produced by intramuscular vaccination would be at least 1.0 IU/mL.

10.4 Farm selection

The district annual livestock statistics records were used to select the animals for this study. All the data were recorded in an Excel sheet (Microsoft USA). Out of the six sub districts, three sub districts were excluded from the study as these sub districts practiced the transhumant system of rearing, which meant that animals were not available for follow up. The remaining three sub districts, Bji, Kartshok and Eusu were included in

the study. As of 2016, these three regions had 523 farms and 3312 cattle. All farms recorded as having less than four cattle in the herd were excluded by manual selection, leaving 271 farms, which as of 2016, were recorded as having approximately 2500 cattle. Twenty-five farms from this list were selected randomly using a random allocation table. Based on the census, these 25 farms had 260 cattle with maximum herd size of 50. All the farms were located within 20 km radius of veterinary hospital which was approximately at the centre of the three sub districts.

10.5 Animal selection

A minimum of four cattle per farm was required to get a final sample of 100 animals from 25 farms. However, since two of the pre-selected farms had only two cattle in their herd, two extra farms that were located near to the pre-selected farms were chosen, so that 100 animals were allocated to the study from 27 farms. Ninety animals were designated for treatment 'A' or treatment 'B, and 10 animals for controls. Each animal in the farm was randomly assigned to treatment 'A' (1 mL intra-muscular route) or treatment 'B' (0.2 mL intra-dermal route) with equal number of treatment A' and 'B' in each farm. Control animals were selected only on farms that had more than 4 cattle in a farm. Control animals were equally distributed across the three selected sub districts. The selected cows were a mixture of breeds including local, Jersey, Jersey cross and Holstein Friesian cross. Only animals of age six months and above were eligible for selection in order to avoid any interference from maternal antibodies.

10.6 Vaccination

Forty-five animals in treatment group 'A' were administered with 1mL of inactivated rabies vaccine into the middle third of the neck intramuscularly. Similarly 45 animals in treatment group 'B' were injected with 0.2 mL of the same vaccine intradermally at the same site (see Table 5) Benisek *et al.* (2006).

An inactivated cell culture vaccine (Raksharab, Indian Immunologicals, India) produced on BHK21 cell line was used for this study. As per manufacturer's data, the potency of the vaccine was >1 IU/ml and was authorised for intramuscular or subcutaneous use in cattle, dogs and cats (Raksharab profile, 2011).

Group ‘A’ (n)	Group ‘B’ (n)	Control (n)
1 mL intramuscular (45)	0.2 mL intra-dermal (45)	No vaccination (10)

Table 5: Dose and route of rabies vaccine and number of cattle in each treatment group

10.7 Animal management

All animals, irrespective of treatment groups were managed as normal by the owners who were blinded to the treatment allocation. On 16 farms cattle were permanently housed during the winter and stall fed, but during summer all adult cattle were released for grazing during the daytime and housed only at night. On 10 farms, cattle were sent for grazing during the daytime in both winter and summer. One farm housed cattle throughout the year.

The study was conducted in late winter (March) through to summer (June). Day 0 sample collection and vaccination was undertaken on 9th to 11th of March. On most farms, animals were housed for the first three sample collections (days 0, 14 and 30), but on days 60 and 90 all farms had started summer grazing except for the one farm which housed their cows throughout the year.

10.8 Sample collection and shipment

Blood samples were collected on day 0 before vaccination and on days 14, 30, 60 and 90 after vaccination. Blood samples were collected in the morning from 6 am to 11am and in the evening at 4pm to 8pm. This was done to avoid grazing times. Animals were restrained manually and approximately 10 mL of blood was collected via jugular venepuncture into plain vacutainers (BD, India). After collection, the blood samples were allowed to settle for 10 to 15 minutes before moving on to the next farm.

