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History, evolution and newer perspectives of rabies vaccines

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Abstract

Rabies continues to be one of the most deadly infectious diseases known to human race since antiquity, with a case fatality rate almost 100 per cent after the onset of clinical disease. The disease still has a significant impact on human and animal living all over the globe. It is found on all continents where terrestrial animals exist, with the bulk of animal and human cases documented in resource-constrained African and Asian countries, where thousands of human deaths are being recorded annually. The disease produces one of the most agonising deaths in humans and it is likely that the global statistic of roughly 59,000 human rabies fatalities per year is an underestimate. Scientific innovations that led to the successful development of several vaccines and immunisation policies in identified 'at risk'human and animal populationshave gained a great reputation in minimising the impact of disease across wide portions of the globe.Vaccines continue to be the most significant triumphs of the combined global efforts of the public and animal health communities and has achieved significant strides in the treatment, prevention, and control of disease. This paper describes the history, evolution, and accomplishments of human ingenuity, scientific endeavour, and the joint global efforts of the public and animal health communities that resulted in evolving an effective prevention and control strategies.

Keywords: *Evolution, history, rabies, vaccination*

Rabies, an ancient zoonotic viral disease associated with man's closest vertebrate companions, dogs, as animal vectors, was first identified and studied over 4000 years ago. It is a preventable yet devastating illness that kills around 60,000 people each year (Hampson *et al*., 2015). However, because of the rampant under reporting of cases, the true number of deaths is likely to be greater. In many countries of Asia and Africa, poor and rural people are disproportionately affected, who bears the burden and majority of deaths happening in children under 15 years. Dog bites cause 99 percent of human rabies infections and hence control of rabies in dogs still remains the key factor in control of the disease. Once symptoms appear, the disease is nearly always lethal with case fatality rate 100 per cent, being the hall mark of infection. According to WHO reports, the disease is extremely lethal, killing one person every nine minutes. The disease was often

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characterised as a neglected tropical zoonotic disease, which has inflated its potential to be a major worldwide public health issue. However, fortunately the disease is one of the few infectious diseases that could be prevented with vaccination even after exposure. Vaccination is critical in preventing fatalities and is the most effective method. Indeed, for more than a century, rabies vaccines have had a significant impact on health through averting deaths.

HISTORY OF RABIES TREATMENT

Man has been terrified by rabid dog bites for millennia.The disease is of great historical significance, having been described approximately four thousand years ago in the pre-Mosaic Eshnunna Code of ancient Mesopotamia as follows: "If a dog is vicious and the ward officials have made known to its owner, but he does not keep it in, and it attacks a man and kills him, the owner of the dog shall pay 2/3 of a mina of (Rosner, 1974)".

The disease has been chronicled perhaps more exhaustively than any otherviral disease. Since the time of Aristotle, a plethora of viewpoints and theories on its aetiology have been recorded. The disease has also been described by poets and thinkers from practically every civilization. Scholars from ancient Babylon, China, India, Persia, Greece, and Rome all wrote about a syndrome linked to disease and animal bites.One common misconception was that rabies was induced by a little "worm" near the base of the tongue. GrattiusFalistcus, an Ovid (1st century BC) contemporary poet, believed the mythological origin of the sublingual 'lyssa' of rabid dogs popularised by Pliny; they believed that extracting the worms totally cured the dog.This worm was also thought to possess magical curative powers in preventing the disease in the person bitten when it was injected, but only after having been carried around a fire (Baer, 2007).

By the Middle Ages, a more nuanced approach to the prescription of cures for the exposed person had evolved. It is unusual in that the time and location of the bite are nearly always known to the patient. However, until the nineteenth century, there was no reliable diagnosis of the disease in humans or animals. There was no isolation of the infectious agent, no animal control, and human treatment (Baer, 2007).

The incurability of the disease has spawned a myriad of superstitions and myths, some of which are still prevalent in specific areas today. Throughout history, the herbal cures for rabies have ranged from filbert nuts to hellebore and rose oil, to camomile tea. Eating a cock's brain, coxcomb, goose fat combined with honey, salted flesh of a rabid dog, and maggots from a dead dog's carcass were among the other treatments (Wilkinson, 1977).

