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Potential antibody titers in non-vaccinated dogs

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Swedish University of Agricultural Sciences, SLU
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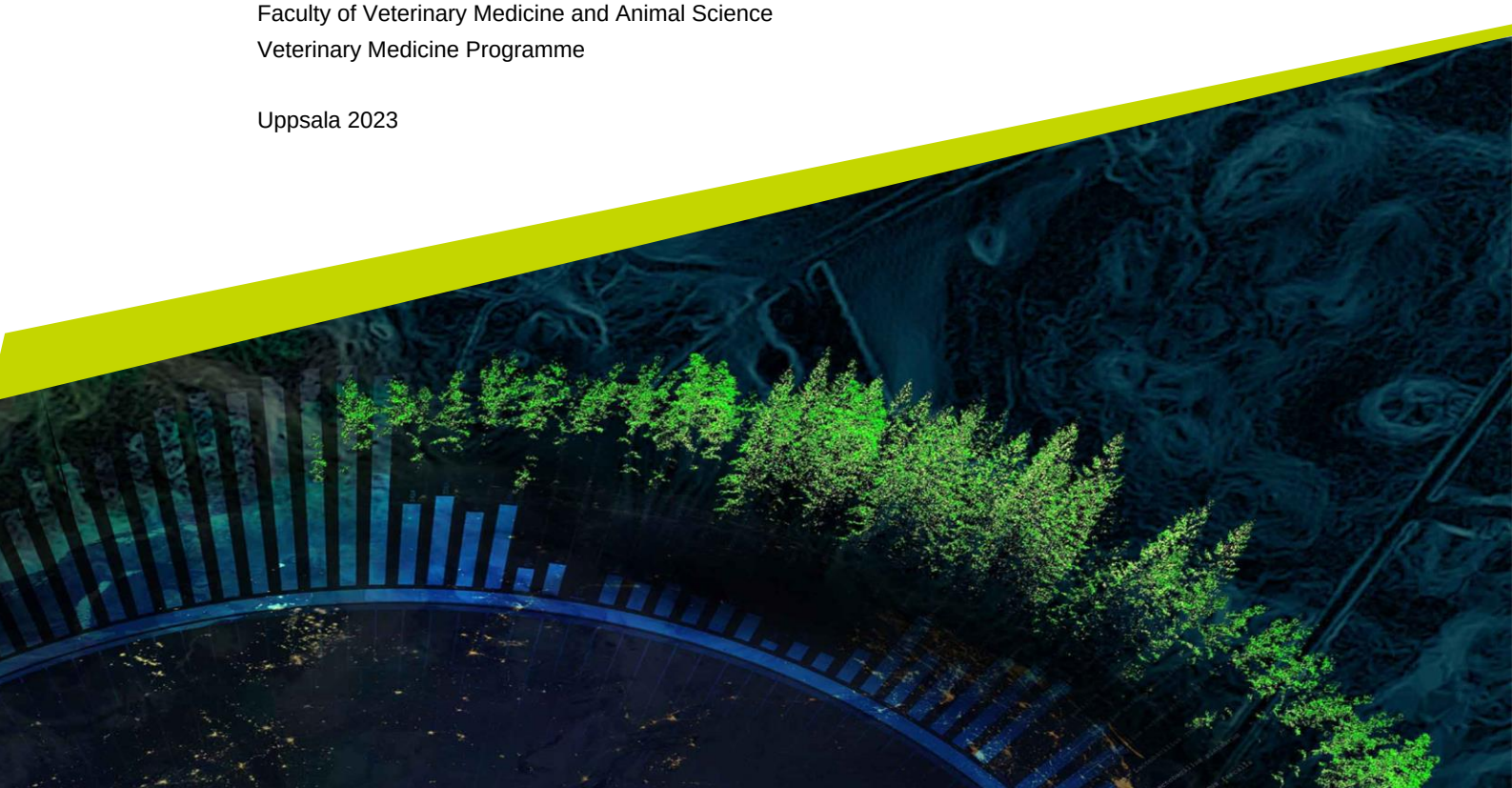

A field study on rabies in dogs in Cambodia: Potential antibody titers in non-vaccinated dogs

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Abstract

Rabies is a highly fatal viral disease, capable of infecting virtually all mammalian species. Once symptoms occur mortality is generally considered to be 100%. Each year the virus is responsible for the loss of approximately 59,000 human lives around the world. The most common route of transmission to humans is bites from domesticated dogs, where the virus is transferred through the infected dog's saliva into the bite victim. Death can, however, be prevented through the use of vaccines or post-exposure treatments in the form of vaccinations and antibody injections to temporarily fight off the virus until the body's own immune system can defeat it.

Despite the near certainty of death if the infection progresses into the symptomatic stage, there have been reports of dogs in rabies endemic areas around the globe who have antibodies against the disease in their serum, despite not having received any rabies vaccines. Since antibodies are not seen in the blood until a few days after the debut of symptoms, these individuals should, based on the mortality rate, not exist, or at the very least be incredibly rare. Some studies have however reported an antibody prevalence of up to 27% in some regions.

In this study the potential of rabies virus neutralising antibodies (RVNA) in non-vaccinated domestic dogs was examined, looking for signs of potential non-lethal infections of the virus. Since the majority of rabies cases caused by domestic dog bites occur in Asia and Africa, this study took place in Cambodia, which has one of the highest estimated cases of rabies mortalities in Asia. Sera was collected from 97 dogs across three different provinces. The samples were then tested for antibodies using an enzyme-linked immunosorbent assay (ELISA) test. The test both detected the samples that were positive for RVNA as well as measured the level of antibodies against the virus. All the positive samples were analysed again in a control run.

The results found that 9% of the dogs in the study were positive, despite there being no report of previous vaccination campaigns performed in the area. The owners also reported that none of the positive dogs had been vaccinated prior to the sampling. The study also found a statistically relevant correlation between age of the dog and testing positive for antibodies, where older dogs were more likely to have antibodies.

The positive dogs in one of the provinces, Kampong Speu, were also resampled a few days later and analysed again. The result was that 3/4 previously positive dogs tested positive once more, further strengthening the result that non-vaccinated RVNA positive dogs do indeed exist in rabies endemic regions.

Keywords: Rabies, antibodies, ELISA, serology, Southeast Asia, non-vaccinated, canine, immunity

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Abbreviations

ABLV	Australian bat lyssavirus
CDC	Centres for Disease Control and Prevention
ELISA	Enzyme-linked Immunosorbent assay
EBLV-1	European bat lyssavirus 1
FAVN	Fluorescent antibody neutralization test
IPC	Institute Pasteur Cambodia
IU	International unit
NAHPRI	National Animal Health and Production Research Intitute
PEP	Post-exposure prophylaxis
PG	Phylogroup
PrEP	Pre-exposure prophylaxis
RAPINA	Rapid neutralizing antibody detection test
RABV	Rabies virus
RFFIT	Rapid fluorescent focus inhibition test
RIG	Rabies Immunoglobulin
RVNA	Rabies virus neutralising antibodies
SLU	Swedish University of Agricultural Sciences
SVA	Swedish National Veterinary Institute
WHO	World Health Organisation

1. Introduction

Rabies is a virtually 100% fatal disease (WHO 2021) that can affect any mammal (CDC 2019a). If the patient develops symptoms it is nearly always a fatal infection (WHO 2021). The disease is 100% vaccine preventable but to this day it is still endemic in different regions of the world, mainly in Africa and Asia.

Rabies causes approximately 59,000 human deaths per year, with 59.6% occurring in Asia (Hampson *et al.* 2015). The majority of human cases of rabies, approximately 99%, are caused by domestic dogs (CDC 2020), most likely due to the vast amount of human interaction with dogs compared to wildlife.

In Cambodia the official reported number of confirmed human deaths due to rabies is a total of 16 cases between 2015-2017 (WHO 2019). However, the rabies situation in the country is largely underestimated due to the lack of hospitalisation (Ly *et al.* 2009). A model based on data from Institute Pasteur in Cambodia estimated that around 810 human rabies deaths would occur during 2007.

Reports of antibodies in nonvaccinated domestic dogs have been seen around the world and in several other species (Gold *et al.* 2020), indicating non-lethal rabies infection in these individuals and challenging the previous belief that virtually all infections are fatal. One study in Laos found that 23.73% of dogs stated to be nonvaccinated by their owners tested positive for rabies virus neutralising antibodies (RVNA) (Fogelberg 2020).

This study aims to investigate the degree of naturally occurring antibodies in non-vaccinated dogs in the Takeo, Kampong Speu and Kampong Chhnang provinces in Cambodia. It also aims to investigate the level of antibodies and whether they reach levels that are protective against future infections.

2. Literature review

2.1 Rabies virus

Rabies virus (RABV) is of the genus *Lyssavirus*, belonging to the *Rhabdoviridae* family (OIE 2018). All lyssaviruses are transmissible to humans and have been shown to cause clinical disease indistinguishable from rabies. However, RABV is found globally and causes the overwhelming majority of reported cases of animal and human rabies. The other lyssaviruses appear more restricted geographically and have a smaller host range, mostly isolated from bats, and have a limited implication on animal and public health.

RABV is a single-stranded, non-segmented, negative sense RNA virus composed of an internal core protein or nucleocapsid, which contains the nucleic acid, and an outer lipid-containing bilayer covered with transmembrane glycoprotein spikes (Rupprecht 1996, 2002). In the virus genome there are five different encoded proteins. A transcriptase (L), a nucleoprotein (N) and a transcriptase-associated protein (NS) comprise a ribonucleoprotein complex, together with the viral RNA. This complex can aggregate in the cytoplasm of infected neurons and form Negri-bodies, characteristic for rabies infection. The two remaining proteins; matrix (M) and glycoprotein (G) are associated with the lipid envelope.