For the blood samples collected in the morning, serum separation was undertaken during the afternoon (1pm-3pm), while for those collected in the evening, serum separation was done at night (9pm-11pm). Blood samples were centrifuged at 1000 g for 15 to 20 minutes, and serum then transferred to screw-capped cryovials before being stored at -20°C (Rodrigues da Silva *et al.*, 2000). Duplicate aliquots were collected for each sample. At the end of the sample collection period (i.e. late June), one serum aliquot per cow was transported by air to the OIE/EU/WHO reference laboratory on

Rabies, Nancy in France for analysis using a FAVN test (Cliquet *et al.* (1998). Each sample from a cow was assigned a sample identification number. The sample identification number was simply the order the samples had been taken in, i.e. the first sample taken was sample 1 and the 75th sample 75. Thus, the laboratory was blinded to the treatment group.

10.9 Sample analysis

The procedure for the FAVN test was as follows. The test was performed on a set of 5 tissue culture microtitre plates each with 96 wells. One microtitre plate was used as the control and the four others to test the sera. Virus stock was prepared by inoculating the suspended BHK-21 cell lines (ATCC number: CCL-10) with rabies seed virus. The infected cells along with growth medium and 10% foetal calf serum were incubated in a 75 mL plastic flask at 37 °C for 40 hours. The virus was then stored in aliquots of 100 µL at -80°C.

Dulbecco's modified Eagle's medium with 10% foetal calf serum and antibiotics (DMEM) was used as diluent. The positive and negative sera controls and the test sera were serially diluted threefold in 100 µL volumes. 50 µL of serum from the first well was added to the adjacent well and diluted four times. Each dilution was mixed at least eight times to make the dilution homogenous. Then 50 µL of challenge virus with 100 TCID₅₀ was added into the wells containing diluted serum. The microplates were then incubated at 36°C±2°C in a 5% carbon dioxide humidified incubator for 1 hour. Following incubation, 50 µL of diluted BHK-21 cell suspension with concentration of 4 X 10⁵ cells/mL was added to each well and further incubated at 36°C±2°C in a 5% carbon dioxide humidified incubator for 48 hours.

Following incubation, the cell culture medium was discarded and the microplates were rinsed once in phosphate buffer solution (pH7.2) and then in 80% acetone. The microplates were then fixed in 80% acetone at room temperature for 30 minutes. After fixation the acetone was drained off and the microplates were air dried at room temperature.

For staining, 50 µL of fluorescein isothiocyanate conjugate was added to each well and incubated at 37°C±2°C for 30 minutes. After incubation, the conjugate was discarded and the microplates rinsed thoroughly using a phosphate buffer solution. The excess

phosphate buffer solution was removed by inverting the microplates on an absorbent paper.

The microplate wells were read using a fluorescent microscope (10X magnification). Starting with the control plate, each well in the microplate was examined for the presence or absence of fluorescing cells. The wells were considered positive if there were any fluorescing cells.

10.10 Calculation of the rabies virus neutralising antibody titres

The 50% endpoint titrations for virus neutralising antibody in the sera and the CVS virus were calculated using the Spearman-Kärber method (Spearman, 1908). The logD50 sera titres were converted to IU/mL using the formula below:

$$\text{Serum titre (IU/mL)} = \frac{[(10^{(\text{serum logD50 value})}) \times \text{theoretical titre of OIE serum } 0.5 \text{ IU/mL}]}{(10^{(\text{theoretical logD50 of OIE serum})})}$$

The theoretical value for OIE logD50 can either be “value of the day” or “mean of the OIE logD50 values”. For this analysis, 1.19 was used as the OIE logD50.

10.12 Test validation

For validation of each test, the current titration results of the challenge virus standard, and the positive and naïve sera were compared to the results of the previous tests done in the laboratory by different laboratory technicians. Each FAVN test was regarded as valid if the values of these controls and the corresponding means from the previous tests did not differ significantly.

10.13 Data analysis

All data were analysed using SPSS statistics 24 (IBM, USA). Three thresholds of antibody titre were used to indicate response to vaccination: ≥ 0.1 IU/mL (minimum titre; European pharmacopoeia, 2013), ≥ 0.24 (potentially protective titre; Aubert, 1992; Côrtes *et al.*, 1993; Cliquet *et al.*, 2000) and ≥ 0.5 IU/mL (indicative protective titre; OIE, 2013; WHO, 2013). The proportion of cattle meeting these thresholds at each time point were compared for the two treatment groups using a generalised linear model with a binomial output and a logit link, with cow as a random effect and time since vaccination and treatment group (and their interaction) as fixed effects.