Traditional procedures were used by non-medical personnel and the general people to halt the spread of the disease. Some physicians thought such techniques ought to be tried or advised them; one physician travelled to the seaside, apparently to bathe in the waves. Hot water or steam baths, cold affusions and Galvanism were frequently recommended for patients who receive dog bites (Carter, 1982).

St. Hubert was considered and worshipped as the patron of rabies and 'dullighedon,' or mental illnesses. People who had been bitten by dogs went to priests for the 'cutting' procedure, in which the priest made a tiny incision and inserted a small portion of the saint's tole.Animals attacked by rabid dogs were also 'treated' in the cult of St. Hubert: a key that St. Hubert got from the hands of the pope which was heated and applied red hot to the wound of the animal. This cauterization was accompanied by five to nine day period of penance placed on the owner, as well as a diet of 'holy' oat bread (Baer, 2007).

During the first century B. C., Cicero called attention to hydrophobia- thirst and fear of water and outlined the precautions to be followed, including quick cauterization of the wound or suction. In 1735, Shrewsbury cited two individuals who advertised their skills as successful 'Dippers of Man and Beast'demanding hefty fees for immersing unfortunate dog bite victims in the tidal waters of the River Severn.

Most nineteenth-century physicians who wrote about rabies therapy suggested that wounds be cauterised as soon as possible - an opinion that may be traced back to classical literature. Some people believed that any sort of cauterization was insufficient, and they advocated for excision or even amputation.

Nonetheless, rabies has intrigued the imaginations of poets and thinkers throughout antiquity. Despite the limited number of victims, the outbreaks have been extensively recorded due to the symptoms in both humans and dogs, the extended suffering of the victim and the unavoidable fatality of the established clinical condition. The alarming symptoms in both humans and dogs, the prolonged suffering of the victim and the unavoidable fatality of the established clinical disease: the distressing syndrome as a whole have meant that outbreaks of the disease have been meticulously out of proportion to the small number of victims claimed in comparison to the major scourges of mankind.

TOWARDS VACCINE DEVELOPMENT: THE WAY FORWARD…

Despite decades of superstitions and inaccurateappraisals, several pre-Pasteurian personalities gradually added more introspection (Table 1) as science finally held more influence than dogmatic repeats of "learned" expert opinion.

However, the first scientific attempt to prevent the development of rabies was made

Table 1. Pre- Pasteurian personalities related to rabies

Sl. No.	Individual	Period	Importance
1.	Aulus Cornelius Celsus	~ 25 AD	Roman author of De Medicina and an early proponent of wound treatment after bite
2.	Pliny the Elder	~ 70 AD	Roman naturalist with attribution in the influence of temperatures on disease and believed dogs were most susceptible to rabies during the hottest seasons of the year, as well as the alleged importance of "tongue worms
3.	Galen of Pergamon	~ 200 AD	Greek physician who advised prompt local treatment and that bite wounds be kept open to avoid viral absorption
4.	IbnSina (Avicenna)	~ 1000 AD	Persian physician who wrote a famous Canon of Medicine
5.	Moses Maimonides	~ 1198	Talmudic scholar and author of a treatise on Poisons and their Antidotes, who described long incubation periods in bitten persons
6.	Girolamo Fracastoro	~ 1546	Italian physician who recognized a clear material basis of contagion for rabies infections
7.	Giovanni Battista Morgani	~ 1769	Italian anatomist and author of On the Seats and Causes of disease, who established a fundamental pathological principle that diseases such as rabies are not dispersed vaguely throughout the body, but originate locally, in specific organs and tissues, such as the nerves
8.	Georg Gottfried Zinke	~ 1804	German investigator who demonstrated that virus could be transmitted by infectious saliva
9.	Apollinaire Bouchardat	~ 1852	French pharmacist who was one of the first to speculate on the potential utility of inoculations against rabies
10.	Pierre-Victor Galtier	~ 1881	French veterinarian who showed pathogen transmission via injection and bite, used rabbits as a research model, developed a concept for an early experimental intravenous vaccine producing immunity in sheep and who had a major influence upon Pasteur's later work

(Adapted from Nagarajan and Rupprecht, 2020)

by Louis Pasteur, one of the doyens in the field of vaccine development who was renowned as the 'Germ Hunter'. Vaccines have evolved from the first generation of crude nerve tissue based products to recombinant vaccines. Cell culture based inactivated vaccines for intramuscular (IM) and in tradermal (ID) use in humans continue to play a pivotal role when it comes to rabies prophylaxis. Parenteral and oral vaccinations have repeatedly proven to be effective strategies for rabies management in both domestic and wild animals.