2.2 Epidemiology

Rabies causes approximately 59,000 human deaths per year, with 59.6% occurring in Asia (Hampson *et al.* 2015). The majority of human cases of rabies, approximately 99%, are caused by domestic dogs (CDC 2020), most likely due to the vast amount of human-dog interaction compared to wildlife. Approximately 40% of all deaths are children under the age of 15 (Rupprecht *et al.* 2010). This is due in part to their curious nature and shorter stature, which makes it more likely that potential bite wounds occur at higher risk locations such as the head (Liu & Cahill 2020).

The global economic cost of rabies is estimated to be around 8.6 billion USD annually, the majority of the cost due to premature deaths leading to loss of productivity (Hampson *et al.* 2015).

2.3 Transmission

RABV is transmitted through direct contact between infected saliva and nervous tissue with broken skin or mucous membranes such as the eyes, or mouth (CDC 2019b).

The most common route of transmission is through bite wounds, but it is possible to be infected just through exposure to infected saliva (CDC 2019b). RABV has not been shown to be contagious through other routes such as contact with faeces or urine.

The virus is fragile and do not persist long in the environment (Rupprecht 2002). The virus becomes non-infectious if dried out or exposed to sunlight (CDC 2019b).

2.4 Pathogenesis

The virus can either enter the peripheral nervous system directly from the wound, or remain in the muscle tissue where it replicates and remains throughout most of the incubation period (Rupprecht 1996). As the infection progresses it spreads to the central nervous system through retrograde axial movement where it causes a fatal encephalomeningitis (WHO 2021). The virus, after extensive replication, then transits to the salivary glands where it is shed, allowing for transmission to other hosts (Rupprecht 2002).

2.5 Development of rabies antibodies

Rabies antibodies in serum appear late in the disease progression, usually 6-7 days after symptoms has appeared, while RVNA in cerebrospinal fluid appear even later in the process (Folkhälsomyndigheten 2019).

Due to the RABV immunoevasive ability to enter the nervous system without triggering apoptosis of the cells, as well as its ability to kill protective, migrating T-cells the immune response and antibody production is delayed (Lafon 2011).

The blood-brain-barrier could also play a part in the delay of the immune response, as well as the fact that the virus only increases its replication once it has reached the central nervous system (Johnson *et al.* 2010). Therefore, the amount of antigen in the periphery is limited. All of this in combination can lead to a delayed presentation of viral antigen to B-cells.

2.6 Prevention

Rabies is a vaccine preventable disease. The most cost effective way to prevent the spread of rabies to humans is through vaccination of domestic dogs, since they are the most common source of human infection (WHO 2021).

2.6.1 Pre exposure prophylaxis (PrEP)

PrEP is a prophylactic vaccination of an individual, but does not eliminate the need for post exposure prophylaxis in humans, but can however reduce the number of vaccine injections and in some cases eliminate the need for rabies immunoglobulin (RIG) injections (Liu & Cahill 2020).

Two types of rabies vaccines exist today: nerve tissue and cell culture vaccines. WHO recommends the cell culture vaccine due to higher efficacy as well as higher safety (WHO 2022). There are two different kinds of cell culture vaccines available: Human diploid cells and purified chick embryo cell vaccine (Liu & Cahill 2020). The vaccines can be administered either intra-muscularly or intradermally, with equally good results (WHO 2022).

Although vaccination of domestic dogs is the most effective way to prevent human cases of rabies (CDC 2020), preexposure prophylaxis should be offered to high-risk populations, such as veterinarians, researchers or travellers spending a long time in endemic regions (Liu & Cahill 2020). Vaccine should be administered in two doses, on day 0 and day 7 (Folkhälsomyndigheten 2019). A serological titre of ≥ 0.5 IU RVNA 14 days after vaccination is considered protective against rabies (Tarantola *et al.* 2019).

Dogs should be vaccinated after 3 months of age, and then receive booster doses according to the vaccine manufacturers recommendations (CDC 2019c). While different vaccine companies have different booster guidelines, many suggest a booster interval of 3 years (Fass Vet 2020, 2021). However, immunity after vaccination in dogs extends well beyond 3 years (Dodds *et al.* 2020) and could still affect the titers in the older dogs who have not gotten a booster dose in time or have been vaccinated earlier in their lives. It should be noted however, that the antibody

responses to vaccinations differ between dogs, not all individuals reach protective levels after vaccination and that different vaccines can differ in the level and duration of protection received (Minke *et al.* 2009).

2.6.2 Post exposure prophylaxis (PEP)

Post-exposure prophylaxis (PEP) is the recommended immediate treatment after a suspected infected bite wound (WHO 2021). The goal is to prevent the virus from reaching the central nervous system. This is achieved through three different steps: extensive washing of the wound, vaccination and injection of RIG if indicated.

PEP of a non-vaccinated individual

With a correct PEP regimen, the prevention rate is virtually 100% (Folkhälsomyndigheten 2019). PEP should be started as soon as possible post exposure. The degree of PEP needed depends on the amount of exposure, which is divided into three different categories, see *table 1*.

Table 1. Degrees of exposure to rabies (Folkhälsomyndigheten 2019; WHO 2021)

Category	Degree of exposure	Example	PEP
1	No exposure	For an example petting or feeding animals, licks on intact skin	No PEP needed, recommended washing of the area
2	Exposure	Abrasions or scratches without bleeding, nibbling of uncovered skin	Wound washing and immediate vaccination
3	Severe exposure	One or more bites through the skin, contamination of mucus membranes with saliva, direct contact with bats	Wound washing and immediate vaccination and administration of RIG

PEP vaccination can be administered according to either the Zagreb schedule or the Essen schedule (Folkhälsomyndigheten 2019). The Essen schedule consists of 1 dose on day 0, 3 and 7 as well as 1 more dose between day 14-28. The Zagreb schedule consists of 2 doses day 0, followed by 1 dose on day 7 and 21. The WHO also have a shorter recommendation of a 2-site intra-dermal injection on day 0, 3 and 7 (O'Brien & Nolan 2019).

The Zagreb schedule is shown to give a faster antibody response compared to the Essen schedule (Ren *et al.* 2015). Serum antibody tests following PEP vaccination

are not indicated in healthy individuals since consistently adequate levels of RVNA has been shown to be produced (Liu & Cahill 2020).

Post exposure of vaccinated individuals

To a person who have received a full PrEP, earlier PEP or have a serum RVNA level of 0.5 IU/ml, no RIG treatment is needed, regardless of exposure level (Folkhälsomyndigheten 2019). The rest of the protocol remains the same.

Post exposure of immunodeficient individuals

Individuals with immunodeficiency or immunosuppression shall, after exposure of category 2-3, receive vaccinations according to the Essen schedule as well as RIG (Folkhälsomyndigheten 2019; O'Brien & Nolan 2019). This is applicable regardless of earlier vaccinations. In these individuals serological control of antibody levels should also be performed 2-4 weeks post vaccination.

2.6.3 Rabies immunoglobulin (RIG)

RIG are rabies antibodies that when transferred to the wound area aids in the neutralisation of RABV in the area (Folkhälsomyndigheten 2019). RIG should be administered only once, preferably at the initiation of PEP, but no later than 7 days post the first rabies vaccination in the immunologically naïve patient (O'Brien & Nolan 2019). 7 days is the period needed for the body's own immune response to start producing antibodies, hence any RIG administration past this point is not indicated (WHO 2018b).

There are currently two different types of immunoglobulins available, human immunoglobulin and equine immunoglobulin (WHO 2018b). Equine immunoglobulin is considered to be less expensive, with most new products being both safe and potent. Anaphylactic reactions are rare and usually treatable.

RIG is however in short supply globally (WHO 2018b). Therefore, the WHO recommends the use of monoclonal antibody cocktails in place of RIG. Testing of a recombinant human monoclonal antibody showed it to be safe with no inferiority to human RIG (Gogtay *et al.* 2018)

2.7 Symptoms

Incubation time varies from 1 week to 1 year, depending on the location of inoculation and the virus load (WHO 2021). The initial symptoms include fever, paraesthesia at the wound site, ataxia, anxiety, altered mentation (Rupprecht 2002; WHO 2021). As the disease progresses it spreads to the central nervous system

through retrograde axial movement, where it causes a fatal encephalomeningitis (WHO 2021). There are two forms of rabies: furious and paralytic.

2.7.1 Furious form in humans

This form is characterised by hydrophobia (Warrell 1976). Also common for this form are inter-mittent episodes of hallucination, excitement, and/or maniacal behaviour. Further signs of the form include hypersalivation, hyperpyrexia and tachycardia. The symptoms culminate in coma and paralysis. This process usually leads to death within a few days due to cardio-respiratory arrest (WHO 2021).

2.7.2 Paralytic form in humans

This form is responsible for around 20% of all human cases (WHO 2021). It causes gradual muscle paralysis, starting at the entry site. This slowly progresses into a coma and eventually death. The process is usually longer than that of the furious form.

2.7.3 Rabies in animals

Symptoms in animals are often like those in humans and include the early non-specific symptoms such as fever, lethargy, vomiting and anorexia (CDC 2019d). These are followed by the more acute neurological symptoms. Including cerebral and cranial nerve dysfunction, ataxia, weakness, paralysis, seizures, difficulty breathing and swallowing, aggression or other behavioural changes, excessive salivation and self-mutilation.