The antibody titre data were significantly right skewed so were log transformed before analysis. A repeat measures mixed model was then used with log VNA titres as the outcome variable, cow as a random effect and time since vaccination and treatment group (and their interaction) as fixed effects. A heterogeneous first-order autoregressive covariance structure was used based on minimising the Akaike information criterion.

10.14 Potency testing of rabies vaccine (Raksharab)

Two vials of 10 mL of the rabies vaccine used for this study was sent to the same laboratory (OIE/EU/WHO reference laboratory on Rabies, Nancy, France) for potency testing. Potency testing of the vaccine was done using National Institutes of Health (NIH) test as described in the European pharmacopoeia monograph 0451 (European pharmacopoeia, 2013).

11.0 Results

11.1 Descriptive data

Data from 100 cattle and 27 farms located in 14 villages were included in the study. Of the 100 cattle, 68 had been born on their current farm and 32 were imported. Three cattle were imported from India about three years ago and rest were bought from neighbouring districts. The minimum herd size was one and maximum was 11 with a median herd size of seven. Of the 27 farms, 24 farms also kept other animals. There were yak, horses, dogs and cats on 2, 8, 21 and 10 farms respectively.

A total of 480 serum samples were collected from 100 study cattle over the 90-day study period. The distribution of age, sex, breed and body condition score (BCS) of the cattle included in each treatment groups (intramuscular and intradermal) and control are presented in Table 6.

Parameters	Intramuscular group	Intradermal group	Control
Age (years)	5.6 (range: 0.5-15)	3.1 (range: 0.5-12)	4.8 (0.5-9)
Male	5	10	0
Female	40	35	10
Jersey cross bred	31	31	6
Local breeds	14	14	4
BCS (scale of 5)	2.5 (range: 2-3)	2.4 (range: 2-3)	2.5 (range: 2-3)

Table 6: Distribution of age, sex, breed and BCS in each treatment group

11.2 Potency of rabies vaccine

The National Institutes of Health (NIH) test showed that the rabies vaccine used in the study had a potency of 8.5 IU/mL (Limits 2.2 to 37.4 IU/ml), significantly greater than the recommended minimum of 1 IU/mL (WHO, 2005).

11.3 Proportion of animals that responded to the vaccination

On day 0, all cattle in all three treatment groups had titres <0.1 IU/mL. Based on this cut-off point, the overall response rate for intramuscular group was 100% and for intradermal group it was 93% (Table 7). The proportion of the cattle with VNA titre ≥ 0.1 IU/mL between the two vaccination groups was neither affected by route of vaccination ($p=0.35$) nor was there any interaction between vaccination route and time (days after vaccination) on that proportion ($p=0.31$). However, there was an effect of time since vaccination ($P<0.001$). Compared to Day 0, the proportion of vaccinated cattle with a titre >0.1 IU/mL was greater on all other sample days ($p<0.001$); this was also the case for days 14 and 30 compared to day 90 ($p\leq 0.02$).

Three cattle in the control group had VNA titres ≥ 0.1 IU/mL during the study. One control animal had two titres ≥ 0.1 IU/mL on days 14 (0.1 IU/mL) and 30 (0.17 IU/mL) while the other control cattle had only one elevated titre on day 14 (0.17 IU/mL).

Days	Route of vaccination		Control
	Intramuscular	Intradermal	
0	0	0	0
14	44/45 (98%)	40/45 (89%)	2/10
30	43/45 (96%)	40/45 (89%)	1/10
60	37/45 (82%)	39/45 (87%)	0
90	33/45 (73%)	29/45 (64%)	0
TOTAL	45/45 (100%)	42/45 (93%)	0

Table 7: Proportion of cattle that responded to rabies vaccination (VNA titre ≥ 0.1 IU/mL)

11.4 Proportion of animals with protective VNA titres

The proportion of vaccinated cattle with lower (≥ 0.24 IU/mL) protective VNA titre is summarised in Table 8.

