Antibodies in non-vaccinated dogs

A field study on rabies in dogs in Laos

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Swedish University of Agricultural Sciences, SLU
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Abstract

Rabies is a viral, zoonotic disease caused by a rhabdovirus. Most rabies cases occur in wild animals such as bats, foxes, raccoons, and skunks, however, any mammal can get the infection. 99% of all human cases are caused by bites from rabid dogs. Vaccination of dogs is the most effective way to prevent rabies in people. Each year, approximately 55,000 people die from rabies, and more than 95% of these mortalities take place in Asia and Africa.

According to previous research, rabies is 100% fatal once clinical symptoms have shown. However, studies have now shown apparently healthy, non-rabies vaccinated dogs, other domestic animals, and other wild mammals seropositive for rabies in Brazil, Kenya, Nigeria, Haiti, and the US among other countries.

This study was conducted in Bolikhamsai province, Vientiane province, and Vientiane capital in Laos. Rabies antibody levels in apparently healthy and non-rabies-vaccinated dogs were investigated in order to identify dogs that may have been exposed to the rabies virus and survived. Our study found 35.6% seropositivity for rabies antibodies in Laos, which is a noticeably higher percentage than in an earlier study in Laos where they found 23.7% seropositivity (Fogelberg 2020). The results are also significantly higher compared to other studies in Kenya, Nigeria, and Haiti where they found 20%, 16.1%, and 9.3% seropositivity, respectively (Wosu & Anyanwu 1990; Kitala *et al.* 2001; Smith *et al.* 2019).

When using the lab method for rabies antibodies, there is no way of knowing if these antibodies derive from rabies infection or rabies vaccination. If the measured antibodies in this study do derive from a previous rabies infection and not rabies vaccination, this means that there are many dogs in Laos that survive rabies infection, many more than previously thought.

Even though this study does not provide absolute proof, it contributes to the research on rabies and its serological responses. In conclusion, further research and work need to be done both in Laos and more importantly, in other countries. This is to receive a deeper understanding of the serological levels of rabies antibodies in apparently healthy and non-rabies-vaccinated dogs, and therefore continue to challenge the previous belief that rabies is a 100% deadly disease once clinical symptoms have shown.

Keywords: Rabies, neglected zoonoses, serology, Laos, canines, ELISA

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Abbreviations

ASEAN	The Association of Southeast Asian Nations
BBB	Blood Brain Barrier
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
dFAT	Direct Fluorescent Antibody Test
ELISA	Enzyme-linked Immunosorbent Assay
ERIG	Equine Rabies Immune Globulin
FAO	Food and Agriculture Organization of the United Nations
FAVN	Fluorescent Antibody Neutralization Test
GARC	Global Alliance for Rabies Control
HRIG	Human Rabies Immune Globulin
ID	Intradermal
IM	Intramuscular
NTD	Neglected Tropical Diseases
PCR	Polymerase Chain Reaction
PDR	People's Democratic Republic
PEP	Post-Exposure Prophylaxis
PrEP	Pre-Exposure Prophylaxis
RFFIT	Rapid Fluorescent Focus Inhibition Test
RNA	Ribonucleic Acid
VNA	Virus Neutralizing Antibodies
WHO	World Health Organization
WOAH	World Organization for Animal Health

1. Introduction

Rabies is a viral, zoonotic disease caused by a rhabdovirus, which is a bullet-shaped RNA virus in the *Rhabdoviridae* family, genus *Lyssavirus* (Hankins & Rosekrans 2004). Most rabies cases occur in wild animals such as bats, foxes, raccoons, and skunks (Centers for Disease Control and Prevention 2020), however, any mammal can get the infection. 99% of all human cases are caused by bites from rabid dogs (Dürr *et al.* 2008; World Health Organization 2021). Vaccination of dogs is the most effective way to prevent rabies in people (World Health Organization 2021). Education for both children and adults, such as preventing and treating dog bites, is an important addition to rabies vaccinations.

Each year, approximately 55,000 people (30,000-70,000) die from rabies, and more than 95% of these mortalities take place in Asia and Africa (Hankins & Rosekrans 2004; Dürr *et al.* 2008; Gnanadurai *et al.* 2013). More than 31,000 of these deaths happen in Asia (Ahmed *et al.* 2015). Children are most at risk, 40% of people with suspected rabid bites are children under the age of 15 (World Health Organization 2021). The predicted number of 55,000 annual human deaths from rabies is grossly underreported, with an estimation of between 20 and 160 times in Asia and Africa, respectively (Knobel *et al.* 2005).

Contrary to the common opinion that rabies is 100% fatal, studies have shown apparently healthy, non-rabies vaccinated dogs, other domestic animals, and other wild mammals seropositive for rabies in Brazil, Kenya, Nigeria, Haiti, and the US, among other countries (Gold *et al.* 2020).

In this study, rabies antibody levels in apparently healthy and non-rabies-vaccinated dogs were investigated to identify dogs that may have been exposed to the rabies virus and survived. The antibody levels were investigated with an enzyme-linked immunosorbent assay (ELISA). The dog owners were interviewed about their dogs' health and vaccination status before the blood sampling. This study, together with other studies from around the world, will contribute to an increased understanding of rabies. The potential finding of rabies antibodies in apparently healthy and non-rabies-vaccinated dogs would change the previous knowledge that rabies is a 100% deadly disease.

2. Literature review

2.1 Etiology and transmission

2.1.1 Etiology

The rabies virus particle consists of two different functional and structural parts, the outer envelope and an inner nucleocapsid (Epiwebb 2013a). The RNA genome encodes five different proteins: glycoprotein (G), polymerase (L), matrix protein (M), nucleoprotein (N), and phosphoprotein (P) (Centers for Disease Control and Prevention 2020). How these proteins are arranged is what determines the structure of the virus. There are several viruses and at least six different serotypes in the genus Lyssavirus that are associated to rabies.

2.1.2 Transmission

The virus is most commonly spread through saliva when an infected animal bites another animal or licks an open wound (Hankins & Rosekrans 2004). It can also be transmitted by scratches and aerosolized virus that enters the respiratory tract (Hankins & Rosekrans 2004; Centers for Disease Control and Prevention 2019c). Contact with non-infectious fluid or tissue (blood, urine, feces) is not associated with a risk of infection (Centers for Disease Control and Prevention 2019c).

2.2 Pathogenesis and pathology

After introduction into a muscle, the rabies virus is transported within the nerves of the body, from the infection site to the brain (Centers for Disease Control and Prevention 2019d). After the virus has reached the brain, it multiplies, which leads to an encephalomyelitis, an inflammation of the brain tissue. Before the virus multiplies in the brain, no clinical signs are shown. The virus then travels to the salivary glands and saliva. The virus is also spread from the central nervous system (CNS) through the peripheral nervous system, to the lungs, kidneys, adrenal glands, heart, etc. (Epiwebb 2013b). Circulating antibodies do not occur until late in the

course of the disease. A study showed that dogs can excrete the virus in the saliva up to 13 days before the onset of symptoms (Fekadu *et al.* 1982). If a dog that bit or licked a human shows no signs of symptoms 14 days after, the dog cannot have transmitted rabies virus infection (Folkhälsomyndigheten 2019).

The incubation time is hard to predict and can vary a lot since the infection is spread to the central nervous system (Hankins & Rosekrans 2004). The incubation period can range from 10 days up to a year, but the average incubation time is 20-60 days. Many factors affect the incubation time, for example, the exposure site, the type of rabies virus and if the exposed person or animal has any type of immunity (Centers for Disease Control and Prevention 2019c).

