COMPARATIVE STUDY OF HISTOCHEMICAL STAINING OF NEGRI BODIES USING LENDRUM'S PHLOXINE-TARTRAZINE AND FUCHSIN SAFRANINE BLUE STAINING METHODS

BY

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DECLARATION

I **Nambajjwe Pamellah** hereby declare that this report is my personal effort; it holds a high degree of originality and has never been submitted to this University or any other higher institute of learning, for any award.

Signature..... Date....4th/12/2019....

APPROVAL

This research project has been submitted under the supervision of my supervisor

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CDL	Central Diagnostic Laboratory
CNS	Central Nervous System
COVAB	College of Veterinary Medicine, Animal Resources and Biosecurity
dFAT	Direct Fluorescence Antibody Test
Drit	Direct Rapid Immunohistochemical Test
H&E	Hematoxylin Eosin
IBs	Inclusion Bodies
OIE	World Organization for Animal Health
PEP	Post Exposure Prophylaxis
RABV	Rabies Virus
RIDT	Rapid Immunodiagnostic Test
RNA	Ribonucleic Acid
RREID	Rapid Rabies Enzyme Immunodiagnostic
SPSS	Statistical Pack For Social Science
SST	Seller's Staining Test
WHO	World Health Organization

ABSTRACT

Rabies is a fatal zoonotic disease caused by infection with the rabies lyssavirus, which causes 55,000 human deaths annually worldwide with 95% occurring in Africa and Asia. Diagnosis of rabies started way back in 1880 with inoculation of rabbits with the rabies virus. Today there are many methods used in rabies diagnosis from routine techniques such as Lendrum's Phloxine-Tartrazine method and Fuchsin Safranine blue method to rapid techniques such as direct Fluorescent Antibody Test (dFAT), Rapid immunodiagnostic test (RIDT) and others with Fluorescent Antibody Test as a gold standard. However, dFAT is expensive and requires experienced personals as well as special equipment which are not usually available in developing countries such as Uganda. The aim of this study was to assess the histochemical staining of Negri bodies using Fuchsin Safranin blue method in comparison to Lendrum's method and demonstrating the structure of Negri bodies. A total of thirty achieved rabies suspected brain samples were sectioned and stained with Lendrum's Phloxine-Tartrazine method and Fuchsin Safranine blue method. The stained sections were observed under a light microscope at x40 for the presence of Negri bodies. Out of 30 brain samples, 17 and 12 were found to be positive for Lendrum's Phloxine-Tartrazine method and Fuchsin Safranin blue staining method respectively. The sensitivity, specificity, predictive value of positive results and predictive value of negative results of Fuchsin Safranin blue staining method in relation Lendrum's Phloxine-Tartrazine method was found to 70.1%, 100%, 100% and 72.2% respectively. In conclusion, the sensitivity of Fuchsin Safranine blue method for rabies is low in relation to Lendrum's method, Fuchsin Safranine blue method has a high specificity for rabies and Lendrum's method gives the best picture of Negri bodies. Therefore Lendrum's method is recommended for diagnosis of rabies and demonstration of Negri bodies in brain samples to confirm rabies.

CHAPTER ONE
INTRODUCTION

1.1 Background

Rabies is a fatal zoonotic disease caused by infection with the rabies lyssavirus Pringle, (1999), which causes 55,000 human deaths annually worldwide with 95% occurring in Africa and Asia (Kamoltham *et al.,* 2003). With an estimate of approximately 30% to 60% in children below 15years of age (Kayali *et al*., 2003). Rabies is an endemic disease which is known for many years. It's found in many animals such as rats, monkeys, cats, bats, foxes and dogs that are mainly responsible for the maintaining of the disease and transmitting it to humans and other animals (Kadam *et al.*, 2011). World Health Organization (WHO), (2018), reported that about 99% of all rabies transmissions to humans is caused by a domestic animal..

These human losses occur despite the existence of effective anti-rabies vaccines for humans and animals and data that supports the feasibility of dog-rabies elimination(Hampson *et al.,* 2015). In areas with high rabies burden, the disease remains largely under reported owing to poor surveillance and misdiagnosis with other common diseases manifesting with nervous disorders such as cerebral malaria (Lembo et al., 2010). Consequently, this has led to a perceived lack of importance for rabies, driving a cycle of neglect for this endemic disease.

Diagnosis of rabies started way back in 1880 with inoculation of rabbits with rabies virus (Duong *et al*., 2016). Thereafter detection of Negri bodies in1903. Today there are many methods used in rabies diagnosis from routine techniques such as Hematoxylin and eosin staining (H$E), Seller's Staining Test (SST), Lendrum's Phloxine-Tartrazine method and Fuchsin Safranine blue method for inclusion Negri body staining to rapid techniques such as direct Fluorescent Antibody Test (dFAT), Rapid immunodiagnostic test (RIDT), direct Rapid Immunohistochemical Test (dRIT), Rapid rabies enzyme immunodiagnostic (RREID) and other techniques that demonstrate the presence of rabies viral antigens. These rapid techniques are quick with high sensitivity and specificity for rabies diagnosis. The dFAT is recommended as a gold standard for rabies diagnosis (WHO, 2018).