2.8 Rabies in Cambodia

Cambodia has an estimated domestic dog population of about 5 million, most of them not being vaccinated against rabies (Institut Pasteur 2017). An estimated 600,000 severe dog bites occur every year in the country, with nearly 60% being children younger than 17 years old. Despite this, only approximately 5% of the cases seek vaccination after the injury.

The rabies situation in Cambodia is largely underestimated due to the lack of hospitalisation (Ly *et al.* 2009). The official reported number of confirmed deaths due to rabies between 2015-2017 is a total of 16 cases (WHO 2019). However an estimated model based on data from Institut Pasteur in Cambodia estimated around 810 human rabies deaths would occur during 2007 (Ly *et al.* 2009).

2.9 Diagnostics

There are currently no suitable diagnostic tools for rabies detection before onset of symptoms (WHO 2021). Clinical diagnosis may be difficult without specific symptoms such as hydro- or aerophobia. Cases of human rabies can be confirmed *intra vitam* and *post mortem* using a variety of diagnostic techniques capable of detecting whole viruses, viral antigens or nucleic acids in infected tissues such as brain, skin or saliva.

2.10 Serological analysis methods

2.10.1 Rapid fluorescent focus inhibition test - RFFIT

RFFIT is a virus neutralisation test which measures the amount of functional, binding antibodies in sera (CDC 2021). The test is considered a gold standard clinical serological assay, recommended by the Advisory Committee of Immunization Practices and WHO.

Sera is diluted to different concentration levels and added to chambers on a slide with a set amount of RABV (CDC 2021). These are then incubated to allow the RVNA in the serum to neutralize the virus, after which the mixture is added to cells, in which the virus can replicate. The samples are then incubated for about 20 hours to allow viral replication. The tests are fixed and stained with immunofluorescent staining and read in a microscope. The different concentrations are compared against a positive and a negative control.

2.10.2 Fluorescent antibody virus neutralisation test - FAVN

FAVN test is a modified version of the RFFIT test (Cliquet & Wasniewski 2015; WHO 2018a). It is conducted on 96-well microplates containing tissue culture and rabies virus. Usually made up of 5 plates of sera and one control plate with a negative and a positive control. It is the other gold standard test for serological evaluation of RVNA recommended by WHO.

The principle of the test is an *in vitro* neutralisation of a set amount of rabies virus (CVS 11 strain), before inoculation in a susceptible cell culture (Ondrejková *et al.* 2002; Cliquet & Wasniewski 2015). Serial dilutions of the sera with a factor of four are prepared directly on the plates, to which a set amount of CVS 11 is added. This solution is then incubated for an hour after which a cell suspension with a concentration of $4x10^5$ cells per cm^3 is added and the tests are incubated for another 48 hours. The medium is poured off, the wells washed with PBS and fixed in

acetone and stained with fluorescein isothiocyanate anti-rabies antibodies and evaluated through microscopic study. FAVN results has shown to be comparable to RFFIT (Briggs *et al.* 1998).

2.10.3 Enzyme linked immunosorbent assay - ELISA

ELISA can be used to detect and quantify different soluble substances. While there are several different kinds of ELISA-tests, the one used in this study is a blocking-ELISA. It detects and measures the levels of RVNA in serum.

It does so through sera containing RVNA being added to specific rabies-antigen covered wells in microplates. Serum is diluted and incubated in the wells. Biotinylated anti-rabies antibodies are then added to the wells, and any rabies-antigen not bound by potential RVNA in the serum will be bound by the coated antibodies, forming antigen-biotinylated antibody complexes. Streptavidin peroxi-dase conjugate, a substance which binds to the previously formed complexes, is then added. The wells are then washed to remove any excess substance. A tetra-methylbenzidine substrate is then added, revealing any biotinylated-complexes. After a set time, stop solution is added, halting the reaction. The optical density is read at 450 nm, calculating the percentage of blocking antibodies. All results are compared against a negative and a positive control.

While not considered a gold standard for clinical evaluation of rabies, since it does not differentiate between functioning, neutralising antibodies and antibodies that just bind in to RABV, ELISA is an appropriate diagnostic tool for research (CDC 2021).

2.10.4 Rapid neutralising antibody detection test - RAPINA

RAPINA is a test based on the principle of immunochromatography (Shiota *et al.* 2009). It measures RVNA quantitatively through the detection of unbound viral particles. The test uses specific RAPINA strips. Serum is mixed with 6 μg of inactivated RABV (iRABV) of the CVS11 strain and incubated. 6 μg has been shown to be the optimal con-centration to bind 0.5 IU/ml.

The sample is then added to a RABV G detection kit where any unconjugated iRABV will bind into the gold-conjugated mAb in the strip and form complexes (Shiota *et al.* 2009; Nishizono *et al.* 2012). These complexes will be bound to a RABV G-specific unconjugated mAb at the test line in the strip and produce a red band, indicating a positive test (RVNA >0.5 IU/ml). When compared to RFFIT the results were shown to correlate strongly (Nishizono *et al.* 2012).

2.11 RVNA in non-vaccinated individuals

Rabies antibodies in serum appear late in the disease progression, usually 6-7 days after symptoms have appeared, while RVNA in cerebrospinal fluid appear even later in the process (Folkhälsomyndigheten 2019). Since the disease is considered nearly 100% fatal once symptoms appear, this would mean that RABV positive individuals, aside from vaccinated individuals should be virtually non-existent.

Despite this, several studies throughout the years have found individuals with rabies antibodies in non-vaccinated populations (Gold *et al.* 2020). One study in Laos found that 23.73% of dogs, stated to be nonvaccinated by their owners, tested positive for RVNA using ELISA-testing (Fogelberg 2020). In Tunisia, Bahoul *et al.* (2005) found a prevalence of 27% in non-vaccinated dogs below the age of 2 years old. Another study in Haiti showed 10/107 (9.3%) non-vaccinated dogs to have RVNA using RFFIT, 2 of these having protective levels >0.5 IU/ml. 7/10 of the dogs were confirmed positive, including the two dogs with protective levels, by the ELISA (Smith *et al.* 2019). Since the mortality rates are nearly 100% after symptoms develop, survivors of the classic route of rabies would not be able to cause the 23.73% positive dogs in the study by Fogelberg *et al.* (2021), pointing at a different explanation for the antibody presence.

2.11.1 Subclinical infections and recovery

An epidemiological study in Tanzania found the probability of a dog developing clinical rabies after a bite from an infectious individual to be 49%, (CI 0.45-0.52) (Hampson *et al.* 2009). Milder symptoms, followed by recovery from infection, have also been observed in experimentally infected dogs (Manickam *et al.* 2008; Gnanadurai *et al.* 2013). This indicates that recovery from infection as well as possible subclinical infections could be a part of the explanation.

2.11.2 False positives

Another explanation could be false positive results from the diagnostic tests. The specificity of ELISA tests, however, seems to be very high.

In a study examining the specificity of the BioPro ELISA kit, 315 non-vaccinated laboratory animals, both dogs and cats, a specificity for 100% was found, and the highest level of PB recorded for the dogs was 32.9% (Wasniewski & Cliquet 2012).

A study comparing ELISA to RFFIT in Tanzania found that ELISA had greater specificity compared to RFFIT, with 0 false positives when tested on a rabies free population, while RFFIT testing found 10.3% of the population to be positive. (Cleaveland *et al.* 1999). The agreement between the two analysis methods is also

poor, a study comparing the BioPro rabies ELISA kit to the FAVN test, the other of the WHO gold standard tests, found a concordance of only 86.2% when comparing known, vaccinated individuals in a laboratory setting (Wasniewski & Cliquet 2012). Similarly in a study from 2005, rabies virus neutralisation tests and ELISA had a concordance of only 30% (Bahloul *et al.* 2005).

This further speaks for ELISA based results to be more likely true positives but could be a partial explanation to antibody positive individuals in RFFIT based studies. However, no studies of specificity were found that was performed on naturally infected individuals, and the results could therefore be different in these individuals.

Another potential risk is the possibility of cross reactivity between viruses. Viruses that are closely related can have similar antigens, therefore the antibodies produced against one virus may also neutralize others. This means that RVNA-positivity potentially could be caused by an infection by another, closely related lyssavirus. Lyssaviruses are divided into different phylogroups, with cross reactivity being limited between groups, but possible within them. Rabies belongs to phylogroup 1 (PG1) (Hayman *et al.* 2016).

While surveillance of *lyssavirus* in Cambodia appears to be limited, a serologic study of bats in Cambodia did find antibodies to four different lyssaviruses, including rabies, speaking for the presence of these viruses in the country (Reynes *et al.* 2004). Out of these, two others were in PG1, European bat lyssavirus 1 (EBLV-1) and Australian bat lyssavirus (ABLV). EBLV-1 has been known to spill over both to humans and other mammals (Fooks *et al.* 2003) and ABLV have shown to cause mild disease as well as seroconversion with RVNA found in sera post infection (McColl *et al.* 2007).

However, since the prevalence of rabies in Cambodia is very high, Institut Pasteur Cambodia (IPC) has since 2000 sampled on average 200 biting dogs every year and found a prevalence of rabies of nearly 50% among these dogs (Institut Pasteur 2017), rabies is the most probable cause of RVNA-positive individuals. The extent of which *lyssavirus* cross-reactivity affects the test results of rabies antibody positive individuals remains unclear.