THE FIRST GENERATION VACCINES

Nerve tissue vaccines

Louis Pasteur created history by developing the first rabies vaccine, which was based on rabbit CNS and physical inactivation of rabies virus by drying. However, due to the presence of nerve tissue and myelin basic protein, this method posed a risk of residual live virus and severe allergic reactions. As a result of this, Sir David Semple developed a newer nerve tissue vaccines (NTV) at Central Research Institute (CRI), Kasauli, India from adult sheep (Semple vaccine). It was made by propagating rabies virus(RABV) in adult sheep brain, followed by phenolic inactivation. It was widely used in numerous nations despite numerous drawbacks, including low efficacy, the necessity for multiple dosages, and severe side effects, including Guillain–Barre Syndrome (GBS) and the danger of transmitting Transmissible Spongiform Encephalopathies (TSE) owing to the presence of myelin, it was widely used by many nations.Because of these disadvantages, the WHO has consistently discouraged its use, leading to its eventual abolition in almost all countries.

The journey for a less reactogenic, better alternative resulted in the development of a newer vaccine derived from the brain of suckling mouse (SMB vaccine) by phenolic inactivation followed by its partial purification (Fuenzalida*et al*., 1964). Due to the lack of myelin in tissues derived from newborn animals, the SMB vaccine is not as reactogenic as the Semple vaccine but is similar to it in that it has a lesser potency and requires multiple doses to be administered. Its decades-long use in nearly all countries came to an end when most national regulatory authorities decided to stop using it in accordance with the WHO recommendation.

Duck embryo vaccines

The purifiedchick/ duck embryo technique was an inexpensive and convenient method for cultivating a wide variety of animal viruses and were being used for vaccine production (Peck *et al*., 1955) and are still used today for the production of vaccines against a variety of agents, including RABV. The ease of availability, handling, presence of naturally sterile environment within the limits of egg components, inability of the embryo to produce antibodies against the viruses used as inoculaand availability of eggs with a relatively uniform genetic constitution popularised its use. Since 1983, the World Health Organization has advocated for the use of embryonated eggs as a manufacturing platform. However, there are a number of drawbacks including the risk of limited supply, time-consuming processes with variable yields, high manufacturing costs and the possibility of allergic reactions to egg components (Montomoli*et al*., 2012).

The purified duck embryo vaccine (PDEV) production entails propagation of the Pitman-Moore (PM) strain of RABV in embryonated duck eggs and the extraction of RABV from the brains of infected embryos under mild conditions to avoid the release of soluble avian antigens that can cause purification issues and adverse reactions. Nonviral lipids are removed by continuous density-gradient centrifugation and the purified RABV is subsequently inactivated using beta-propiolactone (BPL). The improved extraction and purification method enabled the production of purified duck embryo vaccine that was almost completely free of egg proteins and other components such myelin basic protein. It is worth noting that the classic DEV contained myelin basic protein, which has been linked to the development of allergic encephalomyelitis. More than 25 nations have registered and marketed the contemporary DEV. Similar to previous tissue culture-derived human rabies vaccinations, it is immunogenic, safe and well-tolerated.

THE SECOND GENERATION VACCINES

Tissue culture vaccines

One of the first commercial applications of *in vitro* animal cell technology was the production of vaccines utilizing animal cell substrate.The ability to execute successive infectious cycles in cell culture was a critical step for research on viral diseases and vaccine development (Jordan and Sandig, 2014). The cultured cells could act as substrates for the production of vaccines. Currently,numerous cell types are being used by manufacturers for the production of human rabies vaccines. This includes the primary cells produced without passage in tissue culture, diploid cells with a finite life span and passaged in tissue culture and continuous cell lines with an infinite life span and apparently unlimited capacity to replicate.