Days	Route of vaccination	
	Intramuscular	Intradermal
0	0	0
14	38/45 (84%)	29/45 (64%)
30	37/45 (82%)	30/45 (67%)
60	26/45 (58%)	27/45 (60%)
90	18/45 (40%)	14/45 (31%)
TOTAL	41/45 (91%)	40/45 (89%)

Table 8: Proportion of cattle in each vaccination group with at least VNA titre ≥ 0.24 IU/mL at any time point.

As with the proportion of cattle with a titre ≥ 0.1 IU/mL, there was no effect of vaccination route or interaction between vaccination route and time on the odds of a vaccinated animal having a titre ≥ 0.24 IU/mL ($p = 0.67$ and 0.38 , respectively), but there was an effect of time since vaccination ($p < 0.001$). The comparison of VNA titres between days since vaccination is summarised in Figure 1.

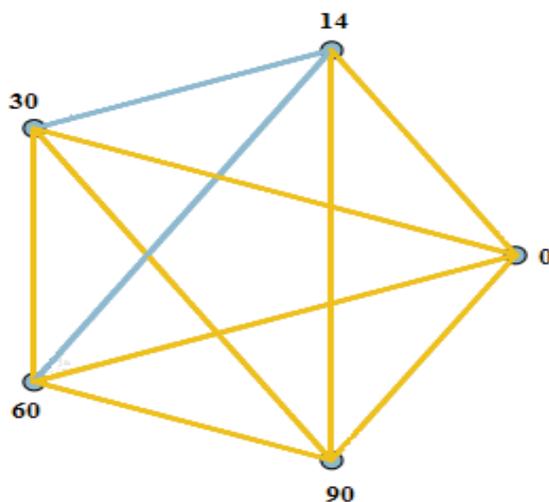


Figure 1: Pairwise comparisons of the effect of time since vaccination on proportion of vaccinated cattle with rabies VNA titres ≥ 0.24 . Blue lines: Comparison between day 14 and day 30 ($p = 0.7$), and comparison between day 14 and day 60 ($p = 0.07$). Gold lines, $p < 0.002$ for all comparisons, except day 60 and day 30 where $p = 0.02$.

For the 0.5 IU/mL threshold, no effect of vaccination route on proportion of titres ≥ 0.5 IU/mL was found ($p= 0.538$). However, there was an effect of the interaction between vaccination route and time and time alone ($p=0.039$ and <0.001 , respectively). This meant that on both day 14 and day 30 the proportion of cattle with a VNA titre ≥ 0.5 IU/mL was lower in the intradermally vaccinated group than in the intramuscularly vaccinated group (Table 9).

Days	Route of vaccination		Comparison between vaccination groups
	Intramuscular	Intradermal	
0	0	0	-
14	35/45 (78%)	16/45 (36%)	$p<0.001$
30	34/45 (76%)	25/45 (56%)	$p=0.02$
60	20/45 (44%)	18/45 (40%)	$p=0.4$
90	11/45 (24%)	9/45 (20%)	$p=0.7$
TOTAL	40/45 (89%)	32/45 (71%)	

Table 9: Proportion of cattle with VNA titre ≥ 0.5 IU/mL at 0,14,30,60 and 90 days

11.5 Effect of vaccination route and time on VNA titres

Time, vaccination route and their interaction were all found to have an effect on VNA titres ($p<0.001$) (see Fig 2). In both groups, VNA titres were higher after vaccination throughout the study period than before vaccination ($p<0.001$ for all comparisons), with the mean VNA titres of both groups peaking on day 30 (though the model found no difference between mean titres on day 14 and day 30 for either group ($p>0.45$)). The decline in titres after the peak was more marked in the intramuscularly vaccinated group than the intradermal group with titres being lower on day 60 than on day 30 in the former group ($p<0.001$), but only by day 90 in the intradermally vaccinated group.

Mean antibody titres were lower on days 14 and 30 in the intradermally vaccinated group than in the intramuscularly vaccinated group. On day 14 the back transformed mean difference between the VNA titres of intramuscularly and intradermally vaccinated animals was 0.62 (95% CI 0.02 to 1.3) IU/mL, whereas on day 30 it was 0.66 (95% CI 0.22 to 1.39) IU/mL. Thus, in terms of the antibody response, the intradermal vaccination was inferior to the intramuscular vaccination.