2.11.3 Factors affecting sensitivity

Repeated freezing and thawing of samples can lead to antibody decay and false negatives (Kostense *et al.* 2012). A study on influenza virus in mallards also showed that multiple factors affect the antibody level in a previously exposed individual, such as variations in immune response and time passed since exposure

to the virus (Pepin *et al.* 2017). While not specifically tested on rabies, this could potentially also be relevant for rabies infection, where naturally infected individuals due to these factors may vary in their antibody response and therefore be negative when analyzed, despite having undergone an infection with the virus. Another factor to take into consideration is antibody decay over time, which has been shown to occur in bat populations experimentally infected with rabies (Turmelle *et al.* 2010).

3. Materials and method

3.1 Study area

The included provinces were chosen based on information regarding reported cases of rabies, reported to IPC, and a previously low activity of vaccination campaigns in the area according to the National Animal Health and Production Research Institute (NAHPRI), which would increase the number of non-vaccinated individuals needed for the study.

The included provinces were Takeo (n=20), Kampong Speu (n=27) and Kampong Chhnang (n= 50), in which two to three villages were included for sampling. After permission from the village leader to conduct the research, the sampling was done in company of a provincial officer, walking on foot between houses, collecting blood from dogs who could be captured and handled by their owners.

3.2 Study population

Participants in the study were all inhabitants of the selected provinces who volunteered for the study. They were informed of the project through their village leader who in term was informed by NAHPRI and the provincial officer.

All participating owners were informed of the sampling process and had given their permission before sampling begun. Only healthy, non-aggressive, non-vaccinated dogs older than 3 months were included in the study. If the owners had more than one dog, all the dogs were included in the study.

All participating dogs received an injection of ivermectin as compensation for their and the owner’s cooperation.

3.3 Collection of data

Survey

A survey was composed in cooperation with the NAHPRI in Cambodia. The survey included questions regarding the ownership and care for the dog as well as the main purpose of keeping the dog. General information such as age and medical history was also included, focusing on previous bite wounds within the last 6 months and vaccination status, to make sure that no previous vaccinations had been performed which would interfere with the results.

Blood samples

After the survey was completed, blood sampling was performed on all dogs matching the inclusion criteria. Total number of included dogs: n=100.

Sampling was done from one of the front legs, through *vena cephalica*. If the collected amount of blood was insufficient or in case sampling failed, the other leg could be used as well. All dogs were muzzled and held by the owner or staff of the project. The blood was collected using a butterfly cannula connected to a vacutainer adapter. All samples were collected in serum tubes and stored in an ice box until centrifugated or naturally separated in the serum tubes. After separation the serum collected was stored in a freezer at -18°C until analysis.

3.4 Laboratory analyses

Analysis of canine sera for the presence of rabies antibodies

The sera were analysed using BioPro Rabies ELISA Ab kit (O.K. SERVIS BioPro, Horni Pocernice, Czech Republic). All samples were thawed and brought to room temperature before use. Out of the 100 samples, 3 were not possible to analyse due to insufficient amounts of sera.

50 microliters of positive, negative, control and sampled sera were added to specific wells on the microplates and diluted with 50 microliters diluent. The plates were then incubated for 18-24 hours at 2-8 degrees Celsius. After incubation the plates were emptied of content and washed 6 times with washing solution after which 100 microliters of biotinylated anti-rabies antibodies were added to each well and allowed to incubate for 30 minutes at 37 degrees Celsius. The plates were emptied and washed 4 times with washing solution. 100 microliters of diluted streptavidin peroxidase conjugate was then added to each well and the plates were once again incubated at 37°C for 30 minutes, emptied and washed 4 times with washing solution.

After this process, 100 microliters of TMB substrate was added and the plates were incubated at 18-25°C for 30 minutes, since none of the incubations were done with an orbital shaker, as per the manufacturer's recommendations. Post incubation, 50 microliters of stop solution was added to the plates and the samples were read with an ELISA reader at an optical density (OD) of 450 nm.

The results were inserted into Microsoft Excel and percentage of blocking (PB) antibodies were calculated using the following formula:
PB%=((ODNc – ODSample)/(ODNc – ODPc))x100

A PB higher than 40% is considered positive for rabies antibodies. A PB higher than 70% is considered as a serum with antibody level equal to, or greater than 0,5 IU/ml, based on FAVN test (BioPro 2013).

All positive samples were analysed in duplicates to further increase the authenticity of the results. Of the 11 positive samples in the first analysis, 9 were positive in the second analysis.

Of the positive samples from the first 2 runs, the 5 positive dogs from Kampong Speu were sampled and analysed a second time. Due to time restraints, it was the only province that could be re-sampled. All samples were run in duplicates. Out of these 5, 3 were positive, one was negative and one was inconclusive, with both a positive and a negative outcome.

3.5 Data analyses

The data from the questionnaires was exported into Microsoft Excel and cleaned. Laboratory results were entered into Microsoft Excel and merged with the data from the questionnaires. Data were analysed descriptively.

4. Results

4.1.1 Positive samples and mean PB levels

Out of 97 analysed samples, 11 were tested positive for RVNA (11%), with PB-levels above 40%. All of the dogs were reported as never having been vaccinated, both by their owners as well as the village leader confirming that no vaccination campaigns had taken place in the village. Out of these 11, 10 were analysed again, the 11th sample having insufficient sera to be analysed again, and a mean PB-value was calculated. After both runs, 8 out of the 10 samples had a mean PB-value of > 40%.

The sample that could only be analysed once due to the low amount of serum had a PB of 67% in the first analysis. Since the PB level was well above the positive level, that sample was also included in the further analyses, resulting in a total of 9 positive samples out of 97 (9%), see *figure 1*.

Out of the 9 positive samples only one were shown to have protective levels of >70 PB at 450nm. This sample had protective PB levels in both the first run and the control run, having 75.3% and 70.7% PB respectively, resulting in a mean PB level of 73%.

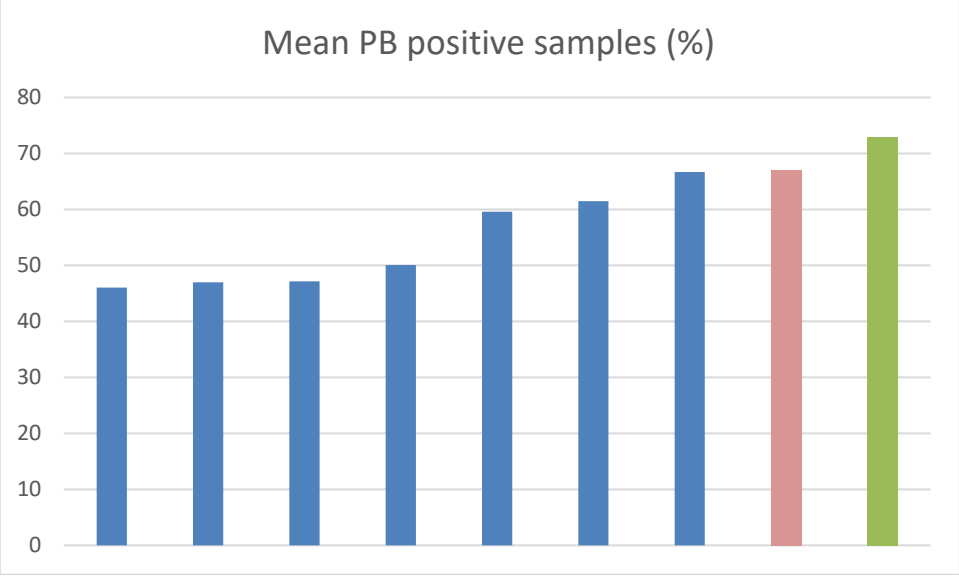

Figure 1. Mean percentage blocking (PB) of positive samples. (Pink – only ran once. Green – protective antibody levels)

4.1.2 Positive dogs based on sampling location

There is a big difference in the percentage of positive dogs between the provinces in the study (Table 2), with Kampong Speu having the highest prevalence of around 15%, almost twice as high as Kampong Chhnang, and three times as high as Takeo, see *table 2*. However, the sample groups are small, even with a Pearson chi-2 test, no statistical correlation could be made regarding prevalence in the different provinces (p=0.44).

Table 2. Number of positive samples and % of positive dogs per province

Province	Total samples	Number of positive samples	Positive %
Takeo	20	1	5
Kampong Speu	27	4	15.38
Kampong Chhnang	50	4	8.16
Total	97	9	9.47

4.1.3 Difference in PB between first analysis and control run

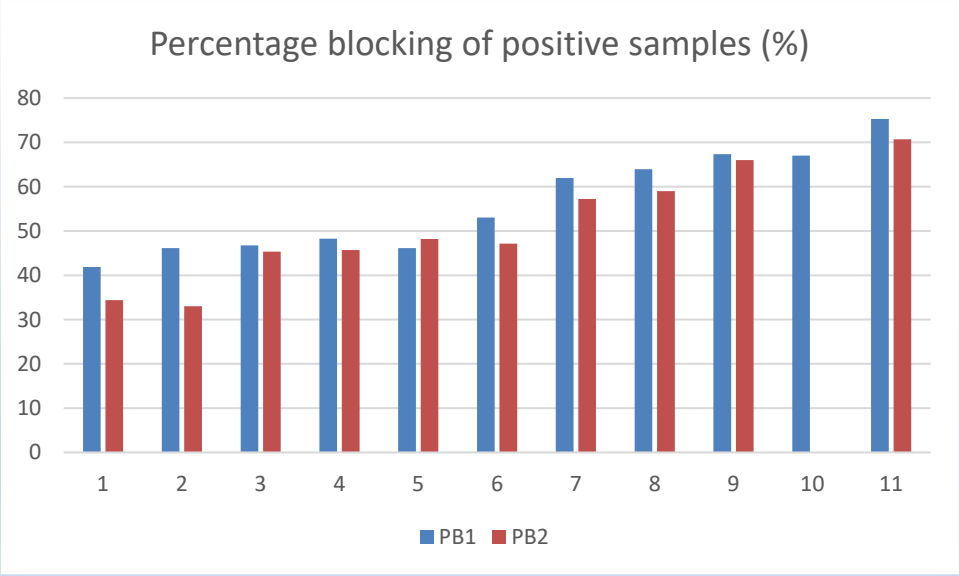

Figure 2. Percentage blockage (PB) of positive dogs for the first run (blue) and control run (red).