However, dFAT is expensive and requires experienced personals as well as special equipment which are usually available only in reference laboratories in developing countries such as

Uganda. There is need to compare the sensitivity and specificity of other diagnostic methods of detecting rabies which are cheap, easy to perform, readily available in peripheral laboratories and can easily be used for demonstrating the picture of Negri bodies to confirm rabies diagnosis. The aim of this study was to assess the histochemical staining of Negri bodies using Fuchsin Safranin Blue method in comparison to Lendrum's method and demonstrating the structure of Negri bodies.

1.2 Problem statement

Diagnosis of rabies in suspected animals requires the recovery of the brain for demonstration of rabies inclusion bodies or antigens. Unfortunately, many of the peripheral and sometimes central diagnostic laboratories Uganda are unable to perform the recommended dFAT due to the use of specific antibodies or fluorescent microscopes which are expensive to obtain and hence lacking in the country. Therefore, histochemical methods such as lendrum's Phloxin Tetrazine staining remain inevitable. Recently, Lendrum staining in the College of Veterinary Medicine Animal Resources and Biosecurity has been affected by the quality of Phloxine and Tetrazine dyes, hence there is a need to try an alternative histochemical staining method to demonstrate rabies Negri inclusion bodies.

1.3 Objectives

1.3.1 General

To assess the histochemical staining of Negri bodies using Fuchsin Safranin Blue method in comparison to Lendrum's method.

1.3.2 Specific objectives

1. To determine the sensitivity and specificity of Fuchsin Safranine Blue method
2. To determine which of Lendrum's method and Fuchsin Safranine Blue method gives the best picture of Negri bodies.

1.4 Research questions

1. What is the sensitivity and specificity of fuchsin safranine blue method in relation to Lendrum's method?

2. Which of Lendrum’s method and fuchsin safranine blue method gives a better picture of Negri bodies?

1.5 Justification and Significance

Rabies is a highly fatal disease with 100% case fatality rate if signs and symptoms appear. It mostly occurs in developing countries with no surveillance mechanism for identification of rabies. In the developing countries, people keep dogs and cats which are unvaccinated against the rabies virus. This puts most of the people in developing countries at a high risk of getting the disease. The diagnosis of rabies in such countries is done by some of the routine diagnostic tests with a relatively low sensitivity as compared to the gold standard which has a higher sensitivity, are more expensive and require special equipment. However, special stains are cheaper and easily performed in the lab Lendrum’s and fuchsin safranine blue methods. If this study is done, the results will be used to decide which routine test to be used in early diagnosis of rabies. This will help in rabies surveillance for the early management of rabies disease.

CHAPTER TWO
LITERATURE REVIEW

2.1 Historical review of rabies

Rabies was first diagnosed in Uganda in 1935 in our laboratory here in Entebbe (Winyi., 1999). The department of veterinary services that had been created in 1909 to control major epidemic diseases vigorously vaccinated the dogs and brought the disease under control. Up to the early 1970's, rabies was only restricted to the border districts of North West and South Western parts of the country (Masiira *et al*., 2016). Other districts were regarded as rabies free. During the period of civil strife of the 1970's and 1980's, there was a breakdown of delivery of veterinary services and there was a resurgence of most of the major epidemics of livestock including rabies. This was accentuated by the increasing number and mobility of both human and dog populations that are usually associated with civil strife and breakdown of the economy (Ministry of Health (MoH), 2015). The economic situation at the time could not regularly avail the vaccines and logistics to enable carrying out meaningful rabies vaccination campaigns. The staff morale was low and our diagnostic capacity literally went to nil.

2.2 Rabies virus

Rabies is caused by negative strand RNA- viruses belonging to the genus Lyssavirus, family Rhabdoviridae of the order Mononegavirales, causing a fatal disease associated with intense viral replication in the central nervous system (Lahaye *et al*., 2009). Lyssaviruses are bullet-shaped with a helical nucleocapsid consisting of the ribonucleic acid (RNA) genome, nucleoprotein (N), phosphoprotein (P, formerly M1 or NS) and polymerase protein (L, large). The nucleocapsid is surrounded by an envelope derived from a host-cell lipoprotein membrane and is studded with viral glycoprotein (G) trimers (Kapdri *et al*, 1996)). A structural membrane protein (M, previously M2) supports the viral envelope (Barrat *et al*., 1988).