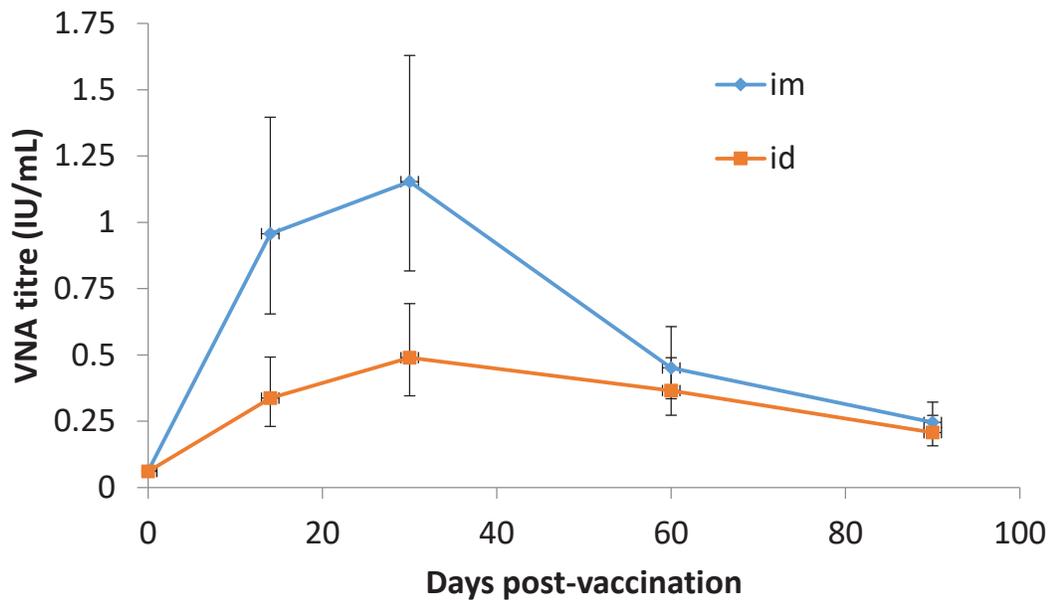


Figure: 2 Geometric mean VNA titres of intramuscularly (im) and intradermally (id) vaccinated cattle on 0, 14, 30, 60 and 90 days post vaccination.

12.0 Discussion

This is the first study to compare the efficacy of intramuscular and intradermal routes of rabies vaccination in cattle in Bhutan, under field conditions. It is also, as far as the author is aware, the first study of intradermal vaccination against rabies in cattle to use more than 10 cattle per treatment group. The study was designed as a non-inferiority trial with the aim of confirming whether the mean VNA titres produced by intradermal vaccination were no more than 0.5 IU/mL lower than the titres produced by the standard intramuscular vaccination. In addition, three thresholds of vaccination response were used in order to further compare the response of the two vaccination routes.

The geometric mean VNA production by intradermal vaccination using 1/5 (0.2mL) of the dose used in standard intramuscular (1mL) route was significantly lower than the standard intramuscular route on days 14 and 30 post vaccination. The back transformed mean difference between intramuscular and intradermal groups was >0.6 IU/mL, indicating that based on the criteria of the study, intradermal vaccination was inferior to intramuscular vaccination. Furthermore, the geometric mean titre in the intradermally vaccinated cattle did not achieve the WHO and OIE recommended threshold titre of

≥ 0.5 IU/mL on any day post vaccination (WHO, 2013; OIE, 2013). However, an overall 71% (32/45) of the intradermally vaccinated cattle had a titre ≥ 0.5 IU/mL on at least one day. These proportions were significantly lower ($P < 0.02$) than the intramuscular group only on days 14 and 30 post vaccination - with 36 and 56% having titres ≥ 0.5 IU/mL on day 14 and 30, respectively compared to the equivalent figures of 78 and 76% in the intramuscular group.