Many viral vaccines are now being produced using cell culture, which has several benefits over nerve tissue vaccines and egg-based manufacture. It offers a proven safety and efficacy profile, as well as a shorter lead time and better process flexibility, regardless of the type of cell substrate, production method, or purification and formulation procedures used (Rupprecht*et al*., 2002). However, the challenge is to strike a balance between the desire for a very efficient production system and the goal of minimizing risks.

CELL SUBSTRATES FOR VIRUS PROPOGATION

Primary cells

Primary cells are directly obtained from an animal. They retain the characteristics of the tissue from which they originate and do not have tumorigenic properties. The most important source of primary cells intended for the production of human rabies vaccines is the cells from avian embryo like chicken embryo fibroblasts (CEFs) (Hernandez *et al*., 2010). Because CEFs have a limited lifespan, the embryonated eggs must be retrieved on a regular basis. Each new preparation carries a certain risk of variation in the permissivity of the target virus, inconsistent starting material and contamination with potential adventitious agents.

In accordance with current pharmacopoeia and WHO regulations, the purified chick embryo cell vaccine (PCECV) is made in CEFs utilising SPF fertilised eggs and the Flury low egg passage (LEP) RABV strain. The FluryLEP RABV is inactivated with Beta-propiolactone, purified via continuous density-gradient centrifugation and stabilised with polygeline (Barth and Franke, 1996).In both animal and human investigations, the PCECV shows comparable immunogenicity and tolerability to the human diploid cell vaccine (HDCV) (Barth *et al*., 1984; Briggs *et al*., 2000). It meets or exceeds the 2.5 IU per single IM dose minimum potency level. Human serum albumin (HSA), a stabiliser and a crucial component in most vaccinations, is known to be very low in it. Rabipur is the brand name for the PCECV, which is sold all over the world, manufactured by Chiron Behring.

Diploid cells

Diploid cells are defined as having a finite *in vitro* life span and contain the full complement of the genetic material. They undergo senescence and are non-tumorigenic (Barret*et al*., 2009).The human diploid cells have several advantages over primary cells because they allow multiple expansion passages of material obtained from well-characterized cryogenically preserved master and working cell banks in essentially a closed system; and screening for the absence of adventitious agents (Jordan and Sandig, 2014). However, they have a number of drawbacks, including cellular aging when serially passaged, difficulties scaling up in bioreactors, particularly when utilising microcarriers, and a requirement for a demanding growth media as well as trouble propagating under serum-free circumstances (Barret*et al*., 2009).Two well-known international human diploid cell reference strains are WI-38 and MRC-5 (Hayflick, 1989).

The original human diploid cell vaccine, which was created at the Wistar Institute in WI-38 human diploid cells approximately 3–4 decades ago, received the most attention,

around 3–4 decades ago. They were derived from a human embryonic lung and have been thoroughly tested and used (Wiktor*et al*., 1964).

PM 1503 3M strain of fixed RABV derived from a strain originally isolated byPasteur and maintained by the National Institutes of Health (NIH), USA was used as the vaccine virus strain.The virus was customised to grow in WI-38 cells in the early 1960s and was propagated for 52 passages. In the mid-1960s, a master seed pool was created, and the seed was transferred to l'InstitutMerieux, a vaccine production laboratory, in 1966. In 1969, the seed strain was supplied to Behringwerke. WI-38 cells were used to make the early batches of Merieux vaccine. However, later batches composedof whole virion preparations were grown in MRC-5 human diploid cells andinactivated with BPL. The Behringwerke vaccine was concentrated and purified.