The macroscopical findings in animals who died of rabies infection are nonspecific (Epiwebb 2013b). The microscopical findings consist of non-purulent encephalomyelitis and ganglioneuritis. There are lesions in different parts of the brain, such as the pons, hippocampus, cerebellum, medulla, and broncata.

2.3 Clinical signs

There are different forms of rabies (see below). Early symptoms are not very specific, starting with fever, vomiting, and anorexia (Gnanadurai *et al.* 2013; Centers for Disease Control and Prevention 2019a). Later, the patient suffers from anxiety, delirium, and agitation. When the virus has reached the CNS, symptoms such as extensive salivation and hydrophobia are shown (Gnanadurai *et al.* 2013). Eventually, the nervous system fails, and death occurs (Hankins & Rosekrans 2004), usually within seven days after clinical signs are shown (Centers for Disease Control and Prevention 2019d).

2.4 Different forms of rabies

2.4.1 The furious form

The furious form, also known as encephalitic form, takes form in aggressiveness, barking, whining and hydrophobia (fear of water) (Gnanadurai *et al.* 2013; World Health Organization 2021). Sometimes the patient displays aerophobia (fear of fresh air or drafts) (World Health Organization 2021). The onset of symptoms is often sudden (Dutta 2014). Death occurs due to cardio-respiratory arrest within a week after symptoms are shown (World Health Organization 2021). The main malfunction in the furious form is in the limbic system and the brain stem (Dutta 2014). The diagnostic method of this form is mostly clinical.

2.4.2 The paralytic form

The paralytic form (also known as the dumb form) takes the form of behavioral changes, lethargy, loss of appetite, salivation, and paralysis (Gnanadurai *et al.* 2013). In humans, paralytic rabies accounts for about 20% of reported cases (World Health Organization 2021). The course of the paralytic form is often longer and not as aggressive as the furious form. There is a slow paralysis of the muscles, eventually leading to a coma and later death.

2.5 Diagnosis

2.5.1 Clinical diagnosis

When the dog displays clinical signs such as hydrophobia or aerophobia, clinical diagnosis can be relatively straightforward (World Health Organization 2021). Otherwise, it is much more difficult.

In humans, to only use clinical diagnosis is relatively unreliable, and therefore every patient should receive laboratory confirmation of rabies (Damodar *et al.* 2019). Ante-mortem diagnostics in humans are possible. PCR on samples like cerebrospinal fluid (CSF), saliva, urine, and hair follicles as a method for diagnosing the disease is more commonly being used in clinical settings. What is important to remember is that a positive validated result indicates rabies virus infection, but a negative result does not necessarily rule out the possibility of rabies virus infection. Analysis of CSF/serum and detection of rabies antibodies is not totally dependable as a diagnostic tool in the early stages of infection since seroconversion happens so late in the course of the infection. However, it can be a useful tool in cases where the patient has survived more than a week.

2.5.2 Post-mortem diagnosis

To diagnose rabies in animals, they must be euthanized (Centers for Disease Control and Prevention 2019b). Any part of the affected brain can be used to diagnose rabies, but to rule out rabies virus infection at least two tissue samples from different parts of the brain should be analyzed. Even if the test itself only takes two hours, it takes a lot more time to prepare the samples and ship them to a certified laboratory.

The most reliable laboratory tool is by direct fluorescent antibody test (dFAT) on post-mortem obtained brain tissue (World Organisation for Animal Health 2018; Damodar *et al.* 2019; SHIWA *et al.* 2019). However, this is not often used in humans because of biosafety and religious reasons. It is widely used in rabies

samples from dogs, recommended by both the World Health Organization (WHO) and the World Organization for Animal Health (WOAH). When the brain samples are fresh, the sensitivity is remarkably high. This becomes a problem in several endemic countries, where the samples often become warm due to the warm climate, rapidly decompose, and the sensitivity is therefore reduced (Albas *et al.* 1999).

2.6 Prevention and prophylaxis

In 1885, almost 140 years ago, Louis Pasteur developed the first rabies vaccine. Despite this world-changing development and the availability of three effective vaccines today, the WHO estimates 30,000-70,000 annual worldwide human deaths from rabies virus infections (Hankins & Rosekrans 2004). In comparison, only 1-3 deaths occur annually in the U.S. (Centers for Disease Control and Prevention 2021).

2.6.1 Pre-exposure prophylaxis in people

Once the patient has developed clinical symptoms of rabies, only palliative treatment is possible (Dutta 2014). People who risk being exposed to rabies are recommended to use pre-exposure prophylaxis immunization (PrEP), a series of rabies vaccine doses most often consisting of three rabies vaccine doses on days 0, 7, and 21 (Hankins & Rosekrans 2004; Dutta 2014; World Health Organization 2021). The vaccine is administered intradermally (ID) or intramuscularly (IM) (Dutta 2014).

These people include veterinarians, laboratory personnel, animal handlers, and people traveling to high-risk areas where rabies is an endemic disease. People who risk being exposed to rabies in their work should also take a serology test after vaccination (Folkhälsomyndigheten 2019). An adequate antibody level after vaccination in the serology test is ≥ 0.5 IU/ml.

2.6.2 Post-exposure prophylaxis

If a human has been bitten by a rabid dog, they can get a post-exposure prophylaxis (PEP) which can prevent the development of rabies (Gnanadurai *et al.* 2013). PEP consists of three parts: thorough wound cleaning, a dose of rabies vaccine, and a dose of HRIG (human rabies immune globulin), a solution of dried globulins from serum or plasma given from adult humans who have high titers of rabies antibodies due to immunization with the rabies vaccine, given on the same day as the rabies exposure (Hankins & Rosekrans 2004; Folkhälsomyndigheten 2019; Centers for Disease Control and Prevention 2022). After this, a dose of rabies vaccine is most commonly given on days 3, 7, and 14. Non-vaccinated people should get both the

rabies vaccine (active vaccine) and HRIG (passive vaccine). This gives the previously unimmunized people passive antibodies until active antibodies develop to the vaccine (Hankins & Rosekrans 2004). As much as possible of the HRIG should be administered in and around the wound (Folkhälsomyndigheten 2019). Vaccinated people (or people receiving pre-exposure prophylaxis) should receive only wound cleaning and rabies vaccine (Folkhälsomyndigheten 2019; Centers for Disease Control and Prevention 2022). In developing countries, all bites from dogs, domestic or wild, should be considered potentially rabid (Hankins & Rosekrans 2004). This means that, in addition to PEP, extensive cleaning with soap and water (Hankins & Rosekrans 2004; World Health Organization 2021), detergent, and povidone-iodine (or other substances effective against rabies virus) should be started immediately and the wound should be cleaned this way for at least 15 minutes (World Health Organization 2021).