There are many lyssavirus genotype in the world which cause rabies. However, four genotype are endemic in Africa, especially in South Africa. The most common is Genotype 1 (Rabies virus, RABV). Others are Genotype 2 (Lagos bat virus, LBV), Genotype 3 (Mokola virus, MOKV) and Genotype 4 (Duvenhage virus, DUVV). Human infections are mostly due to the canine biotype of RABV (World Health Organization (WHO), 2013).

2.3 Transmission and pathogenesis of the rabies

Rabies is an invariably fatal illness. Diagnosis in the majority of cases is based on the characteristic features of hydrophobia and most of the times do not warrant clinical acumen. Since the disease is endemic in all the countries (Costa et al., 2018). There are weak reporting and surveillance system, this has resulted into failure in the creation of a reliable database and hence the figures being made available by countries are only estimates. Accordingly, it is believed that around 35 000 people die of hydrophobia every year in the countries of the South-East Asia Region. In addition, about 1.5 million people undergo antirabies vaccination. The country-wise estimates (WHO, 2013).

Rabies is caused by neurotropic virus that affects the CNS, particularly causing inflammation of the brains (WHO, 2018). The virus first infects the muscle cells close to the site of infection where they are able to replicate without being noticed by the hosts' immune system. Once enough virus has been replicated, they begin to bind acetylcholine receptors in the neuromuscular junction. The virus is highly neurotropic and once it enters the body through a break in the skin or mucous membrane, it migrates along the nerves from the site of infection to the brain where it causes fatal encephalitis. The body organs are shut down when the virus travels from the brain back out to the organs (Jackson., 2011).

Rabies is a fatal viral disease largely transmitted to humans from bites and scratches of rabid animals. Carnivorous of the family canidae are the most transmitters of rabies to humans and other animals with about 99% cases (Gongal and Wright, 2011). Transmission by wild animals such as bats, foxes, cats and wolves is also reported (Johnson *et al.*, 2014).

Usually, person to person transmission is rare but precautions should be taken to prevent exposure to the saliva of diseased person and tissues from diseased individuals must not be used in transplant procedures (Johnson, 2017). The rabies virus enters the host through broken skin or mucous membranes. The successful transmission is dependent on several factors including the type of exposure, which can be categorized into bite and non-bite. Bite exposure is the most common and significant method of rabies transmission, with the introduction of virus laden saliva directly into the host. More extensive bites are considered higher risk as there is potentially more viral interaction with a wider network of nervous tissue with greater viral dissemination toward the CNS (Johnson et al., 2014).

Non-bite exposure is mainly attributed to the aerosolized virus, splash exposures while handling and butchering of infected carcasses, tissue and organ transplants and contamination of open wounds or mucous membranes by infected material. After inoculation, the virus replicates locally in affected muscle fibers then accesses the peripheral motor axons by binding to acetylcholine receptors at the neuromuscular junction for travel to the CNS (Lepine *et al.,* 2012). The virus further replicates in motor neurons of the spinal cord and local dorsal root ganglia before ascending to the brain. Centrifugal spread then occurs from the CNS via slow anterograde axoplasmic flow (passive) along peripheral nerves to the salivary glands, skin, cornea, and other end organs. Viremia does not occur for rabies infection.

The virus leads to supprative ancephelitis which is responsible for rabies signs and death.

2.4 Signs and symptoms

World Health Organization, (2017) reported that the incubation period for rabies usually varies from 1-3 months, but may also vary from less than a week to greater than a year depending on the age, location of the bite and the rabies viral load on the human. Initial symptoms of rabies include confusion, agitation, nausea, vomiting, headache, excessive salivation, insomnia, partial paralysis and fever with pain and unusual or unexplained tingling, pricking, or burning sensation at the wound site. As the virus spreads to the central nervous system, a progressive and fatal inflammation of the brain and spinal cord develops. Rabies virus infection induces the formation of cytoplasmic inclusion bodies (IBs) called Negri bodies (Harris, 1903). The intracytoplasmic inclusion bodies specific to rabies encephalitis vary in size from small as 3µm to as large as 30µm (Mani and Madhusudana, 2013) . These Negri bodies are usually circular or oval and deeply eosinophilic with characteristic basophilic granules arranged in the form of a rosette within the eosinophilic matrix (Lahaye et al., 2009).

Furious and the paralytic rabies are the two main forms of clinical presentation of human rabies observed. People with furious rabies exhibit signs of hyperactivity, excitable behavior, hydrophobia and sometimes aerophobia. Death occurs after a few days due to cardiorespiratory arrest. Paralytic accounts for nearly one third or approximately 30% and the rest of the cases are due to furious rabies which is about 67%. Paralytic rabies cases cause paralysis of the limbs and respiratory muscles whereas furious is characterized by hydrophobia, aerophobia, reduced consciousness, delirium, excited behavior, hyperactivity and occasionally seizures (Depani *et al*.,

2012). Death occurs within 5-7 days after the onset of symptoms in case human rabies is not treated and the survival span of patients is prolonged by 133 days when treatment is given. Respiratory and cardiac failure is the major cause of death in human rabies cases. It is important to note that by the time any symptoms, rabies cannot be successfully treated (Hampson et al., 2015).