Continuous cell lines

Increasing demands for vaccine production, yields and safety have prompted the development of safer, less expensive, and more efficient cell substrates. Continuous cell lines derived from animal tissues are critical cell substrates for the synthesis of numerous types of biological pharmaceuticals. They have the ability to cause tumours and have an unlimited lifespan.Despite this, a growing body of evidence suggests that cells below a certain passage number are not tumorogenic. They can also be cultivated in large-scale fermentors on microcarriers, which contributes to the standardization, safety, and upscaling of the production system resulting in consistent yields. The Vero cell line, which was developed from the kidney tissue of an African green monkey, is one of the most commonly used mammalian cell lines for vaccine manufacturing. It is a continuous cell line that has been approved by regulatory agencies for the production of viral vaccines. At low passage numbers, it displays pseudo-diploid karyotypes and is non-tumorigenic. It was chosen primarily because it produces high viral yields and batches that are free of adventitious agents. The Vero cell line as a cell substrate is distinguished in terms of cell culture technology by certain limitations. Because of its anchorage-dependent nature, it necessitates cell culture systems with large culture surfaces, such as roller bottles, microcarriers, Cell Factories, CellSTACK, CellCubes, and fixed bed bioreactors.

A significant advancement in rabies prevention was the introduction of purified vero cell-derived rabies vaccine (PVRV) which has a superior industrial scalability.Prior to PVRV, the world depended mainly on eitherofHDCV, PCECV, or NTV.

Over 40 million doses of PVRV (Verorab) have been provided in over 100 countries. The immunogenicity of Verorab has been evaluated in a variety of clinical scenarios and research using a 0.5 IU/mL antibody titer as the cutoff point. This level is highly correlated with clinical rabies protection. Verorab induces acceptable VNA titers when delivered by IM or ID for pre-exposure prophylaxis (PrEP), albeit levels tend to be lower following ID immunisation. Raksharab is an inactivated rabies virus vaccine manufactured by Indian Immunologicalswhichcontaintissue culture rabies virus, CVS strain, propogated on BHK21 cell line, and inactivated with aziridine compound. Aluminium hydroxide gel is used as adjuvant is an commonly used vaccine in veterinary practice

NEWER PARADIGM IN RABIES VACCINE DEVELOPMENT

The persistent high prevalence of rabies in developing countries, the economic burden of post exposure prophylaxis (PEP), and the global scarcity of RIG necessitate the development of innovative, cost-effective rabies vaccines for preventive vaccination or PEP. Virologic developments in the twentieth century resulted in better rabies vaccinations and treatments. Diagnostic, antigenic, and genetic breakthroughs along with a focus on isolation of infected animals, and adaptation from an etiological and pathogenic standpoint provided a solid foundation for better therapeutic and prophylactic strategies. In this section, we will go through some of the more interesting prototypes and their prospective applications in humans.Almost every vaccine prototype, from peptides to plant-derived vaccines, has

been tested in experimental animals and has shown efficacy in numerous circumstances. Monoclonal antibodies are being generated from either human B cells or mouse hybridomas, with the latter being genetically modified to humanise the antibodies constant region are being developed, which could eventually replace rabies immunoglobulin (Smith *et al*., 2011).

Inactivated 'enhanced' traditional rabies vaccines

Inactivated rabies vaccinations are less immunogenic, and repeated doses are required to produce protective VNA titers. In the United States, for example, rabies vaccines are devoid of adjuvants like alum. Unlike alum-fixed vaccines, rabies vaccine formulations incorporating CpG-oligodeoxynucleotides resulted in greater and faster VNA responses in mice (Wang *et al*., 2008).

Protein and peptide vaccines

The viral glycoprotein, a 65-k Da protein, is the basis for protein or peptide vaccinations. Following synthesis, the protein forms trimers and is mildly N-glycosylated at one of three possible locations. However, the proper folding into the natural trimeric structure of the rabies virus glycoprotein, which is required for the generation of neutralising antibodies, remains a challenge with protein vaccines. Although extremely safe, peptide vaccinations are mildly immunogenic and induce very limited B cell responses. Rabies virus glycoprotein have been produced in a variety of plant cells (maize, carrot, spinach or *Nicotianatobaccum* plant cells), insect cells (*Spodopterafrugiperda* (Sf-9) cells (Ramya*et al*., 2011,7) or in*Drosophila melanogaster* Schneider 2 cells (Astray *et al*., 2014)or yeast cells. A peptide expressing a linear epitope of the rabies virus glycoprotein only produced modest VNA titers that failed to neutralise an escape mutant in one research (Niederhauser*et al*., 2008). However, given that rabies vaccinations must elicit a wide antibody response against numerous isolates and genotypes, this method is unlikely to succeed in replacing present vaccines.