2.7 Development of antibodies

When the rabies virus reaches the CNS it induces the expression of pro-inflammatory cytokines via an innate immune response (Roy & Hooper 2008). When an individual is infected with rabies, the majority of infected cells are nerve cells in the CNS, which means that the virus has to pass through the blood-brain barrier, BBB (Hooper *et al.* 2011). This is an especially important step in the infection. The passage through the BBB can cause a problem for the immune response since the BBB is meant to keep cells out of the CNS. The rabies virus can be classified into three broad groups: (1) poorly neuroinvasive attenuated viruses that are associated with no required changes in the permeability of the BBB due to a CNS-targeted immune response, (2) neuroinvasive attenuated viruses that reach the CNS but with an increased BBB permeability which allows immune effectors to cross and clear the virus, and (3) lethal, neuroinvasive viruses where there is no proof of permeability changes in the BBB and therefore insignificant invasion of immune effectors into the CNS (Roy & Hooper 2008). The lethality of a rabies virus infection depends on both viral attributes and the host. An early development of CNS immunity is thought to be protective while a late development can actually be harmful due to virus spread.

The primary protection in rabies infections is the presence of virus-neutralizing antibodies (VNA) (Johnson *et al.* 2010). It is unclear whether it is the virus circulating in the periphery or the virus in the CNS that generates the development of these antibodies (Roy & Hooper 2008). Antibodies are normally found in the serum, but the serum antibodies cannot always reach the CNS and are therefore not able to eliminate the virus from the CNS. The development of rabies antibodies comes late in the course of the disease, at earliest six to seven days after the onset

of symptoms (Folkhälsomyndigheten 2019). This means that many patients die before developing antibodies. If the patient has survived a week, analysis of antibodies is a potentially useful tool.

After a rabies vaccination, it takes about a week before the immune system has produced an adequate amount of VNA (Folkhälsomyndigheten 2019). To gain immediate protection, rabies immunoglobulin (RIG) is given immediately after exposure. RIG is passive transmitted VNA. Some countries use human RIG (HRIG), but several endemic countries use equine RIG from horses, ERIG. Both are equally effective. HRIG is administered in doses of 20 IU/kg and ERIG in doses of 40 IU/kg.

2.8 Detection methods for rabies antibodies

2.8.1 Rapid Fluorescent Focus Inhibition Test (RFFIT)

The rapid fluorescent focus inhibition test (RFFIT) was first described in 1973 (Smith *et al.* 1973). The RFFIT is regarded as a standard rabies virus neutralization assay in diagnostic laboratories (Kostense *et al.* 2012), and detects all classes of antibodies (Gold *et al.* 2020). It evaluates the level of *in vitro* VNA in animal or human serum by mixing a constant amount of rabies virus with different serum dilutions on slides, whereafter the slides are incubated at 35°C for 90 minutes in a controlled humidity carbon dioxide chamber (Smith *et al.* 1973). Whatever VNA is present will neutralize the rabies virus. After this, culture cells are added to each slide and the slides with serum/virus/cells are incubated in the chamber once more, this time for 24 hours. Whatever rabies virus that has not been neutralized by VNA will infect the cells which allows any active rabies virus to replicate. After specific staining, the cells are read with a fluorescent microscope. This is to detect any rabies virus production.

The level of antibodies provides an indication of the immune response to a rabies infection (Kansas State Veterinary Diagnostic Laboratory 2022). This test cannot differentiate whether the immune response is due to rabies vaccination or rabies exposure.

2.8.2 Fluorescent Antibody Virus Neutralization (FAVN)

Fluorescent antibody virus neutralization (FAVN) is the gold standard for testing dog and cat serum for rabies-neutralizing antibodies (Wasniewski & Cliquet 2012). This method was developed in 1997, and is an adaption of the original RFFIT (Cliquet *et al.* 1998). The technique is conducted on 96-well tissue culture micro-

plates including positive and negative control serum. The suspended cells are infected with the virus and then incubated in plastic flasks containing growth medium. All serum samples, including the positive and negative control, are diluted four times and then incubated again. After this, the plates are first rinsed and then stained with anti-rabies serum. The plates are then analyzed using an appropriate microscope (Cliquet *et al.* 1998; Ondrejková *et al.* 2012).

FAVN is comparable to RFFIT (Ondrejková *et al.* 2012). This method is however more complicated and has longer incubations (total of 51 hours) than the RFFIT (24-48 hours).

2.8.3 Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA), in contrast to FAVN and RFFIT, does not measure neutralization (Gold *et al.* 2020). ELISA estimates the antibody concentration in serum samples that is able to bind to rabies antigens specifically. ELISA does not require live rabies virus or cell-culture facilities and is faster and easier to run than RFFIT. RFFIT can also detect false positives while ELISA seems to be more specific when it comes to nonlethal rabies exposures (Cleaveland *et al.* 1999). In neutralizing tests, cytotoxicity can occur if the serum samples are of poor quality which results in false seropositives (Cliquet *et al.* 2003). ELISA usually measures a single class of antibody (Moore & Hanlon 2010).

2.9 Antibodies in non-rabies vaccinated individuals

Previous studies have shown apparently healthy and non-rabies-vaccinated dogs and other wild mammals seropositive for rabies virus in Nigeria, Kenya, Brazil, and Haiti among other countries.

In Nigeria, rabies antibodies were found in apparently healthy and non-rabies-vaccinated dogs. Serum samples from 254 dogs were analyzed (with the hem-agglutination-inhibition technique) and 16.1% had a prevalence of rabies antibodies (Wosu & Anyanwu 1990). The prevalence rates of the antibodies in three age groups: over six months, three-six months, and less than three months old dogs were 22.8%, 7.3%, and 17.5%, respectively. According to the publication, these antibodies were suspected to be due to rabies-related virus strains or a non-virulent prototype of rabies.

In Kenya, a study was conducted where 197 serum samples from dogs were screened for rabies antibodies (Kitala *et al.* 2001). 32% were from reportedly rabies-vaccinated dogs. The proportion of dogs with a history of rabies vaccination

with detectable antibodies was 48%, compared to 20% for dogs with no previous history of rabies vaccination. Of the 26 unvaccinated dogs with detectable rabies antibodies, 20 were over one year old and the rest were under one year old. 17 of these dogs were alive one year later while nine had disappeared or died and five of these were euthanized by their owners because of suspected rabies infection).

In Brazil, 100 wild mammals were tested for rabies antibodies with RFFIT, and among these, five animals (5%) were seropositive including three animals (two wild canids and one primate) with VNA >0.5 IU/ml (Campos *et al.* 2020).

In Haiti, a study was conducted to evaluate the efficiency of oral rabies vaccination (ORV) in dogs (Smith *et al.* 2019). Before vaccination, serum samples were collected from 107 dogs, all reportedly non-rabies vaccinated, to evaluate possible antibody levels. The dogs were between 3-12 months old. 10 out of 107 (9.3%) had pre-vaccination antibody levels (RFFIT >0.05 IU/mL), and after using ELISA, seven of these serum samples were also seropositive.

In Laos in 2019, rabies antibodies were found in apparently healthy and non-rabies-vaccinated dogs (Fogelberg 2020). Out of 375 samples, 89 (23.73%) had detectable rabies antibodies using ELISA.

There is substantial evidence that it is possible to survive rabies virus exposure (Gold *et al.* 2020). However, it is hard to estimate the true prevalence of nonlethal rabies virus exposures if the serology studies do not use the correct cutoffs and controls. It is well established that nonlethal rabies virus exposure occurs regularly in bats, but it is not as well established in other mammals.