2.5 Prevention and treatment

Efforts must be focused on preventing the rabies disease since it cannot be treated. Rabies is a vaccine preventable disease. Vaccinating dogs is the most cost effective strategy for preventing rabies in people (Hampson et al., 2015). Japan initiated the first urban dog vaccination program in the world and eliminated rabies in 1954 by reducing the urban dog population and by vaccinating dogs (Kurosawa *et al*., 1991). Dog vaccination reduces deaths attributable to rabies and the need for PEP as a part of dog bite patient care.

Post exposure treatment of the person and vaccination of the animals that transmit the disease are included in the disease prevention measures. World Health Organization,(2018) reported that pre exposure immunization of people with a high risk of being bitten by dogs or highly exposed individuals such as veterinary doctors, game rangers, laboratory workers handling live rabies and rabies related viruses should be done. Also long term travelers and expatriates moving to rabies affected remote areas who plan to spend a lot of time outdoors should also be immunized.

Dogs suspected to be having rabies should be killed and burnt or buried immediately to prevent further exposure to other animals and people. Creating awareness on rabies through education on dog behavior and bite prevention for both children and adults is an essential extension of a rabies vaccination program and can decrease both the incidence of human rabies and the financial burden of treating dog bites (Lembo et al., 2010).

Increasing awareness of rabies prevention and control in communities includes education and information on responsible pet ownership, how to prevent dog bites, and immediate care measures after a bite. Engagement and ownership of the program at the community level increase the reach and uptake of key messages (Kurosawa et al., 1991).

2.6 Burden of the disease

Rabies is a fatal zoonosis which remains a public health threat in developing countries with a case fatality rate of nearly 100% in both animals and humans(Apanga *et al*., 2016) reported that rabies affects all warm blooded animals and it is prevalent throughout the world and endemic in many countries except in Austria and Antarctica. This is attributed to their unavailing climate conditions in the islands (Singh et al., 2017).

It is estimated that 95%cases of human rabies death occur in Africa and Asia. A total of 35172 humans die every year in Asia where India accounts for 59.9% of the rabies deaths in Asia and 35% globally. In Africa, it is estimated that 21476 human deaths occur each year due to dog mediated rabies (Gongal and Wright, 2011)**.** World Health Organization,(2018) reported that the annual cost of rabies is US$ 6 billion, with almost US$ 2 billion spent directly on post exposure prophylaxis. Rabies also affects other animals such as cattle which causes more loses to farmers and the economy of the country.

2.7 Diagnosis

The development of rabies diagnostic tests began with routine inoculation of rabbits with rabies virus (RABV)-infected brain and saliva samples in 1880. This was followed with the identification of Negri bodies after 1903 (Lendrum, 1943). Today, several different assays and diagnostic techniques are now available which include rapid and routine diagnostic techniques (Tekki et al., 2016). According to the WHO standards rapid viral diagnostic techniques are categorized into four; radioimmunoassay, electron microscopy, enzyme techniques and dFAT which is mostly used in developed countries. Due to the lack of required equipment, reagents, and trained personal, the above techniques are rarely used in developing countries. Diagnosis of rabies can be done using different samples such as saliva, CSF, skin biopsy, serum and brain.

In most of the developing countries, routine diagnostic techniques are used for detection of rabies and these include seller’s test, Lendrum's Phloxine-Tartrazine method and Fuchsin Safranine blue method (Meslin *et al*., 1973).

2.7.1 Inoculation Test

Historically and in the research setting, RABV infection is identified by infecting cells and detecting the virus. This can be done either through the mouse inoculation test (MIT) or by

inoculation of samples onto cultures of murine neuroblastoma or other cells (rapid tissue culture infection test, RTCT). Following intracerebral inoculation of mice aged 3–4 weeks, MIT test results are available after an incubation period of up to 28 days. Some strains are associated with a longer incubation period. In laboratories with cell culture facilities and an appropriate level of bio-containment, the RTCT provides results within 24–48 h, which is far quicker than intracerebral inoculation (Dacheux and Bourhy, 2015).Although it is more sensitive to toxic or bacterial contaminants, its sensitivity is comparable to that of the MIT (Barrat et al., 1988). As RABV does not cause any cytopathic effect, the detection of the virus must, therefore, be evidenced by direct fluorescent antibody testing (DFAT, see below). In addition, MIT requires animal facilities to produce mice (or a supplier that can quickly provide animals of suitable age in sufficient numbers), as well as animal facilities with a high level of bio-containment (ASL3) to maintain the inoculated animals. Animal ethics regulations also recommend avoiding the use of animals when an efficient cell culture system exists. As results are usually required urgently and due to animal protection issues, the World Health Organization (WHO) (Meslin et al., 1973) as well as the World Organization for Animal Health (OIE), now recommend replacing the MIT with the isolation of RABV in cell culture whenever possible.