This finding is in contrast to the findings of Asokkumar *et al.* (2016) that used the same vaccine brand as used in this study. They reported intradermal vaccination produced titres equivalent to those produced by the intramuscular route despite using 1/5 of the dose (see Table 10). However, in addition to being a small study (8 cattle per treatment group), this was a post-exposure prophylaxis study, so cattle were vaccinated on days 0,3,7,14 and 28, significantly increasing the chance of a response. Furthermore as a post-exposure study with no untreated controls, it is not clear whether any of the response was due to exposure to wild-type virus.

Days after vaccination	Intramuscular group	Intradermal group
0	0.07	0.07
14	0.89 \pm 0.07	0.6 \pm 0.08
28	2.81 \pm 0.4	2.54 \pm 0.32

Table 10. Effect of vaccine route on mean (SEM) VNA titres in cattle. (Adapted from Asokkumar *et al.* (2016).

A more directly comparable study is that by Benisek *et al.* (2006) who in unexposed cattle reported that the VNA antibody response in their intradermally vaccinated group was significantly greater than intramuscular group despite using only 1/5th of the dose in the former group (see Table 11).

Vaccine route	Day 0	Day 14	Day 35	Day 90
Intramuscular	0	0.51 \pm 0.11	1.07 \pm 0.15	0.94 \pm 0.20
Intradermal	0	0.73 \pm 0.14	1.64 \pm 0.63	1.40 \pm 0.45

Table 11. Effect in cattle of vaccine route on mean (SD) VNA titres (adapted from Benisek *et al.* (2006).

It is unclear why Benisek *et al.* (2006) found different results from this study. They used a different brand of vaccine from that used in this study; however, both vaccines were inactivated rabies vaccines with an aluminium hydroxide adjuvant. The response to the intramuscular vaccination reported by Benisek *et al.* (2006) was different from that seen in this study. One potential difference is in the immunogenicity of the vaccine. While the immunogenicity of the vaccine used in this study was 8.5 IU/mL that is much higher than the WHO requirement for tissue culture vaccine for use in animals (WHO, 2005), Benisek *et al.* (2006) did not report the immunogenicity of the vaccine used in their study. However, the mean VNA titres in their study were still >0.5 IU/mL 180 days after intramuscular vaccination compared to this study in which mean VNA titres after intramuscular vaccination were <0.5 IU/mL within 90 days. Furthermore, the proportion of cattle with a titre ≥ 0.5 IU/mL on days 14 and 30 in the intramuscularly vaccinated group in this study were lower than the WHO targets for tissue culture rabies vaccine of almost 100% (Sudarshan *et al.*, 2011).

The results of this study also seem to be in contrast to the undoubted efficacy of intradermal rabies vaccination in humans (Madhusudana & Mani, 2014). However, in humans pre-exposure vaccination is a multidose regimen that requires three to four doses of vaccine (Khawplod *et al.*, 2006; Permpalung *et al.*, 2013). Furthermore, the results of the current study are consistent with the statement made by WHO (2013) that ‘antibody titres are higher and more sustained after intramuscular injection’.

One potential issue is that intradermal vaccination is more difficult, so some vaccine could have been incorrectly administered into adipose or subcutaneous tissue. However, satisfaction with vaccination was recorded as part of the study (data not shown) and $>90\%$ of vaccinations were recorded as definitively going intradermally. Another potential issue was that as there was no licensed rabies vaccine for intradermal use in cattle, a 10 mL vaccine vial was used for this study. Repeated drawing of vaccine from this multidose vial could have resulted in some animals receiving a dose less than 0.2 mL. Finally, cattle were released for grazing after vaccination and were not monitored afterwards. As intradermal administration can cause irritation at the vaccination site (Vescovo *et al.*, 2017), rubbing induced by irritation at the injection site could have led to leakage of vaccine before being absorbed into the system. Thus, it is plausible that despite a sufficient dose being given intradermally, the vaccine was not retained long enough in the dermal tissue to be absorbed.