According to previous research, when the virus reaches the brain, virus clearance is impossible (Gnanadurai *et al.* 2013). However, new research shows increasing evidence of non-lethal rabies virus infections in humans and various animal species. Exactly how this works is not yet completely clear. Gnanadurai *et al.* (2013) suggested that the non-lethal rabies virus infections may have something to do with the presence of high levels of VNA in the CSF that has supposedly crossed the BBB. In lethal rabies virus infections, there are no or very low levels of VNA in the CSF (<0.5 IU). However, there have been cases where previously rabies-vaccinated dogs have resisted infection with high levels of VNA in the serum, but without the presence of VNA in the CSF. If you administer VNA intravenously, it can clear the rabies virus from the CNS (Dietzschold *et al.* 1992). And if the permeability of the BBB is increased, immune effectors can enter the CNS and clear the rabies virus. Research has shown that for this to be effective and give some protection, the permeability of the BBB and the levels of VNA must be combined.

Surveillance of rabies is very important in order to try to monitor the progress of eradication and serology is a good method since it is possible to test live animals and animals without an active rabies virus infection (Gold *et al.* 2020).

2.10 The effects on humans and the society

Over 80% of rabies cases occur in rural areas where access to treatment and education is limited or non-existent (World Organisation for Animal Health 2022). According to previous research, rabies is a 100% deadly disease after clinical symptoms have appeared. Rabies counts as one of the neglected tropical diseases (NTD) which affects vulnerable and poor populations living mostly in rural areas (World Health Organization 2021). Even though effective human vaccines and immunoglobulins for rabies exist, those who really need them cannot access them. A study made by Knobel *et al.* (2005) predicts that there are five times more cases of rabies deaths in rural areas compared to urban areas.

The average PEP treatment costs approximately US$ 49.41 in Asia and US$ 39.57 in Africa, which is a substantial part of the annual per capita GNI (gross national income): 3.87% for someone living in Asia and 5.80% for the average person living in Africa, meaning that many people cannot afford PEP treatment (Knobel *et al.* 2005).

2.10.1 Rabies in Lao PDR

Laos is a country surrounded by five rabies-endemic countries (Ahmed *et al.* 2015). During 2010-2016, 415 brain samples from dogs were submitted for diagnosis and of these, 284 cases (68.4%) were positive for rabies (Douangngeun *et al.* 2017). During the dry season (November-April), the number of cases increased. Dogs are the main reservoir of rabies, causing an average of 8,528 bites annually (Kamsing *et al.* 2012). 99% of the people that are bitten are given wound care and rabies vaccination as PEP. Only 30% receive full rabies vaccination consisting of five doses and immunoglobulin is not available in Laos (Kamsing *et al.* 2012). There are between 20 and 90 reported annual human deaths due to rabies virus infections in Laos (World Health Organization 2018). This is a very low number compared to for example China (1,800-8,100 annual deaths) and India (>8,100 annual deaths). However, when comparing deaths per 100,000 population, Laos and China are on the same level: 0.19-0.6 deaths per 100,000 population. In comparison, India has 1.5-3 deaths per 100,000 population.

The Association of South-East Asian Nations (ASEAN) is working towards economic development and consists of 10 member states, where seven states are rabies-endemic: Vietnam, Cambodia, Thailand, Indonesia, the Philippines, Myanmar, and Laos (World Health Organization - Regional Office for South-East Asia 2012). Among the rabies-endemic countries, Laos has the lowest number of reported human deaths from rabies infections (World Health Organization - Regional Office for South-East Asia 2012). However, it is difficult to collect and analyze data from remote areas and there is also a lacking data system (Ahmed *et al.* 2015). Ahmed *et al.* (2015) conducted a study to evaluate the rabies situation in Laos. From 2004 to 2009, the percentage of rabies-positive samples in Laos increased from 40.5% to 60.2%. The samples collected were mainly from dogs (99.2%), followed by cats (0.6%) and monkeys (0.3%). The number of humans being bitten by animals increased from 8,277 in 2008 to 14,156 in 2011. The circulating rabies viruses in Laos are closely related to those from the neighboring countries (Bourhy *et al.* 2008).

Laos is part of the global strategic plan called "Zero by 30", by United Against Rabies (World Health Organization *et al.* 2019). The goal is to lower the number of human deaths by rabid dogs to zero by the year 2030. United Against Rabies is a collaboration between the Food and Agriculture Organization of the United Nations (FAO), the Global Alliance for Rabies Control (GARC), the World Health Organization (WHO), and the World Organisation for Animal Health (WOAH). The plan consists of three main parts: Availability and access to rabies vaccines, improved treatment of dog bites and human rabies (PEP), and better education. In Western Europe, North America, Japan, and much of Central and Southern America, mass dog vaccinations have helped eliminate rabies from our domestic animals (Hampson 2020). But in Asia and Africa, where the disease is widespread, mass vaccination has barely started. Vaccinating 70% of dog populations in areas where rabies is a high-risk disease is enough to break the transmission cycle.

3. Material and Methods

The practical parts of this study (sampling and laboratory analyses) were done together with veterinary student, Elsa Holmström, whose thesis focused on another aspect of rabies in Laos. The participants in the study answered a questionnaire collecting information on knowledge about rabies, opinions of oral vaccination etc., as well as information about the participating dogs. In this thesis, only the questions about the information of the dogs were used as underlying material.

3.1 Study area and participants

The field study was conducted in three provinces in Laos: Bolikhamsai Province (two districts, four villages), Vientiane Province (two districts, four villages), and Vientiane capital (two districts, four villages). If one of the villages did not have enough dogs or people who were willing to participate in the study, more villages were visited, with the aim to reach 300 dogs. The sampling period was September-October 2022.

The sample size was calculated assuming that 24% of the dogs that have never been vaccinated have antibodies, building on the findings of a previous study in Laos in 2019 made by Fogelberg *et al.* (2020), the appropriate sample size was calculated to 281 dogs. The sample size was deemed to be enough to estimate the prevalence with a precision of 5% and a confidence interval of 95%. With this in mind, the aim of the material collection part of the study was to collect 300 samples. Since the mechanism of potential non-fatal rabies infection is unknown, it was difficult to estimate any intra-cluster (village) correlation, but the author of this paper is expecting this to be negligible as most dogs are kept with limited exposure, and there is a high degree of trade between villages for puppies, meaning that the genetic correlation of dogs likely is low.

Only apparently healthy, non-rabies-vaccinated dogs above the age of three months were included in the study. Aggressive dogs were eliminated from participating in the study. Dogs below the age of three months were not included in the study due to the possible presence of maternal antibodies against rabies.

3.2 Animal owner survey and sampling of serum samples

Before going out in the field to the provinces, our personnel from the partner university in Laos talked to the heads of the provinces to get consent to visit the districts and the villages. After confirmation, the field study was conducted, one province and district at a time. The village leaders were contacted and interviewed and summoned the inhabitants of the village through the village speaker system. Owners who were not capable of getting to the gathering point were visited in their houses instead.

The owners gave written consent to participate in the study and were interviewed before taking the blood samples from the dogs. The owners were asked questions about their dogs, including age, history of previous vaccinations, the main purpose of the dog, and health status.

Butterfly cannulas and serum tubes were used for the blood sampling. All dogs wore a muzzle and were restrained by veterinary students. After the sampling, the dogs received antiparasitics (Ivermectin) given as a subcutaneous injection as compensation for being a part of the study. Dogs under the age of three months were given Helminticide-L (containing pyrantel pamoate, febantel, and praziquantel), but were not sampled. The idea was to give the dogs a dose of rabies vaccine but since there was a possibility that some dogs might have to be sampled again due to seropositivity, rabies vaccines were given to the province's veterinarian who later, after the study had ended, administered the rabies vaccines to the dogs in the villages. A total of 340 doses of rabies vaccines were provided.