2.7.2 The Hematoxylin and Eosin

Although historically used, there have been great laboratory changes in Hematoxylin stains; nearly all tissue specimens are treated with Hematoxylin and Eosin today (Bancroft *et al.*, 1983). In addition, various Hematoxylin methods have been developed but all follow the same approach of staining tissue specimens in a hematoxylin, alcohol and tap or alkaline water to clear argentaffin agents. It has been found that most histopathological processes could be studied using Hematoxylin and Eosin procedures (Alturkistani *et al.*, 2015). In the same line, the method is quick to execute, cheap and can be altered. However, the Hematoxylin and Eosin are inefficient in that not all features of a substance can be received and special stains must be used (Musumeci, 2014).

2.7.3 Lendrum's Phloxine-Tartrazine Method

This was invented by Lendrum in 1947, it consists of Phloxine, calcium chloride, distilled water and saturated solution of Tartrazine in 2 Ethoxyetha. Since the Negri bodies have acidophilic

nucleus and basophilic cytoplasm, Lendrum's uses this contrasting acid and basic dyes to exploit these differences in charges on the inclusion body and the host cell (Lendrum, 1947).

2.7.4 Fuchsin Safranine Blue Test

This consists of basic Fuchsin, 50% solution of ethanol and an aqueous solution of Safranine. The stoma stains very pale pink with nerve fibers staining with deeper pink, neuroglia and leukocytes stain purplish blue, neurons are stained light blue, nuclear inclusions and oxyphilic substances are stained bright red and Negri bodies take up the red to mauve pink with the internal structure lilac (Woods and Walker., 1996).

2.7.3 Seller's test

Seller's test was invented by seller in 1920 and since then it has been used for identification of Negri bodies. A lot of research is being done comparing its activity and the available rapid diagnostic tests. The most characteristic feature of Negri bodies is its internal structure considered to be essential for positive identification of rabies (Woods and Walker, 1996). Matrix of inclusion bodies has an acidophilic staining reaction contained within the magenta red stained structure and the basophilic granules that stain dark blue to black. Seller's is composed of methylene blue solution, methanol and basic Fuchsin (Percival *et al.*, 1913)

2.7.4 Direct fluorescent antibody testing (dFAT)

Direct fluorescent antibody testing is the main assay used worldwide; it is the WHO and OIE recommended gold standard for the diagnosis of rabies in fresh or frozen brain samples. The latter are important in tropical countries, as preserving fresh samples at 4 8C is often a challenge (Hanlon and Nadin, 2013). This assay is based on attaching fluorescein isothiocyanate (FITC) to polyclonal antibodies targeting the RABV ribonucleocapsid, or monoclonal antibodies targeting the rabies virus nucleoprotein (N). If the targeted rabies virus antigen is present in the sample fixed on a slide, antibodies attach to it, remain attached despite washing, and can be observed using a fluorescence microscope (Mani, 2013). Results are available within 1–2 h and are expressed as positive or negative. The sensitivity and specificity of dFAT near 99% in an experienced laboratory but is extremely observer-dependent. This test is best performed on fresh brain samples; the reliability of this assay to diagnose rabies in degraded animal brain samples or corneal smears is low (Dacheux et al., 2008).

CHAPTER THREE
METHODS AND MATERIALS

3.1 Study Area and Study Subject

The study was conducted in the pathology laboratory of Makerere University, College of Veterinary Medicine, Animal Resources and Biosecurity. Archived blocks containing samples suspected of rabies were used in the study.

3.2 Study Design

This was a retrospective study done on archived samples in the pathology laboratory.

3.3 Sample identification and retrieval

The record books were checked and all identification numbers for rabies suspected brain samples were obtained. Using these identification numbers samples were looked for in block cabinet and transferred to the refrigerator for an overnight ready for sectioning.

3.4 Sectioning

This was done using a microtome. The blocked samples were placed on melting ice for 5 minutes, then fixed in the microtome chuk and sectioning began. The excessive layer of wax was removed to expose the tissues. A series of thin sections of same thickness 5-10 micrometers as set on the microtome were obtained. The sections were removed using camel brush and floated on warm water at 44^0C in a water bath. The sections were then mounted individually on a labeled microscope slide. The slides were placed in slide rank and then dried in an oven for overnight.

3.4 Reagent Preparation

3.5.2 Fuchsin Safranin Blue Stains

This consists of two solutions one and two and both were prepared as earlier done by (Meslin *et al.*, 1996). Briefly, in solution 1 1g of basic fuchsin was dissolved in 200ml of ethanol 50% solution and solution 2 contained safranin 0.2% aqueous solution. Each solution was properly identified and stored in well labeled bottles.