The PB levels between the two runs for the most part had quite similar results, with the first run tending to show a bit higher PB levels, having a mean value of 56%, while the control run gave slightly lower values, with a mean of 49%, see *figure 2*. The largest difference between the two runs being 13.1 %. The total mean differ-

rence of the runs was 3.8%. Only one sample had a higher blockage percentage in the control run than in the first test. Two of the previously positive samples that were close to the cut-off point of PB 40% came up as negative in the control run and were therefore excluded from further analysis.

4.1.4 Correlating factors to antibody positivity

When analyzing the age of the dogs, there was a statistically significant correlation between the age of the dog and positive test results. The mean age for antibody positive dogs was 5.22 years, which was significantly higher than the age of the antibody negative dogs, which was 2.85 years ($p = 0.0239$).

Other factors were also analysed, such as the main use of the dog, where all the positive dogs were stated to be guard dogs. However, out of the 94 dogs whose main use had been stated, all except one were held as guard dogs, with the exception being kept for company. Therefore, no statistical significance could be found.

Another factor that was examined was the correlation between bite wounds in the last 6 months and the prevalence of antibodies. Once again bite status had only been stated for 94 of the dogs. Out of the 9 positive dogs, only 3 of them had been bitten within this period. For the negative ones the prevalence was 25/85 (29%). No statistical significance could be found using Pearson's chi-2 test ($p=0.81$).

Lastly, previous vaccination status was examined as a possible correlating factor. Out of the 9 positive dogs, 0 were reported as having previously received any form of vaccine, while 3/85 of the negative dogs stated that they previously had been vaccinated. Of these three, one was stated to have received vaccination for rabies and meningitis on a yearly basis, one did not know which vaccine the dog had received and the last did not answer the question. No statistical significance could be found ($p=0.57$). The dog stated as vaccinated against rabies on a yearly basis was negative in the first analysis, with a PB of only 28% and therefore not analyzed further. Given the long persistence of antibodies after rabies vaccination and the reported frequency of vaccinations, it does not seem that the dog would be properly vaccinated, which could be due to improper handling of the vaccine, the owner having gotten another vaccine (the dog was also reportedly vaccinated against meningitis, so there might be some kind of misunderstanding), or the result of a miscommunication or misunderstanding during the interview.

4.1.5 Re-sample of positive dogs from Kampong Speu

Based on the results from the previous two tests, a resampling was done in the province of Kampong Speu where all the dogs who had been positive in any of the

previous tests, either the first run or the control run, were sampled and analyzed again. There were five dogs in total meeting this criterion that were included in the resample. All five individuals were able to be sampled again, approximately 3 weeks after the first sample, and new sera were successfully gathered from each one.

The samples were then run in duplicates. Out of the 5 dogs, 3 were positive (60%), one was inconclusive (20%), with both a negative and a positive result, and one had two negative results (20%). The inconclusive sample had previously been below the threshold also in the control run, further strengthening the result to be negative.

The sample with two negative results had however been positive in both previous runs where it had an average PB level of 59.6%, well above the cut-off level. The average PB of the resampled sera of the individual was 37%, resulting in a difference of 22.6%. The owner only had one dog and confirmed that it was the same dog that had been sampled previously before new blood was drawn for the resampling, making a mix up of dogs very unlikely.

The mean PB levels of the resample was 46.4%, compared to the mean for the average of the previous two runs for the same individuals, which was 55.2%, resulting in a difference of 8.6 % lower average PB for the resample, see *figure 3*.

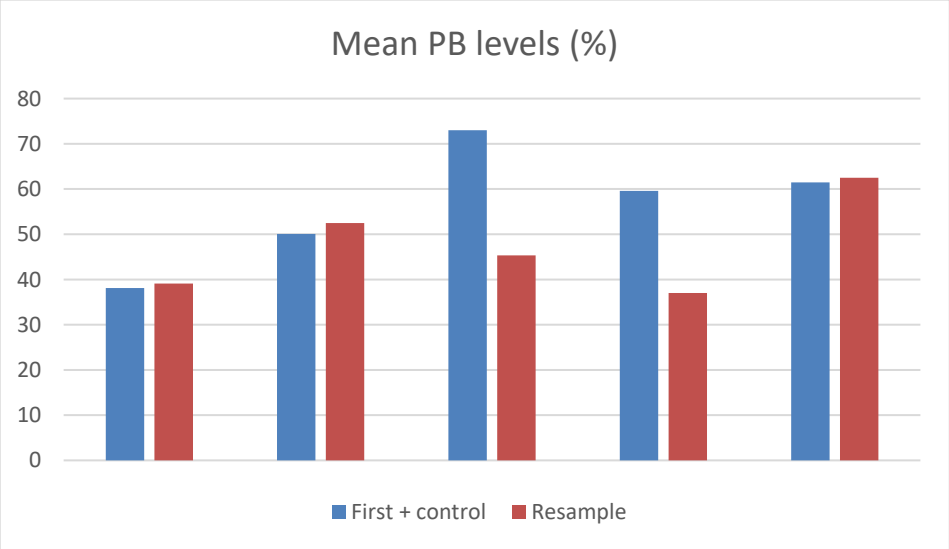

Figure 3. Mean percentage blockage (PB) values of previous tests compared to the resample, for the five dogs from Kampong Speu province.

5. Discussion

RVNA was found in both the first sample batch and the control run as well as in the control re-sampling of the positive dogs in the Kampong Speu province. This seems to indicate the presence of naturally occurring antibodies in the dog populations. However, both the levels of antibodies found and the number of dogs classified as positive varied between tests. Therefore, the prevalence of antibody positive individuals as well as the antibody levels found in this study should be interpreted with caution and no conclusion regarding these two parameters can be drawn with any form of certainty.

5.1.1 Positive samples and mean PB levels.

While several studies regarding the presence and prevalence of RVNA in non-vaccinated domestic dogs have been performed around the world (Gold *et al.* 2020), the majority of these have been analyzed using RFFIT. The correlation between RFFIT and ELISA have proven to be quite poor in field conditions, having a concordance of only 30% (Bahloul *et al.* 2005). Similarly, a study comparing the BioPro ELISA kit, which was utilised in this study, to a FAVN test, the other WHO gold standard test, on known vaccinated individuals found a concordance of 82% (Wasniewski & Cliquet 2012). Comparing results between studies utilising different methods of detection is therefore very difficult and uncertain.

However, other ELISA based studies on non-vaccinated domestic dogs have found a similar or higher prevalence of antibodies than what was found in this study. A study on dogs in Haiti found 9.3% of the dogs to be positive for RVNA (Smith *et al.* 2019), while a study in Laos and one in Tunisia found a higher percentage of seroconverted dogs, 23.6% and 27% respectively (Bahloul *et al.* 2005; Fogelberg 2020).

In a study examining the specificity of the BioPro ELISA kit, 315 non-vaccinated laboratory animals, both dogs and cats, a specificity of 100% was found. The cut-off point for positive individuals was set to 70% PB, corresponding to an antibody titer of >0.5 IU or protective levels of antibodies. However, the highest level of PB value recorded for the negative dogs was 32.9%, hence making the result applicable also for this study with a cut-off point of 40% (Wasniewski & Cliquet 2012). This corresponds to the results found by Cleaveland *et al.* (1999) in Tanzania, where 162 known rabies free, non-vaccinated dogs were tested for antibodies using a liquid phase blocking ELISA, similar to the BioPro ELISA. Zero of these dogs tested positive for antibodies, pointing to a very high specificity for the ELISA test.

This indicates that the test positive individuals should be correctly identified as RVNA positive. Taking into consideration that rabies is such a prevalent disease in Cambodia and that the villages included were selected in consultation with NAHPRI and IPC, confirming that no prior vaccination campaigns had taken place there, the results are further supported. However, there is always a possibility of recollection bias among the owners, or miscommunication during the interview. Thus, the possibility that some of the positive dogs might have been vaccinated cannot be fully excluded.

5.1.2 Positive dogs based on sampling location

While it is interesting that the difference in prevalence of rabies positive individuals is a large as it is between the provinces, very little can be said regarding these results as the sample sizes in two out of three provinces are too small to establish any form of statistical correlation. For the central limit theorem to be applicable, the sample size must be >30. However, the much higher prevalence in the province of Kampong Speu still sticks out. While the sample size is not 30, it is very close at 27, and has almost double the prevalence in the other provinces. This could of course just be due to chance and the small number of dogs sampled, giving a false high prevalence in the district, while the true prevalence might be a lot lower. It might also be that the villages that were chosen in the province had a higher endemic prevalence of rabies than other villages in the province, or that the province has a higher prevalence of rabies compared to the other provinces in this study. A difference in rabies prevalence between provinces has been indicated by the estimated model for the rabies situation in Cambodia made by Ly *et al.* (2009). However, no studies researching the prevalence in the provinces could be found.