Twelve villages were visited in total. 289 blood samples were taken in total, distributed in Bolikhamsai Province: 89 samples; Vientiane Province: 103 samples; and Vientiane capital: 97 samples.

The samples were handled differently depending on how far away the province was from the university. During the field studies, there was no access to a centrifuge, and plasma was pipetted from the tubes, which according to the manufacturer would work as well as serum.

If longer than 48 hours between sampling and access to a centrifuge and after being stored in a cooling box for about 24 hours, plasma was extracted from the blood sample tubes with single-use pipettes and put into Eppendorf tubes as long as the plasma had separated from the blood clot. The tubes were put in a cooling box until

return to the university from the field and were then marked and put in a freezer (-20°C) until lab analysis.

If shorter than 48 hours between sampling and access to a centrifuge: the samples were centrifuged as soon as possible after return to the university from the field and serum was extracted from the blood sample tubes with single-use pipettes and put into Eppendorf tubes. These were marked and put in a freezer (-20°C) until lab analysis. Hemolytic samples were handled the same way since hemolysis should not affect the result, according to the company that distributes the ELISA kits (BioPro).

The initial plan was to contact the owners of the seropositive dogs to see if they were still alive and if they showed any symptoms of rabies and to go back to the villages to sample and analyze the seropositive dogs again. However, due to unexpected circumstances not connected to the study, this was no longer a possibility.

3.3 Detection methods for rabies antibodies

For this study, ELISA (BioPro Rabies ELISA Ab Kit, Prague, Czech Republic) was used as a detection method for rabies antibodies. The manufacturer's instructions were followed, but the process is explained in brief below.

Before analysis, the microplates and a wash solution were brought to room temperature (18-25°C). Working dilution of biotinylated anti-rabies antibodies was prepared by diluting concentrated biotinylated anti-rabies antibodies to 1/100 in diluent for biotinylated antibodies. Working dilution of streptavidin peroxidase conjugate was prepared by diluting streptavidin peroxidase conjugate to 1/100 in the provided diluent.

The serum samples (and positive control serum, negative control serum, and control sera (1-3)) were diluted to 1/2 in sample diluent by dispensing 50µl sample diluent per well and then dispensing 50µl of positive control serum, negative control serum, and control sera (1-3) in the appropriated wells. Then 50µl of the serum samples were dispensed in the remaining wells. After this, the plate was covered with adhesive foil and was incubated at 2-8°C overnight (18-24 hours).

After the incubation, the content was emptied and washed six times with the washing solution. Then 100µl of the diluted biotinylated anti-rabies antibody was dispensed to each well, and the plate was incubated for 30 minutes at 37°C. This procedure was repeated but with diluted streptavidin peroxidase conjugate (this

time the plate was washed four times instead of six). After the incubation, the procedure was repeated but with TMB substrate, and this time, the plate was incubated for 30 minutes at room temperature.

After the last incubation, 50µl of stop solution was dispensed in each well, and the optical density (OD) was read at 450nm.

Due to special circumstances outside of the study, the lab analysis was made by personnel from the National University of Laos (NUoL). 79 out of 82 positive samples in the first run, and 67 out of the 223 negative in the first run were rerun to ensure the authenticity of the detection method. The mean value of duplicated test results was then calculated for a final result for the dog. Three dogs with very deviating results were excluded from further analyses.

3.3.1 Validation criteria

To assure that the test is working in optical conditions, the percentage of blocking for control serums 1, 2, and 3 should be between 45-70%, 25-45%, and lower than 30%, respectively.

3.3.2 Interpretation

The percentage of blocking (PB) was calculated for each sample with the formula below:

$$PB\% = \frac{OD_{NC} - OD_{sample}}{OD_{NC} - OD_{PC}} \times 100$$

Serum sample with PB lower than 40% = negative for rabies antibodies. Serum sample with PB equal to or higher than 40% = positive for rabies antibodies. Serum sample with PB equal or higher than 70% = antibody level equal or higher than 0.5 IU/ml based on FAVN test; assumed protective level.

3.4 Data analyses

For statistical analyses, any dog that might have been sampled in spite of the owner reporting rabies vaccination, was removed from the data set. When samples were analyzed repeatedly, the average results of the runs were used as the final results. The association between seropositivity and categorical variables were analyzed using Chi2 and exact Fisher's test. The difference in means was assessed using Student's T-test. Statistical analyses were done in STATA 14.2 (StataCorp, College Station, Texas).

4. Results

A total of 289 dogs (belonging to 162 dog owners) were included in this study. Presented in Table 1, out of the 289 dogs, 103 (35.64%) were seropositive for rabies antibodies. Out of these 103 seropositive samples, 64 (62.14%) dogs had protective levels of rabies antibodies. Presented in Table 2, the average age for all dogs was 2.13 years. For the seropositive dogs, the average age was 2.5 years, which is significantly higher than the average age for the seronegative dogs; 1.91 years ($p = 0.0419$). When comparing the rerun results with the ones from the first run, there were three samples that deviated a lot. These three samples were excluded from the study.

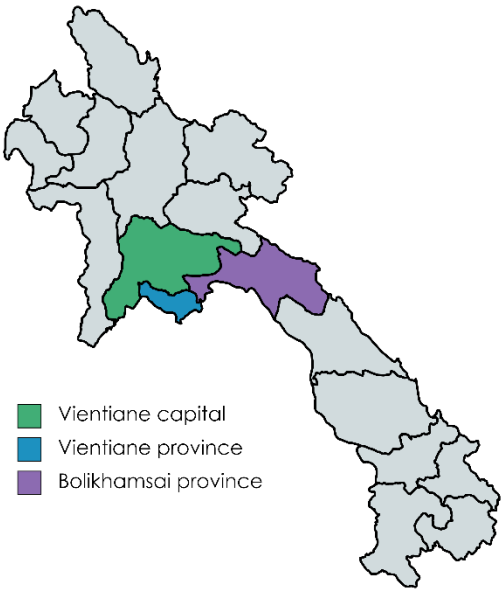

Figure 1. Map of Laos. The three provinces visited are marked in color. Mapchart.net. Laos. https://www.mapchart.net/asia-detailed.html [2022-12-21]

Figure 1 displays a map of Laos and the three provinces visited. The distribution between the different provinces and districts can be seen in Figure 2. The district with the highest number of seropositive dogs was Xaythany district in Vientiane capital with 59.79% seropositivity, where 63.79% of these were on a protective level (PB >70%). This was followed by Viengkham district in Vientiane province

with 37.21% seropositivity, where 68.75% of these were on a protective level (PB >70%). There was a significant difference between the districts (p <0.001).