3.5.3 Unnas polychrome methylene blue

This was prepared as earlier done by (Clinch, 1899). Briefly, 0.3g of methylene blue powder was dissolved in 30ml of 95% ethyl alcohol. 0.1g of potassium hydroxide was weighed and added to the solution, followed by 100ml of distilled water. The solution was stored in a well labeled covered bottle.

3.5.3 Lendrum's Phloxine-tartrazine Method

This stain consists of Mayer's hematoxylin solution, phloxine stain and tartrazine stain and all were prepared as earlier done by (Lendrum, 1947). Briefly, 1g of hematoxylin powder was dissolved in 1000ml of distilled water, 0.2g of sodium iodate was weighed and added, followed by addition of 50g of potassium alum. Then 1g of citric acid and 50g of chloral hydrate was added to the mixture to make up Mayer's hematoxylin solution. To prepare Phloxine stain, 1g of Phloxine was dissolved in 200ml of 70% alcohol, followed by addition 1g of calcium chloride. To prepare Tartrazine stain, 2.5g of Tartrazine powder was weighed and dissolved in 100ml of ethylene glycol monoethyl ether. The different preparations were put in well labeled glass bottles for storage.

3.6 Laboratory Analysis

3.6.2 Fuchsin Safranine Blue Method

Staining was performed according to the technique described by (Rupprecht, 1996). The section was stained for 10minutes under a mixture of equal parts of solution 1 and 2. The section was then washed quickly with a mixture of ethanol and acetone which removed the excess stain. The section was stained with Unna's polychrome methylene blue for 15seconds. The section was then differentiated in ethanol- acetone for a few seconds and then washed immediately in running tap water to remove excess stain, then followed with a second treatment with ethanol-acetone. Rapidly, complete dehydration in absolute alcohol was done and then alcohol was carefully removed by passing through several changes of xylene.

3.6.3 Lendrum's Staining Method

This staining method was performed as earlier described by (Lendrum, 1947). Briefly, the sections were put in xylene and then rehydrated through absolute and 95% alcohol and distilled water for 3minutes in each. Then the rehydrated sections were stained for 5minutes using Mayer's hematoxylin solution and blued in running water. The sections were stained with

phloxine solution for 30minutes and rinsed briefly in distilled water. These sections were then covered with tartrazine stain from a dropping bottle. The sections were dehydrated through 60%, 95% and absolute alcohol, cleared with xylene at two changes. Then the stained sections were mounted with DPX.

3.7 Data Analysis and Presentation

All data collected was entered into excel, then imported into Statistical Package for Social Sciences (SPSS) version 20 using the crosstab function to determine sensitivity and specificity.

3.8 Limitation

The cost of some reagents was so high and only large quantities are supplied yet small quantities were needed.

The stains cannot be used when freshly prepared for better results. They need a long period of time after their preparation before use.

CHAPTER FOUR

4.0 Results

4.1Detection of rabies Negri inclusion bodies using fuchsin safranin blue staining method and Lendrum's Phloxine-Tartrazine method.

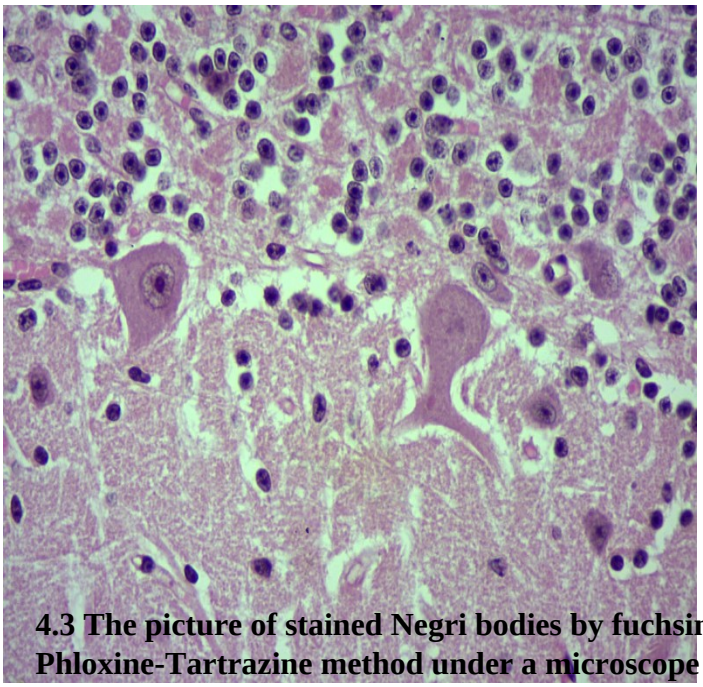
In the present study comparison of the two diagnostic methods (Lendrum's Phloxine-Tartrazine method and Fuchsin Safranin blue staining method) was carried out with a total of 30 suspected rabies brain achieved samples. Out of 30 brain samples, 17 and 12 were found to be positive by Lendrum's Phloxine-Tartrazine method and Fuchsin Safranin blue staining method respectively. According to the results, there were no false positive results obtained. However, 5 samples were found to be false negative for fuchsin safranin blue staining method relative to Lendrum's Phloxine-Tartrazine method as shown in table 1.