To be able to determine whether there actually exists a difference in RVNA prevalence in the different provinces, larger sample sizes from each province are required.

5.1.3 Difference in PB between first analysis and control run

Although the PB levels in the first run and the control run were similar, the mean PB of the control run was slightly lower compared to the first run. This could be explained by a possible error in the process, for example in the dilution of sera or in the steps of complex formation, as described in the methods section. All these steps were performed by pipetting by hand and although the procedure was done under utmost care, the human factor is still a possible cause for error and could explain some of the difference in PB levels between the two runs.

Another possibility is the denaturation of antibodies from undergoing several cycles of thawing and refreezing, as described by Kostense *et al.* (2012). Since the samples by the control run had been stored on ice, brought to room temp, frozen, thawed, frozen and thawed out again before analysis this could potentially have lowered the number of intact antibodies. It does, however, not explain the sample in which the PB was higher in the control run. That could on the other hand be caused by human error in the analysis, as described above.

5.1.4 Correlation between age and antibody positivity

The only significant correlation found among the factors examined from the questionnaire was that of the age of the dog and RVNA positivity. This has also been found in a previous study by Fogelberg *et al.* (2020). The study by Bahloul *et al.* (2005) also seems to point at a strong correlation between age and seropositivity, finding a conversion of 18% in dogs <2 years old, and of 58% in dogs >2 years. The result found by Bahloul *et al.* for the dogs >2 years is uncertain due to lack of knowledge of previous vaccination campaigns in the sample area prior to 3 years before the study took place. This could therefore be a falsely high conversion percentage in older dogs, resulting from previous vaccination campaigns.

While no study on immunity duration in dogs that have been naturally exposed to rabies virus seems to have been made to this date, immunity in vaccinated dogs extend well beyond 3 years (Dodds *et al.* 2020) and could still affect the titers in the older dogs. On the other hand, a study by Prager *et al.* (2012) found no difference in seropositivity among age groups in 290 domestic dogs in Kenya. In order to determine whether or not a correlation truly exists between age and seropositivity, more research needs to be done.

5.1.5 Re-sampling of positive dogs from Kampong Speu

While most of the results from the resampling was in concordance with the previous results, one of the dogs previously tested well above the positive cut-off value with a PB of 59.6%, surprisingly fell below the line in the resampling. Due to the large difference in antibody levels in such a short time, the most likely explanation is that there was a mix up of dogs or a fault in the lab. However, all the control values were correct for the test and the other resampled dogs gave results similar to that of the first samples. This of course does not exclude the possibility of a lab error but does speak against it. The owner was also present during both samplings, confirming the dog's identity, and it was the only dog in the family, making a mix-up of dogs unlikely. There could possibly have been a miscommunication or a misunderstanding during the first sample where the sera could have been mis-

labeled or the wrong owner reported for the positive dog, resulting in the wrong individual being resampled.

Another possibility is that of a false positive result from the first sample. Although the ELISA is claimed to have a specificity of 100%, compared to the gold standard FAVN test (Wasniewski & Cliquet 2012), no tests are perfect and the possibility of a false positive result cannot be completely ruled out. It is however unlikely both due to the high specificity as well as the samples being run in duplicates.

Since the sample size is so small, no significant conclusions can be drawn from the result. No previous studies that had performed a resample of the tested individuals in this manner could be found.

5.1.6 Prevalence of RVNA

While the total prevalence of RVNA positive dogs after the control run indicate a prevalence of 9/97 (9%) dogs, the results from the resampling in Kampong Speu raises the question whether this result can be trusted, since only 3/4 previously positive dogs were positive in the resample. This results in a concordance of 75% between the first sample and the resample.

While all the positive individuals from the first two runs would be expected to be positive in the resample as well, considering the high specificity found by Wasniewski and Cliquet (2012) as well as Cleaveland *et al.* (1999), the large percentual difference is only caused by one dog. This could once again be caused by the small sample size, mistakes in the lab, misunderstandings or any of the other error sources discussed in 5.1.5 above. In order to get a better true estimate of the concordance, a larger sample group is needed.

When looking at the prevalence of antibody positive dogs, one should also take into consideration the possibility of diminishing antibody levels over time shown by Turmelle *et al.* (2010) and the difference in antibody response found by Pepin *et al.* (2017) as factors that might affect the prevalence in the study. While none of these studies were performed on dogs, these factors could play a part in dogs as well, based on the varying antibody response to vaccination shown by Minke *et al.* (2009) and the drop in immunity over time shown in a study by Dodds *et al.* (2020) and could hence affect the prevalence of positive dogs.

Another factor that could affect the prevalence is the possibility of cross reactivity between lyssaviruses of PG 1, since Reynes *et al.* (2004) found serologic evidence of European bat lyssavirus 1 (EBLV-1) and Australian bat lyssavirus (ABLV) in the country, both of which belong to PG 1. Since EBLV-1 has been shown to spill

over and cause disease in other mammals (Fooks *et al.* 2003) and McColl *et al.* (2007) have shown that experimental infection of ABLV in dogs can cause both disease and antibody titers of RVNA, cross reactivity should not be overlooked. However, the prevalence of these viruses in the country as well as the extent of the spillover from bat populations to domestic dogs is not clear. Because of this and the high known prevalence of rabies among domestic dogs in the country as shown in the model by Ly *et al.* (2009), RABV infection is most likely the probable cause of the positive antibody levels found in the study.

However, the confirmation of 75% of the resampled dogs indicates that there indeed exist non-vaccinated dogs with naturally occurring antibodies against rabies, which in turn indicates a possibility of non-lethal infection. To determine the prevalence of, as well as the level of protection provided from these antibodies, more studies are required, looking at larger sampling sizes across more provinces.

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Popular science summary

Rabies is a viral disease, caused by the rabies virus (OIE 2018). The virus is capable of infecting any mammal, including humans (CDC 2019a). Once symptoms develop, rabies is nearly 100% fatal. However, the disease is 100% preventable through vaccination (WHO 2021). Protection can be achieved using either pre- or post-exposure prophylaxis, if performed at the right time (Folkhälsomyndigheten 2019).

Despite this, rabies is still well established in different regions of the world, mainly in Africa and Asia, where it causes approximately 59,000 human casualties each year. Over 56.6% of these deaths occur in Asia (Hampson *et al.* 2015). Over 99% of all human cases of rabies are caused by infection transmitted from domestic dogs (CDC 2020).

Cambodia, in Southeast Asia, has one of the highest estimated numbers of rabies cases per year, with an approximate of 810 annual deaths in the country. The actual rabies death toll in the country is hard to determine due to lack of hospitalization of infected individuals (Ly *et al.* 2009).

Rabies is mainly spread through bite wounds or contact with the saliva of an infected animal on a skin lesion or mucus membranes (CDC 2019b). The virus then either directly enters the nerve cells in the area or remains in the muscle tissue where it replicates and remains during the majority of the incubation period (Rupprecht 1996). After replication, the virus then spreads via the nerves to the central nervous system, causing a fatal inflammation of the brain and the meninges (WHO 2021). The virus also spreads to the salivary glands, where it is shed, enabling the infection of other hosts (Rupprecht 2002). Rabies antibodies usually develop 6-7 days after the debut of symptoms (Folkhälsomyndigheten 2019). This, in combination with the nearly 100% fatality rate of rabies once symptoms start to show, should mean that antibodies existing in individuals who have not been vaccinated should be extremely rare. Despite this, several studies have found anti-rabies antibodies in non-vaccinated individuals around the globe, both domestic dogs and wildlife species (Gold *et al.* 2020). In this study, 97 non-vaccinated, domestic dogs in the

provinces of Kampong Chhnang, Kampong Speu and Takeo were examined for the possible presence of anti-rabies antibodies in serum.

Our results found that 9% of the dogs were positive for antibodies despite never having received any rabies vaccines. This was confirmed both by the owners as well as the institute who is responsible for the vaccination campaigns in the country. This was on the lower end of the results of previous studies, which had found antibody levels ranging from 9.3% all the way up to 27% (Bahloul *et al.* 2005; Smith *et al.* 2019). However, none of the sampled dogs reached protective levels of antibodies.

While it cannot be entirely excluded that some of the positive dogs are false positives caused by insensitivities of the lab tests, earlier studies examining the same test found that no dogs included in the studies were falsely labelled as positive by the test (Cleaveland *et al.* 1999; Wasniewski & Cliquet 2012).

Another factor that could potentially cause rabies antibodies in non-vaccinated individuals is a cross reaction with other types of similar viruses. These viruses are divided into different groups based on similarity, and within these groups, antibodies against one of the viruses can cross react and attach to other viruses within the same group, therefore making them positive on tests despite not having been infected with the virus that was analysed for (Hayman *et al.* 2016). Since signs of similar viruses have been detected in Cambodia (Reynes *et al.* 2004) it is possible that this could affect the results in the current study.

The results found in this study could also in part be affected by the relatively small sample size and the true percentage of positive dogs may vary in both directions.

All the positive dogs from the province of Kampong Speu were also resampled and the new sera were analysed as a control. Out of the 4 dogs positive in the first analysis, 3 were positive in the resample. Considering the huge difference in antibody levels between the two samples, the dog testing negative in the resampling is most likely a result of miscommunication during the interview or mislabelling of the samples, causing the wrong dog to be resampled for new analysis.