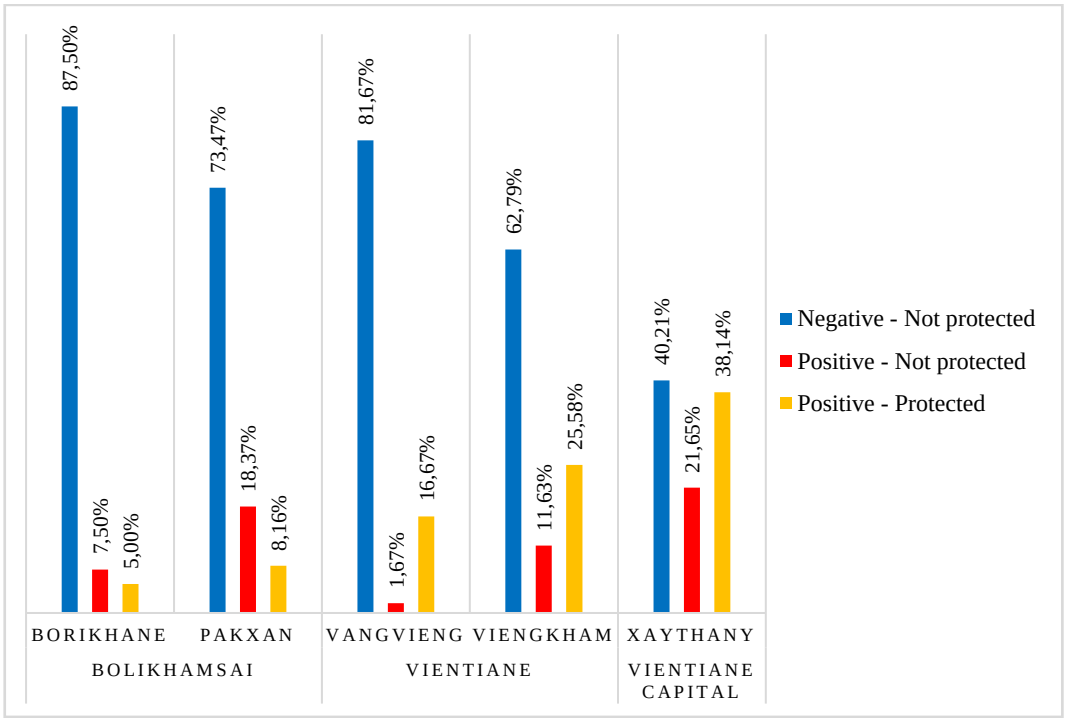

Figure 2. Comparison of rabies serological status among domesticated dogs in different provinces and districts in Laos.

Based on the answers of the owners, 285 out of 289 dogs had not shown any signs of aggression. The owner of two dogs answered “don’t know”. One dog had shown signs of aggression towards only dogs, and one dog had shown signs of aggression toward both dogs and humans. These four dogs were all seronegative for rabies antibodies.

Only four out of 289 dogs had gotten bite wounds in the last six months, and there was no association with seropositivity (p = 0.890). One of the bitten dogs was seropositive on a protective level (PB >70%), the others were seronegative.

Thirteen out of 162 animal owners knew of at least one dog that had rabies in their area: nine in Bolikhamsai province, two in Vientiane province, and two in Vientiane capital. In addition, seven out of 162 knew of at least one human that had rabies in their area: four in Bolikhamsai province, two in Vientiane province, and one in Vientiane capital.

There were no significant differences in seropositivity or protected levels depending on how the dogs were kept. The majority of the dogs were kept as guard dogs, 264 out of 289. Out of these, 35.98% were seropositive for rabies antibodies,

and 64.21% of those had a protective level (PB >70%). 23 dogs were kept as company, and 34.78% of these were seropositive, with 37.50% of these seropositive dogs on a protective level (PB >70%). Figure 2 shows comparison of the serological status among domesticated dogs with different living situations in Laos.

Table 1. Serological results (numbers and percentage) for rabies antibodies in domesticated dogs in Laos, compared with different factors (Province/District, vaccination status, age of the dog, living situation, the purpose of the dog and bite wounds in the last six months).

	Total	**Negative**	**P-value**	**Positive**		
		Total		**Total**	**Not protected**	**Protected**
Province/District	289	186 (64.36%)	<0.001	103 (35.64%)	39 (13.49%)	64 (22.15%)
Bolikhamsai	89	71 (79.78%)		18 (20.22%)	12 (13.48%)	6 (6.74%)
- Borikhane	40	35 (87.50%)		5 (12.50%)	3 (7.50%)	2 (5.00%)
- Pakxan	49	36 (73.79%)		13 (26.53%)	9 (18.37%)	4 (8.16%)
Vientiane	103	76 (73.79%)		27 (26.21%)	6 (5.83%)	21 (20.39%)
- Vangvieng	60	49 (81.67%)		11 (18.33%)	1 (1.67%)	10 (16.67%)
- Viengkham	43	27 (62.79%)		16 (37.21%)	5 (11.63%)	11 (25.58%)
Vientiane capital	97	39 (40.21%)		58 (59.79%)	21 (21.65%)	37 (38.14%)
- Xhaythany	97	39 (40.21%)		58 (59.79%)	21 (21.65%)	37 (38.14%)
Age of the dog (mean. in years)	2.13	1.91	0.0419	2.50	2.31	2.62
Living situation			0.319			
Both indoor and outside. when outside in a leash	17	10 (58.82%)		7 (41.18%)	3 (17.65%)	4 (23.53%)
Both indoor and outside. when outside loose	81	47 (58.02%)		34 (41.98%)	16 (19.75%)	18 (22.22%)
Only indoor	13	7 (53.85%)		6 (46.15%)	1 (7.69%)	5 (38.46%)
Only outside in a leash	13	12 (92.31%)		1 (7.69%)	1 (7.69%)	0 (0.00%)
Only outside loose	121	80 (66.12%)		41 (33.88%)	13 (10.74%)	28 (23.14%)
Only outside loose but in a fenced area	44	30 (68.18%)		14 (31.82%)	5 (11.36%)	9 (20.45%)
Purpose of the dog			0.751			
Guard	264	169 (64.02%)		95 (35.98%)	34 (12.88%)	61 (23.11%)
Company	23	15 (65.22%)		8 (34.78%)	5 (21.74%)	3 (13.04%)
Guard and company	1	1 (100%)		0 (0.00%)	0 (0.00%)	0 (0.00%)
Don't know	1	-		-	-	-
Bite wounds in the last six months			0.890			
Yes	4	3 (75.00%)		1 (25.00%)	0 (0.00%)	1 (25.00%)
No	285	183 (64.21%)		102 (35.79%)	39 (13.68%)	63 (22.11%)