Nonetheless, all of these issues would affect individual cows, thereby increasing variability in VNA response between animals not vaccinated correctly and those, which were. However, there was no evidence of variability in this study, in contrast to Benisek *et al.* (2006) in which intradermal vaccination was associated with an increase in variability of VNA titres. Thus, the most plausible rationale for the difference between the results of this study and that of Benisek *et al.* (2006) is differences between the vaccines used in the two studies or between the cattle. In regard to the latter, while Benisek *et al.* (2006) used young bulls weighing 250-300kg, cattle of different breeds, age and sex were used in this study.

Although, actual VNA titres were lower in the intradermally vaccinated group, the differences between the two routes may not have been as great as suggested in Figure 2. Firstly, when a titre of ≥ 0.1 IU/mL was used to indicate a response to vaccination (ignoring whether that response was protective), there was no difference between the groups in the proportion of cattle responding. In the intradermal group 43/45 responded compared to 45/45 in the intramuscular group. In contrast, only 3/10 of the control unvaccinated group had a titre ≥ 0.1 IU/mL. Each of these cattle had only one titre ≥ 0.1 IU/mL (one of which was on Day 30), rather than multiple titres which the vaccinated cattle had. Of the three cattle, one was imported from India and the two others were home-bred. These data combined with there being no evidence of rabies in Haa province during the study period means that these titres in the control are non-specific false positives.

Secondly, when the cut-off for a protective titre was reduced from ≥ 0.5 to ≥ 0.24 IU/mL, there was no difference in response between the two routes and geometric mean VNA titres in both groups were ≥ 0.24 IU/mL throughout the duration of the study following vaccination. This cut-off point was chosen based on the challenge studies conducted in cattle, dogs, cats (Aubert, 1992; Côrtes *et al.*, 1993) and foxes (Cliquet *et al.*, 2000). Côrtes *et al.* (1993) reported a protection rate of 80% in cattle with titre ≥ 0.24 IU/mL when challenged with a virulent strain of rabies virus. However, there was only 5% increase in the proportion protected with titre ≥ 0.5 IU/mL.

Thus, although a higher VNA titre is preferred because a higher titre is associated with higher chances of protection from infection (Aubert, 1992; Côrtes *et al.*, 1993), failure to meet the 0.5 IU/mL threshold does not mean that vaccination has been completely

ineffective. Moreover, any seroconversion following vaccination indicates some degree of protection (Aubert, 1992; Darkaoui *et al.*, 2016), particularly against natural infection, which is usually less severe than the experimental infection used to set thresholds (Larghi & Nebel, 1985; Aubert, 1992). However, much of this is based on data from dogs, which are reservoir hosts and there may therefore be a certain degree of host-virus adaptation that reduces the risk of infection in dogs compared to dead end hosts such as cattle (Aubert, 1992).

Thus, the results of this study do not support the routine use of intradermal vaccination of cattle using the Raksharab at a dose rate of 0.2 mL. Further studies are needed. Challenge studies could be used to confirm whether the titres produced are protective, but these are likely to be expensive and even if they are, it will be difficult to persuade the authorities to use a vaccine route and dose that has resulted in levels below WHO recommendations. An alternative is to develop booster programmes, which result in higher VNA titres for longer duration. The data from this study suggest a booster vaccination 60 days after primary vaccination as titres decreased between day 30 and day 60. However, multiple vaccination programmes may be impractical especially in Bhutan where more than 60% of the cattle graze freely in forest (MoAF, 2016). The final alternative is testing a higher dose. This will increase costs but not as much as multiple dose regimes may do. Increasing the intradermal dose is easier in cattle than humans as cattle skin is relatively thick and it is therefore easier to administer larger quantities of vaccine at one site (Itzchak *et al.*, 1992).

13.0 Conclusion

Intradermal vaccination produced protective titres (≥ 0.5 IU/mL) in 71% of cattle, despite using 1/5 of the recommended dose. However, the proportion of cattle with a protective titre was significantly lower than for cattle given the standard dose (1mL) intramuscularly (71 vs 89%). In addition, mean antibody titres in the intradermally vaccinated cattle were significantly lower than the intramuscularly vaccinated cattle on days 14 and 30 post vaccination. Intradermal vaccination using 1/5 dose was significantly inferior to vaccination intramuscularly using the standard dose. However, the response seen in this study was good enough to support further testing of intradermal vaccination with an increased dose.

14.0 References

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