The study also showed that there was a correlation between age and antibody positivity, where older individuals were more likely to have antibodies than younger ones. This has also been found by a previous study in Laos.

In conclusion, the results speak for the presence of naturally occurring antibodies in non-vaccinated, domestic dogs in the provinces and that age seems to be a

relevant factor for positivity. However, due to small sample sizes and differences in results between the different analysis runs, no certain conclusions regarding the prevalence of antibody positive individuals can be determined. More studies need to be performed on the subject to properly determine the true prevalence of naturally occurring anti-rabies antibodies in domestic dogs in Cambodia.

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Appendix 1

គណៈកម្មាធិការក្រមសីលធម៌ជាតិ សម្រាប់ការស្រាវជ្រាវសុខភាពនៅកម្ពុជា

កម្រោងស្រាវជ្រាវ “យុទ្ធសាស្ត្រចាក់វ៉ាក់សាំងការពារជម្ងឺឆ្កែឆ្កួតក្នុងប្រទេសកម្ពុជា

By Johanna Lindalh and Tum Sothyra *et al.*

National Ethics Committee for Health Research (NECHR) in Cambodia

“Rabies vaccination strategies in Cambodia” research project

By Johanna Lindalh, Tum Sothyra, Chea Rortana *et al.*

បញ្ជីសំនួរ

Questionnaire

បញ្ជីសំនួរ- ជម្ងឺឆ្កែឆ្កួត សម្រាប់ម្ចាស់សត្វ

Questionnaire – rabies for dog owners

លេខកូដ បញ្ជីសំនួរ **/ Questionnaire code:**

ឈ្មោះអ្នកចូលរួម **Name of participant:**

លេខទូរស័ព្ទ **Phone number:**

1. ទីតាំង **Location:**

១.១ តើស្រុក និង ខេត្តណាមួយដែលអ្នករស់នៅ?

1.1 Which district and province do you live in:

២. ពត៌មានអំពីម្ចាស់សត្វឆ្កែ **Information about the dog owner:**

2.1 ភេទ/Gender	ស្រី/Female ប្រុស/Male
2.2 អាយុ (ឆ្នាំ)/Age (years)	0-15 16-25 26-35 36 and above
2.3 កម្រិតវប្បធម៌/Education level	មិនមានការអប់រំ/No education កម្រិតបឋម/Primary ថ្នាក់ទី ៥ ដល់ ទី១០/Class 5-10 កម្រិតមធ្យមសិក្សា/Higher secondary បញ្ចប់ការសិក្សា និងលើសពីនេះ/Graduation and above
២.៤ តើអ្នកជាម្ចាស់សត្វឆ្កែនេះមែនទេ?/ **Are you the owner of the dog?**	បាទ/ចាស/Yes ទេ/No ប្រសិនបើ ទេ, តើនេះជាឆ្កែរបស់អ្នកណា?/If no, whose dog is it?
២.៥	បាទ/ចាស/Yes

តើអ្នកធ្លាប់បរិភោគសាច់ឆ្កែដែរឬទេ?/ **Do you ever consume dog meat**	ទេ/No
២.៦ ប្រសិនបើធ្លាប់, តើញឹកញាប់ប៉ុណ្ណា?/ **If yes, how often?**	រាល់ថ្ងៃ/Every day ម្តងក្នុង១សប្តាហ៍/Once a week ម្តងក្នុង១ខែ/Once a month មិនញឹកញាប់ទេ/Less often
២.៧ ប្រសិនបើ ទេ, ហេតុអ្វី?/ **If no, why not?**	

៣. សំនួរទូទៅ/General questions

៣.១ តើអ្នកដឹងថាសត្វឆ្កែអាចចម្លងជម្ងឺដល់មនុស្សដែរទេ? **Do you know if dogs can transmit diseases to humans?**	បាទ/ចាស/Yes ទេ/ No
៣.២ ប្រសិនបើ បាទ/ចាស, តើជម្ងឺអ្វីដែលអ្នកដឹងថាសត្វឆ្កែអាចនឹងចម្លងដល់មនុស្ស?/ **If yes, which diseases do you know of that dogs could transmit to humans?**	
តើអ្នកដឹងថា	បាទ/ចាស/Yes

មានវ៉ាក់សាំងសម្រាប់សត្វឆ្កែ?/**Do you know there are vaccines for dogs?**	ទេ/No
ប្រសិនបើអ្នកដឹង, តើជម្ងឺណាមួយដែលអ្នកដឹងថាអាចចាក់វ៉ាក់សាំងប្រឆាំងបាន?/**If yes, which diseases do you know it is possible to vaccinate against?**	

4. ជម្ងឺឆ្កែឆ្កួត/**Rabies**

៤.១ តើអ្នកដឹងថាជម្ងឺឆ្កែឆ្កួតជាអ្វីដែរទេ?/ **Do you know what Rabies is?**	បាទ/ចាស/Yes ទេ/No
៤.២ តើធ្កដឹងថាជម្ងឺឆ្កែឆ្កួតឆ្លងដោយរបៀបណាដែរទេ?/**Do you know how rabies is transmitted?**	បាទ/ចាស/Yes ទេ/No
៤.៣ ប្រសិនបើអ្នកដឹង, តើជម្ងឺឆ្កែឆ្កួតឆ្លងដោយរបៀបណា? (អនុញ្ញាតឲ្យរើសជម្រើស **If yes, how is Rabies transmitted?** Multiple options allowed:	មូស/Mosquitoes លាមក/Faeces ខាំ/Bites ប៉ះពាល់ឈាម/Blood contact ប៉ះពាល់ជាមួយទឹកមាត់ឆ្កែ/Contact with dog saliva អាហារ/Food ផ្សេងៗ/Other, explain:

៤.៤ តើអ្នកណាដែលអាចឆ្លងជម្ងឺឆ្កែឆ្កួត? (អាចជ្រើសរើសជម្រើសលើសពី១)/ **Who can get Rabies?** **(More than one option can be selected)**	មនុស្ស/Humans ឆ្កែ/Dogs ឆ្មា/Cats សត្វពាហនៈ/Cattle បក្សី/Birds ទាំអស់/All ផ្សេងៗ/Other, explain: ខ្ញុំមិនដឹងទេ/Don't know
៤.៥ អាការៈជម្ងឺឆ្កែឆ្កួតនៅក្នុងមនុស្ស (អាចរើសជម្រើសលើសពីមួយ)/ **Symptoms of rabies in humans** **(More than one option can be selected)**	ក្តៅខ្លួន/Fever ក្អួត/រាគ/Vomiting / Diarrhoea ឆេវឆាវមិនអាចគ្រប់គ្រង/Aggressiveness ហៀរទឹកមាត់/Salivation រលូតកូន/Abortion រន្ធត់ខ្លាំង/Staggering ពិបាកដកដង្ហើម/Difficulty breathing ស្រកទម្ងន់/Weightloss អស់កម្លាំង/Fatigue ដំបៅស្បែក/Skin lesions មិនដឹងទេ/Don't know ផ្សេងៗ, សូមពន្យល់/Other, explain:
4.5 អាការៈជម្ងឺឆ្កែឆ្កួត	ក្តៅខ្លួន/Fever

នៅក្នុងសត្វឆ្កែ(អាចរើសជម្រើសលើសពីមួយ)/bSymptoms of rabies in dogs **(More than one option can be selected)**	ក្អួត/រាគ/Vomiting / Diarrhoea ឆេវឆាវមិនអាចគ្រប់គ្រង/Aggressiveness ហៀរទឹកមាត់/Salivation រលូតកូន/Abortion រន្ធត់ខ្លាំង/Staggering ពិបាកដកដង្ហើម/Difficulty breathing ស្រកទម្ងន់/Weightloss អស់កម្លាំង/Fatigue ដំបៅស្បែក/Skin lesions មិនដឹងទេ/Don't know ផ្សេងៗ, សូមពន្យល់/Other, explain:
៤.៦ តើអ្នកគិតថាជម្ងឺឆ្កែឆ្កួតធ្ងន់ធ្ងរប៉ុណ្ណាសម្រាប់សត្វឆ្កែ?/ **How serious do you think rabies is for dogs?**	ភាគច្រើនរស់, ប៉ុន្តែខ្លះងាប់/Most survive, but some dies ភាគច្រើនងាប់ ប៉ុន្តែខ្លះរស់/Most will die, but some survives ឆ្កែទាំងនោះតែងតែងាប់/They always die
៤.៦ តើអ្នកគិតថាជម្ងឺឆ្កែឆ្កួតធ្ងន់ធ្ងរប៉ុណ្ណាសម្រាប់មនុស្ស?/**How serious do you think rabies is for**	ភាគច្រើនរស់ ប៉ុន្តែខ្លះស្លាប់/Most survive, but some dies ភាគច្រើនស្លាប់

humans?	ប៉ុន្តែខ្លះរស់/Most will die, but some survives ពួកគេតែងតែស្លាប់/They always die
៤.៨ តើអ្នកធ្វើយ៉ាងដូចម្តេចប្រសិនបើអ្នកសង្ស័យជម្ងឺឆ្កែឆ្កួតមាននៅក្នុងសត្វឆ្កែ?/ **What do you do if you suspect rabies in a dog?**	
៤.៩ តើអ្នកធ្វើយ៉ាងដូចម្តេចប្រសិនបើអ្នកសង្ស័យជម្ងឺឆ្កែឆ្កួតមាននៅក្នុងមនុស្ស? **/ What do you do if you suspect rabies in a human?**	
៤.១០ តើនៅក្នុងតំបន់នេះមានសត្វឆ្កែមានជម្ងឺឆ្កែឆ្កួតដែរឬទេ?/ Have any dogs in the area had rabies (that you know of)?	បាទ/ចាស មាន/Yes មិនមានទេ/No
៤.១១ តើមាននរណាម្នាក់នៅក្នុងតំបន់នេះមានជម្ងឺឆ្កែឆ្កួតដែរឬទេ?/Has any person in the area had rabies (that you know of)?	បាទ/ចាស មាន/Yes មិនមានទេ/No