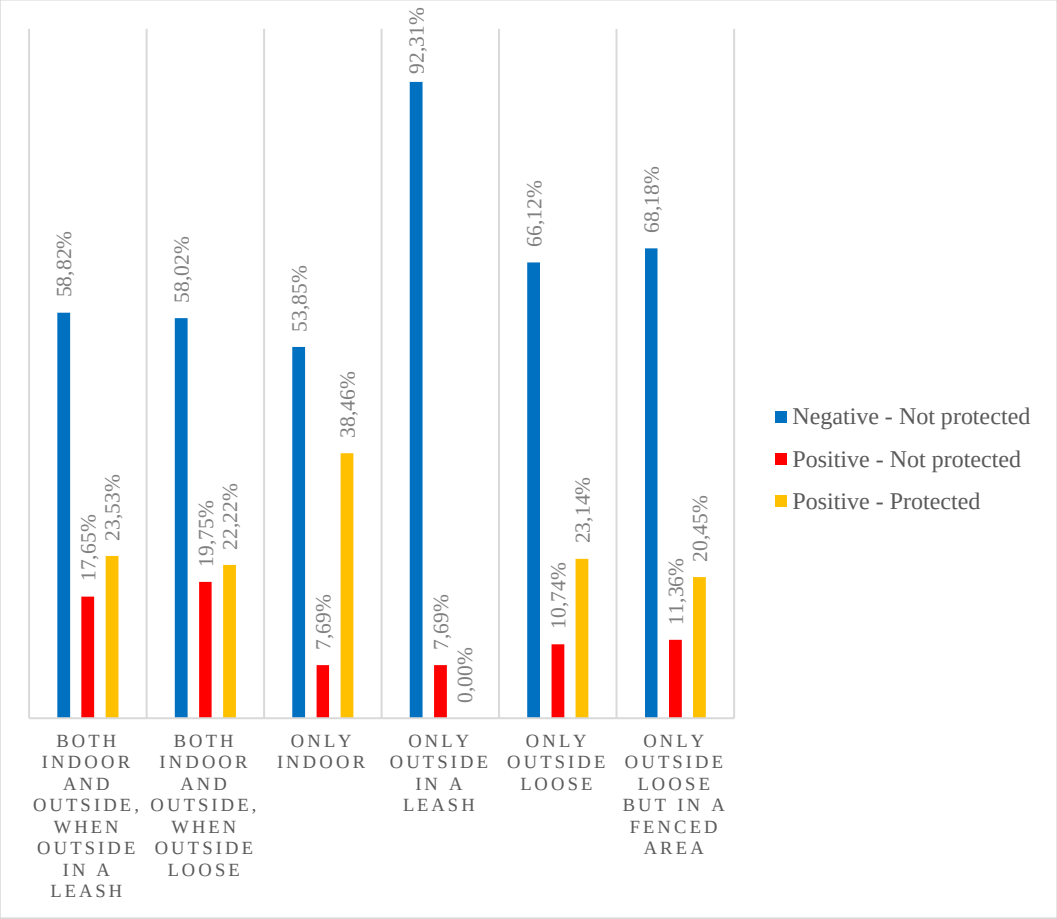

Figure 2. Comparison of rabies serological status among domesticated dogs with different living situations in Laos.

Table 2. Average and standard deviation (SD) of age (in years) of the participating dogs.

	Average	SD
Negative	**1,91**	**2,03**
Positive	**2,50**	**2,50**
Not protected	2,31	2,35
Protected	2,62	2,60
Total	**2,13**	**2,23**

5. Discussion

This study was conducted in Bolikhamsai province, Vientiane province and Vientiane capital in Laos. Rabies antibody levels in apparently healthy and non-rabies-vaccinated dogs were investigated in order to identify dogs that may have been exposed to the rabies virus and survived. Our study found 35.64% seropositivity for rabies antibodies in Laos.

The overall seropositivity found in this study was significantly higher than what was found in Kenya, Nigeria, and Haiti where the result was 20%, 16.1%, and 9.3% seropositivity, respectively (Wosu & Anyanwu 1990; Kitala *et al.* 2001; Smith *et al.* 2019).

Even when comparing this study's overall result with the previous one made in Laos in 2019 which measured 23.73% seropositivity (Fogelberg 2020), the difference is noticeable. 375 dogs were reportedly not rabies-vaccinated and 89 of these were seropositive. The samples were also analyzed with BioPro Rabies ELISA Ab kit, the same as in this study. The study in 2019 was conducted in Vientiane prefecture. Is it just a coincidence that Xaythany district, which was also investigated in 2020, had the highest seropositivity in this study? Are the same dogs sampled again, meaning that the majority of the seropositive samples in this study are from rabies-vaccinated dogs? But that would mean that both the heads of the provinces, the provincial and district veterinarians, village leaders, and animal owners had forgotten about the vaccinations, and this does not seem likely since it has only been three years between these field studies. Also, the majority of the personnel from the National University of Laos were the same people in 2022 as in 2019, so these people should remember which districts/villages were visited in 2019.

There was a significant difference in seropositivity between the different districts and provinces included in this study ($p < 0.001$). Xhaythany district in Vientiane capital had the highest level of seropositivity in this study: 59.79%, where 63.79% of the positive animals were on a protective level. This was followed by Viengkham district in Vientiane province with 37.21% seropositivity, where 68.75% of the positive dogs were on a protective level. The district with the lowest level of

seropositivity was Borikhane district in Bolikhamsai province: 12.50%, where 40% of these were on a protective level. The results vary a lot and there can be different reasons for this. For example, there may have been a vaccination campaign in Xhaythany district that people are not aware of, meaning that the serological result may be a response to vaccination and not an actual rabies virus infection. There is also a possibility that there has been a circulating rabies virus in that area, but of either a low-pathological virus strain type that does not cause a deadly infection, or a virus strain causing a subclinical rabies infection. If this study truly has sampled different dogs from other villages than in the last study made in Laos in 2019, this means that the overall seropositivity really is higher than before, and that the antibodies most probably derive from infection and not vaccination.

There were no significant differences in seropositivity, nor having protective levels, depending on the dogs' living situation ($p = 0.319$), main use ($p = 0.466$) or bite wounds in the last six months ($p = 0.890$).

5.1 The ELISA method used

The detection method used for rabies antibodies in this study was ELISA. All control serums (1, 2, and 3) were in the correct intervals, meaning that the ELISA worked as expected. A relatively low number of samples had very conflicting results when re-run, which indicates that the laboratory processes worked well and there was repeatability.

The FAVN test and the RFFIT, both neutralization methods, are referenced by the WHO, WOAH, and the European Commission (Wasniewski & Cliquet 2012). These are however expensive, advanced, and time-consuming, which means that many labs will not have the capacity to run these analyses. Therefore, other methods such as ELISA have been developed. Wasniewski and Cliquet evaluated the BioPro ELISA kit in 2012. The specificity was 100% and the overall agreement with neutralization tests was 86%. Two false positives were found out of 701 tested serum samples. In non-vaccinated animals, the specificity was 100%. Since ELISA is a quite easy technique and not time-consuming, it is the preferred method for field surveys and samples of poor quality. Because of this research and that the control serums were in the right intervals, the risk of false positives is considered low in this study.

5.2 Sources of errors

In this study, there are several potential sources of errors. There may have been a vaccination campaign in a certain area even though both the heads of the provinces, the provincial and district veterinarians, village leaders, and animal owners were asked about it. However, this seems unlikely since there were so many groups of people interviewed and no information about any campaigns of the type was found. There is a small risk that some dog owners may have answered that their dog/dogs are not vaccinated to be allowed to participate. There is no way of knowing if this was the case, but all dogs (except pregnant ones) irrespective of earlier vaccination status, received Ivermectin and the province's veterinarian received rabies vaccine doses to be handed out after the study had ended. There is also the possibility that the owner has forgotten or does not know about their dog/dogs being vaccinated. This is due to several factors such as if the dog/dogs have changed owner, if the owner being interviewed is not the one responsible for the dog's healthcare or if the overall knowledge about vaccines is poor, leading to the belief that every shot received is of another type of medicine, for example antibiotics.

Several factors may have had some effect on the blood samples. For example, since this field study was conducted in Laos, the temperature and humidity were high. This, in combination with limited cooling possibilities, may have affected the quality of the blood samples. Due to the lack of a centrifuge in the field, some samples had to wait longer than others to get centrifuged. If longer than 48 hours between sampling and access to a centrifuge, the plasma was extracted from the blood and was used instead of serum. Hemolytic samples were marked and then analyzed the same way as the other samples. According to the company that distributes the ELISA kits (BioPro), one purpose of the BioPro Rabies ELISA Ab Kit is to analyze wildlife samples that are often hemolytic or of poor quality. Because of this, the probability for the sample handling to be a source of error is deemed to be low.