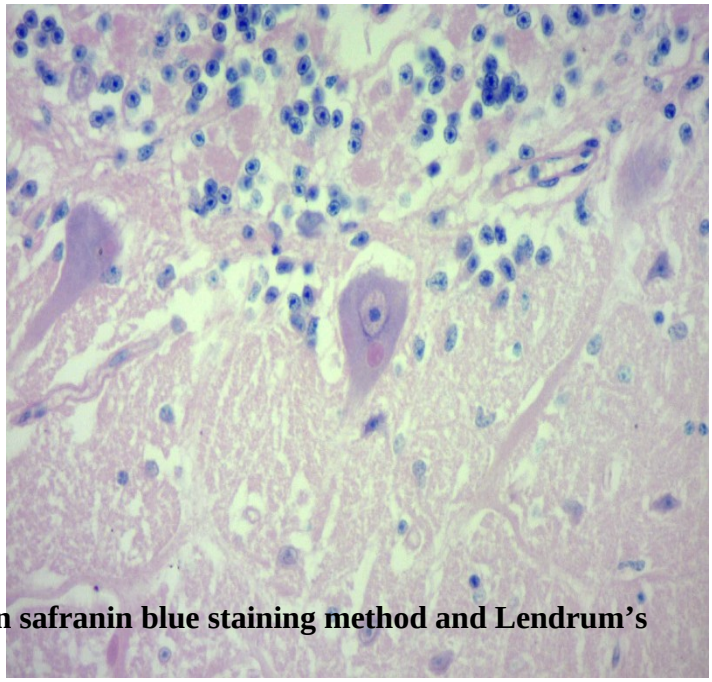
Table 1: Comparative evaluation of tests performed for the rabies diagnosis with Lendrum's Phloxine-Tartrazine method and fuchsin safranin blue staining method

Technique		**Lendrum's Phloxine-Tartrazine method**		
	Result	positive	negative	**Total**
Safranin blue	Positive	12	0	**12**
	negative	5	13	**18**
Total		**17**	**13**	**30**

4.2 Sensitivity and specificity of fuchsin safranin blue staining method.

The sensitivity, specificity, predictive value of positive results and predictive value of negative results of Fuchsin Safranin blue staining method was calculated in relation Lendrum's Phloxine-Tartrazine method was 70.1%, 100%, 100% and 72.2% respectively.





4.3 The picture of stained Negri bodies by fuchsin safranin blue staining method and Lendrum's Phloxine-Tartrazine method under a microscope

Figure 1: Microphotograph showing Negri bodies stained by Fuchsin Safranin blue method at X40.