៤.១២ តើអ្នកដឹងថាមានវ៉ាក់សាំងប្រឆាំងនឹងជម្ងឺឆ្កែឆ្កួតដែរឬទេ?/**Do you know if there is a vaccine against Rabies?**	ទេ/No មិនដឹងទេ/Don't know បាទ/ចាសមាន, សម្រាប់ឆ្កែ/Yes, for dogs បាទ/ចាសមាន, សម្រាប់មនុស្ស/Yes, for humans បាទ/ចាសមាន, សម្រាប់ទាំងមនុស្ស និងសត្វឆ្កែ/Yes, for both dogs and humans
៤.១៣ តើអ្នកចង់ចាក់វ៉ាក់សាំងដល់ឆ្កែរបស់អ្នកសម្រាប់ជម្ងឺឆ្កែឆ្កួតដែរឬទេ?/**Would you want to vaccinate your dog for rabies?**	ទេ/**No** ប្រសិនទេ,ហេតុអ្វី?/**If, no why not?** បាទ/ចាស/**Yes** ប្រសិនបើអ្នកចង់, តើអ្នកនឹងព្រមបង់ថ្លៃសម្រាប់វ៉ាក់ដែរទេ?/**If yes, would you be willing to pay for the vaccine?** តើថ្លៃប៉ុន្មាន?/**How much:**
៤.១៤ ប្រសិនបើអ្នកនឹងចាក់វ៉ាក់សាំងឆ្កែរបស់អ្នកប្រឆាំងនឹងជម្ងឺឆ្កែឆ្កួត, តើអ្នកចូលចិត្តមួយណាជា	ការចាក់/**Injection** ផ្តល់វ៉ាក់សាំងនៅក្នុងអាហារ/**Give vaccine in food**

ង?/ **If you were going to vaccinate you dog against rabies, what would you prefer**	
៤.១៥ តើអ្នកគិតយ៉ាងដូចម្តេចប្រសិនបើវ៉ាក់សាំងនឹងត្រូវបានផ្តល់ឲ្យសត្វឆ្កែនៅក្នុងភូមិទាំងមូលដោយការផ្តល់វ៉ាក់សាំងតាមរយៈដុំអាហារ?/**What would you think if vaccines were given to dogs in the whole village by given them vaccines through pieces of food?**	មិនមែនជាគំនិតល្អទាល់តែសោះ/**Not a good idea at all** មិនប្រាកដ/**Not sure** ពិតជាគំនិតល្អខ្លាំងណាស់/**Very good idea**

៥. ពត៌មានសម្រាប់សត្វឆ្កែ (១សន្លឹក សម្រាប់ឆ្កែ១ក្បាល)**Dog information one sheet per dog:**

លេខកូដសំណាក (លេខកូដសំនួរ និង សំនួនសត្វឆ្កែ)៖/**Sample code (questionnaire code plus number of the dog):**

៥.១ អាយុសត្វឆ្កែ/ **Age of the dog**	
៥.២ ពូជ/ **Breed**	
៥.៣ ការប្រើប្រាស់ចម្បងរបស់សត្វឆ្កែ/ **The dogs' main use**	យាម/Guard កំដរ/Company សាច់/Meat ផ្សេងៗ, សូមពន្យល់/Other, explain:
៥.៥ អ្នកមើលថែចម្បង/**Main caregiver**	មនុស្សពេញវ័យនៅក្នុងគ្រួសារ/ Adult in family ក្មេងនៅក្នុងគ្រួសារ/Child in family ផ្សេងៗ, សូមពន្យល់/Other, explain:
៥.៥ ស្ថានភាពរស់នៅ/ **Living situation**	នៅតែខាងក្រៅដោយសេរី/Only outside loose នៅតែខាងក្រៅដោយជាប់ចំណង/Only outside in a leash

	នៅតែខាងក្រៅដោយសេរី ប៉ុន្តែក្នុងតំបន់មានរបង/Only outside loose but in a fenced area នៅតែក្នុងផ្ទះ/Only indoor ទាំងក្នុង និងក្រៅ, ពេលនៅក្រៅមានចំណង/Both indoor and outside, when outside in a leash ទាំងក្នុង និងក្រៅ, ពេលនៅក្រៅក៍សេរី/Both indoor and outside, when outside loose រស់នៅជាមួយគ្រួសារ/Lives with family
៥.៦តើអ្នកបានឆ្កែមកចិញ្ចឹមដោយរបៀបណា?/ **How did you come to own the dog?**	ទិញ/Bought ការដូរ/A gift ជាកូនឆ្កែដែលបានមកពីឆ្កែមុនៗ/A puppy from previous dog ផ្សេងៗ, សូមពន្យល់/Other, explain:
៥.៧ តើអ្នកដែលយកឆ្កែរបស់អ្នកទៅជួបពេទ្យសត្វ ឬអ្នកជំនាញពេទ្យសត្វដែរឬទេ? / **Have your dog ever visited a veterinarian or veterinary technician?**	ទេ/No បាទ/ចាស/Yes ប្រសិនបើធ្លាប់, តើយកទៅដើម្បីអ្វី?/If yes, what for:
៥.៨ តើឆ្កែរបស់អ្នកដែលមានរបួសដោយសារខាំក្នុងរយៈពេល៦ខែមុនដែរឬទេ? **/Did your dog have any bite wounds the last six month?**	បាទ,ចាស/Yes ទេ/No
៥.៩ តើឆ្កែរបស់មានសញ្ញានៃការឆេវឆាវដែរឬទេ?/**Has your dog ever shown signs of aggression?**	បាទ,ចាស/Yes ទេ/No ប្រសិនបើមាន, តើវាសំដៅទៅមនុស្ស ឬសត្វផ្សេងៗ?ឬទាំងពីរ/If yes, towards humans or other animals or both?

6. ស្ថានភាព សុខភាពរបស់សត្វឆ្កែ/**Health status of the dog:**

៦.១ តើសត្វឆ្កែនេះធ្លាប់ទទួលបានវ៉ាក់សាំងដែរឬទេ?/ **Has the dog ever gotten vaccinated?**	បាទ,ចាស/Yes ទេ/No (បន្តទៅសំនួរ ៦.៤) (continue to 6.4) **មិនដឹងទេ**/Don't know
៦.២ តើញឹកញាប់ប៉ុណ្ណាដែលសត្វឆ្កែនេះទទួលបានវ៉ាក់សាំង?/ **How often does the dog get vaccination?**	ម្តងគត់/One time ម្តង/១ឆ្នាំ 1 time / year ម្តង/៣ឆ្នាំ 1 time / 3 years ផ្សេងៗ/Other: មិនដឹងទេ/Don't know
៦.៣ តើប្រឆាំងនឹងជម្ងឺអ្វីដែរ?/ **Against which illnesses?**	
៦.៤ ប្រវត្តិនៃជម្ងឺរបស់សត្វឆ្កែ (អាចរើសបានជម្រើសលើសពីមួយ)/ **History of illness of the dog** **(More than one option can be selected)**	របួសដោយសារខាំ/Bite wounds ក្អួត/រាគVomiting/diarrhoea ប៉ារ៉ាសិត/Parasites ពិការភាព (ខ្វិន)/Lameness ផ្សេងៗ, សូមពន្យល់/Other, explain:

ការសង្កេតពីការទទួលយកនៃនុយ (ត្រូវបំពេញដោយអ្នកសង្កេត)

Observation of bait acceptance (to be filled by the observer)

៧. តើនុយមួយណាដែលបានផ្តល់ដំបូងគេ?/Which bait was given first? _____

	នុយទី១Bait 1			នុយទី២Bait 2		
	ទេ/No	បាទ,ចាស Yes	មិនប្រាកដ Uncertain	ទេ/No	បាទ,ចាស Yes	មិនប្រាកដ Uncertain
ឆ្កែបង្ហាញពីការចាប់អារម្មណ៍ទៅលើនុយ (សម្លឹងមើល,ហិតក្លិន) /Dog shows interest in bait (looks at, sniffs at)						

ឆ្កែលិឧនុយ/Dog licks at bait						
ឆ្កែខាំនុយនៅក្នុងមាត់វា/Dog takes the bait into it's mouth						
ឆ្កែទំពានុយ/Dog chews the bait						
ឆ្កែលេបផ្នែកខ្លះរបស់នុយ/Dog swallows part of the bait						
ឆ្កែលេបនុយទាំងអស់/Dog swallows the whole bait						

Questions for village leaders:

1. Do you know if there has ever been any vaccination campaigns for rabies in your villages? In that case, how did it happen, and when was it?
2. What do you think about rabies vaccine campaigns? Do you think they work?
3. How many dogs approximately are in your village? Is it common with people eating dogs? Are there many dogs that no one owns?
4. Is there a problem with aggressive dogs or wild dogs?
5. If there was a campaign using oral vaccination, what would you think about that? Do you have any concerns?

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