Lastly, there are several potential sources of errors with the lab analysis. ELISA requires certain instruments and environments to function properly. Due to the before-mentioned unexpected circumstances, the lab analysis was conducted by the National University of Laos. The lab did not have the most optimal resources and instruments for this method; some instruments were broken, and some were not calibrated right. Several factors could affect the precision. There is always a risk that the instructions were not followed correctly all the way, and that the required amount of fluids/antibodies may not have been precise. For example, if the amount of pipetted antibodies was too high, the percentage of blocking (PB) is falsely increased. However, the control serums (1, 2, and 3) were all in the correct intervals, indicating that the ELISA worked as expected. And most of the rerun samples had

results that were similar to the first run, except a few that were excluded from the study. And even if for example the pipette used is not calibrated right, the dogs who had protective levels of rabies antibodies should still be seropositive since the interval is so big (≥40% PB = seropositive, not protected and ≥70% = seropositive, protected).

Even if the result from this study shows 35.64% seropositivity, there is no way of knowing for sure if these antibodies come from an actual rabies infection or rabies vaccination since ELISA cannot differentiate between these two. Right now, there is no single detection method for rabies antibodies that can do this. Many other vaccines are produced without a certain protein as a marker to differentiate between vaccine antibodies and infection antibodies (Tumpey *et al.* 2005), but this is not yet available for rabies.

To increase the credibility of the results from a field study like this one, a test that could differentiate between infection antibodies and vaccine antibodies would be optimal. But since this does not exist yet, it is particularly important to try to ensure that the dogs included in the study are apparently healthy and non-rabies vaccinated. This was ensured by interviewing province leaders, district veterinarians, village leaders, and dog owners. In this study, a questionnaire was written in English which was translated into Lao by colleagues at the National University of Laos.

After the interviews, the questionnaires were translated back into English to be analyzed. This means that there is a risk of misinterpretation and therefore the answers may not fully represent the truth. There were several questions where the answers did not make sense, which indicates a misinterpretation somewhere down the line. The person translating might not have fully understood the questions in English, or the dog owner might have not understood the question in Lao, or there might have been an error in the translation back to English. To minimize this risk in future studies of the same sort, it would be recommended to hire a professional translator to translate the questionnaires and later test the questions on some people before the field study. It would also be in the study's best interest to bring the professional translator to the field, making it possible to do the interviews more direct.

Overall, there have been a few struggles with this study. Firstly, the communication with the partners for the study in Laos before arrival was difficult and therefore challenging to prepare properly before arriving at the university in Laos. The language barrier was also a challenge. When conducting a field study, things can always be improved. For example, hiring a professional translator, and making sure

the translations of the questions are complete and ready upon arrival would be an important improvement to make. Having more planned meetings with everyone involved before going out in the field would also be a good idea, minimizing any confusion about the goal of the study. It would also be preferred to perform the lab work internally instead of handing it over to the partner university, therefore ensuring that the instructions, etc. are followed correctly and that the lab work is done on time. However, this was not possible due to unexpected circumstances outside of this study which could not have been accounted for beforehand.

6. Conclusion

The results from this study does not provide proof of seropositivity in healthy and non-rabies-vaccinated dogs in Laos, since there is no method to differentiate vaccination antibodies from rabies infection antibodies. There is a small risk that the sampled dogs actually had an active rabies infection, even though they appeared healthy. There were 35.64% seropositive dogs in this study, which is a significantly higher percentage than in other previously investigated countries. Even when comparing to a previous study in Laos from 2019, there is a noticeably higher seropositivity in apparently healthy and non-rabies-vaccinated domesticated dogs in Laos in 2022. If these antibodies do derive from a previous rabies infection and not rabies vaccination, this means that there are many dogs in Laos that survive rabies infection, many more than previously thought.

Even though this study does not provide absolute proof, it contributes to the research on rabies and its serological responses. In conclusion, further research and work need to be done in Laos but more importantly, in other countries. This is to receive a deeper understanding of the serological levels of rabies antibodies in apparently healthy and non-rabies-vaccinated dogs, and therefore continue to challenge the previous belief that rabies is a 100% deadly disease once clinical symptoms have shown.

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Popular Science Summary

Rabies is a disease caused by a virus (rabies virus). It is a zoonotic disease, meaning that rabies can be transferred between animals and humans. Most rabies cases occur in wild animals such as bats, foxes, raccoons, and skunks (Centers for Disease Control and Prevention 2020), however, any mammal can get the infection. There are two forms of rabies: a furious form and a paralytic form. The furious form, also known as the encephalitic form, takes form in aggressiveness, barking, whining and hydrophobia (fear of water) (Gnanadurai *et al.* 2013; World Health Organization 2021). The paralytic form (also known as the dumb form) takes the form of behavioral changes, lethargy, loss of appetite, salivation, and paralysis (Gnanadurai *et al.* 2013). 99% of all human cases are caused by bites from rabid dogs (Dürr *et al.* 2008; World Health Organization 2021). Vaccination of dogs is the most effective way to prevent rabies in people (World Health Organization 2021). Education for both children and adults, such as preventing and treating dog bites, is an important addition to rabies vaccinations.

Each year, approximately 55,000 people die from rabies, mostly in Asia and Africa (Hankins & Rosekrans 2004; Dürr *et al.* 2008; Gnanadurai *et al.* 2013). More than 31,000 of these deaths happen in Asia (Ahmed *et al.* 2015). Children are most at risk, 4/10 of people with suspected rabid bites are children under the age of 15 (World Health Organization 2021). The predicted number of 55,000 annual human deaths from rabies is grossly underreported of between 20 times in Asia and 160 times in Africa (Knobel *et al.* 2005).

According to previous research, rabies is 100% fatal once clinical symptoms have shown. However, studies have now shown apparently healthy, non-rabies vaccinated dogs, other domestic animals, and other wild mammals seropositive (positive for rabies antibodies) for rabies in Brazil, Kenya, Nigeria, Haiti, and the US among other countries (Gold *et al.* 2020).

This study was conducted in Bolikhamsai province, Vientiane province and Vientiane capital in Laos. Rabies antibody levels in apparently healthy and non-rabies-vaccinated dogs were investigated in order to identify dogs that may have been exposed to the rabies virus and survived. Our study found 35.64% sero-

positivity for rabies antibodies in Laos, which is a noticeable higher percentage than in an earlier study in Laos where they found 23.73% seropositivity (Fogelberg 2020). The results are also significantly higher compared to other studies in Kenya, Nigeria, and Haiti where they found 20%, 16.1%, and 9.3% seropositivity, respectively (Wosu & Anyanwu 1990; Kitala *et al.* 2001; Smith *et al.* 2019).

When using the lab method for rabies antibodies, there is no way of knowing if these antibodies derive from rabies infection or rabies vaccination. If the measured antibodies in this study do derive from a previous rabies infection and not rabies vaccination, this means that there are many dogs in Laos that survive rabies infection, many more than previously thought.

Even though this study does not provide absolute proof, it contributes to the research on rabies and its serological responses. In conclusion, further research and work need to be done both in Laos and more importantly, in other countries. This is to receive a deeper understanding of the serological levels of rabies antibodies in apparently healthy and non-rabies-vaccinated dogs, and therefore continue to challenge the previous belief that rabies is a 100% deadly disease once clinical symptoms have shown.

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