Nucleolus

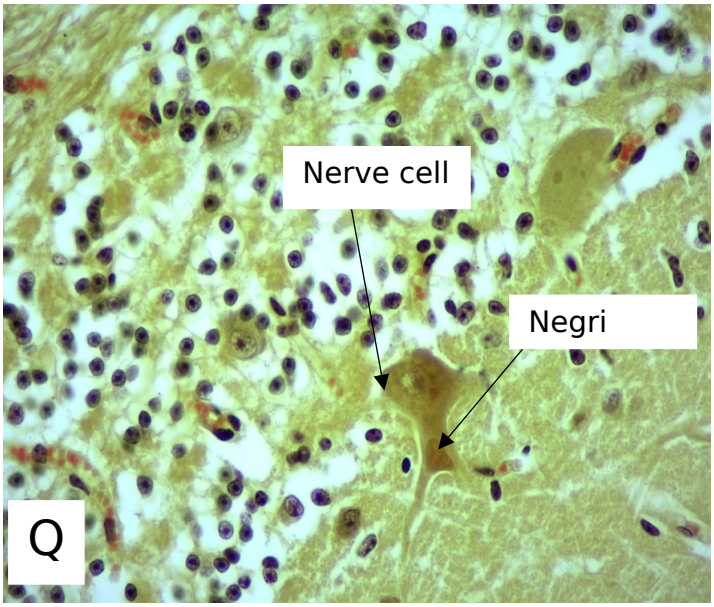
Nerve

Nerve

Negri

Negri

A B



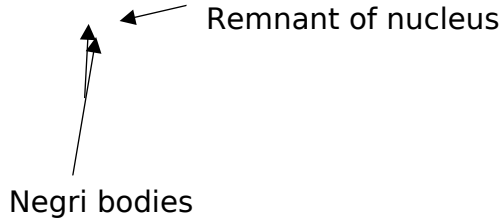


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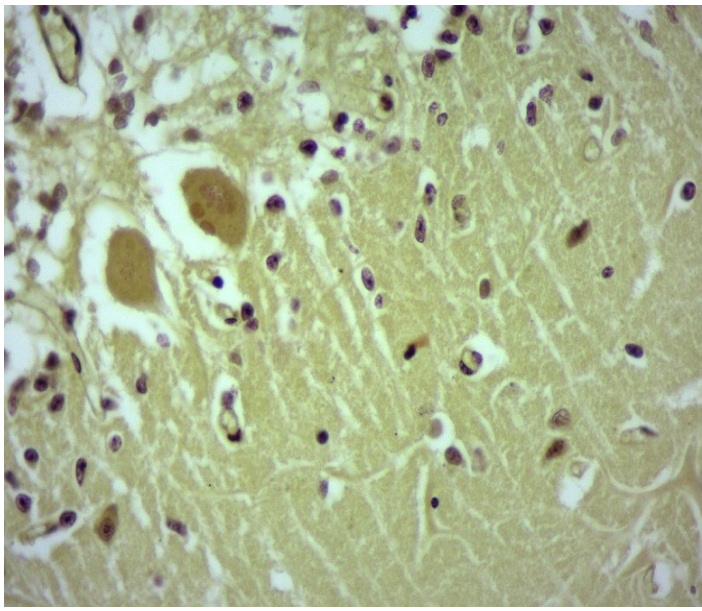
Figure 2: Microphotograph showing Negri bodies as stained by Lendrum's Phloxine-Tartrazine method X40.



CHAPTER FIVE

5.0 Discussion

The sensitivity of Fuchsin Safranin blue staining method in relation to the "gold standard" Lendrum's Phloxine-Tartrazine method was 72.1% (95% CI; 37.7% – 74. 0%), while the

specificity was 100% (95% CI; 71% - 100%). This showed that Fuchsin Safranin blue staining method missed Negri bodies in some positive samples in the study resulting in false negative results. However, the high specificity of the Fuchsin Safranin blue staining method means there were no false positive results from samples that tested positive by the Fuchsin Safranin blue staining method. Therefore, a positive result guarantees that the sample is truly positive while a negative result does not. This further implies that the Fuchsin Safranin blue staining method is only most useful to the clinician when the test result of rabies suspect animal is positive, but doubtful when the result is negative. Given that rabies is a deadly disease with 100% case fatality rate once the virus has entered the central nervous system (CNS), a highly sensitive, reliable and standardized technique is desirable for its accurate diagnosis. However, Yang *et al*.,(2012), reported that false negative results may occur in some infected materials and false positive results may occur if nonspecific inclusion bodies are present in tissue samples tested.

This study was done using special stains of Lendrum's Phloxine-Tartrazine method and Fuchsin Safranin blue staining method to stain the inclusion bodies in brain tissue. The result of this study revealed that Lendrum's Phloxine-Tartrazine method and Fuchsin Safranin blue staining method gives a good picture of Negri bodies as shown in figure 1 and 2. However, Lendrum's Phloxine-Tartrazine method showed a better picture of stained Negri bodies (Q and A of figure 2 and 1 respectively). This was in agreement with Lendrum, (1943) who reported that Lendrum's Phloxine-Tartrazine method gave a good picture of Negri bodies. This implies that special stains are good in demonstrating Negri bodies and can be used in teaching.

This study showed that Fuchsin Safranin blue staining method is 72.1% sensitive and 100% specific. However, the sensitivity was slightly higher while specificity was lower in the study done by Pavithrani *et al.,* (2014) for this method, Lendrum's Phloxine-Tartrazine method has been reported to be 90% sensitive and 100% specific this shows that Lendrum's method is better than Fuchsin Safranin blue staining method.

Although Negri bodies are specific for rabies virus infection, Fuchsin Safranin blue staining method is less sensitive and specific as it only detects affinity of Negri bodies for acidophilic stains (Mallewa *et al*., 2007). It has also been shown that Negri bodies are not developed in all infected individuals Robles and Miranda, (1992) and cannot be demonstrated by both Lendrum's Phloxine-Tartrazine method and Fuchsin Safranin blue staining method all animals from which

the virus can be isolated (Goldwasser *et al.*, 1959). Therefore Lendrum's Phloxine-Tartrazine method and Fuchsin Safranin blue staining method cannot be used as a confirmatory test for rabies.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.0 Conclusions

- The sensitivity of fuchsin safranine blue method for rabies is low in relation to Lendrum's method
- Fuchsin safranine blue method has a high specificity for rabies.
- Lendrum's method gives the best picture of Negri bodies.

6.1 Recommendations

Lendrum's method for diagnosis of rabies should be embraced in teaching institution for demonstration of the virus to students and in diagnostic laboratories.

More research should be done on special stains in rabies diagnosis to ensure good documentation.

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