Virus particle vaccine

Some viruses can be altered to express the rabies virus glycoprotein on the virion's surface. Here, the protein is immediately available for inducing immunological responses, perhaps permitting its usage in PEPs.Pre-existing immunity to the parent virus or low amounts of the rabies virus glycoprotein, which could decrease immunological responses, are both potential drawbacks. The New Castle disease virus (Ge*et al*., 2011), baculovirus (Wu *et al*., 2014) and Parainfluenza 5 (Chen *et al*., 2013) viruses are the common pseudotyped viruses employed as vaccines to rabies virus.

Genetically altered vaccines

Reverse genetics can be used to alter the rabies virus. As a result, highly attenuated rabies virus and/or virus with increased immunogenicity have been developed, which could be used for animal or human vaccination. The rabies virus is weakened when the P gene, which encodes a component of the viral polymerase, is knocked out.Even when injected intracerebrally into adult or suckling immunocompetent mice or immunodeficient mice, the virus is apathogenic, though the P gene-deleted vaccine moved from the periphery into the central nervous system in the latter. A virus-neutralizing antibody response is induced by the P gene deleted rabies viruses(Morimoto *et al*., 2005). The response begins slowly but eventually outperforms that of an inactivated vaccine based on wild-type virus, indicating that such a design could be considered for PrEPbut not PEP. The P-gene deleted rabies vaccine was further modified to express two copies of the viral glycoprotein gene to improve immunogenicity. This vaccine produced rabies virus neutralising antibodies more quickly.

VACCINATION

Pre-exposure prophylaxis

Fortunately, the fatalities associated with rabies can be avoided with immunizations. The virus clearance prior to the onset of sickness,is crucial, which in turn, is dependent on the presence of VNAs. As a result,

rabies prevention relies mostly on rabies vaccines capable of rapidly producing VNA. A combination of local viral neutralisation by antibodies or antibody mediated clearance of virus infected cells provides protection. Pre exposure prophylaxis is commonly administered to high risk groups on days 0, 7, 21 or 28 by intramuscularly (IM) route. However, ID vaccination is considered by the WHO to be a cost-effective and acceptable alternative to IM immunisation, although it is technically more difficult, requiring sufficient staff training and skilled medical expertise.

Post exposure prophylaxis

Worldwide, millions of exposures to rabies are registered, resulting in tens of thousandsof human deaths, with most occurring in Asia and Africa. Based on the types ofinteraction with suspected rabid animals, exposure is broadly classified into threecategories I, II, and III. All exposures determined to represent a risk for rabies requirePEP, which includes immediate local treatment of all bite wounds and scratches withthorough washing and disinfection, local wound infiltration with RIG (for categoryIII alone) and vaccination. The main purpose of PEP is to prevent the developmentof clinical rabies after exposure has occurred. The combination of active and passiveimmunization is considered for PEP, except for those persons whohave been previously immunized with a rabies vaccine via the WHO approved vaccination regimen.

Intramuscular versus intradermal vaccination

A conservative cost estimate for human rabies prophylaxis of 4 million patients utilising IM versus ID regimens over a five-year period in Southeast Asia reveals that the cost of PEP can be considerably lowered if the (IM) regimen is gradually replaced with the ID regimen. The ID method of rabies vaccination was initially approved in the United States in 1986 for pre-exposure immunisation. As a result, in 1991, the WHO Expert Committee endorsed intradermal injection of current rabies vaccines (Gongal and Sampath, 2019).

Later on, throughout the previous three decades, intradermal multisite regimen for post-exposure prophylaxis(PEP) was developed.The eight site regimen, also known as the Oxford regimen (Warrell*et al*., 1984), was introduced in the 1980s, but due to the large number of inoculations required on days 0 and 7, it was difficult to persuade patients, particularly children, to complete the full vaccination course, and as a result, the regimen did not become popular. In 1986, the Queen Saobabha Memorial Institute of Thailand introduced another low-cost, multi-site ID immunisation approach called as the Thai Red Cross (TRC) regimen. It is a 2-site intradermal regimen which is administered as 2-2-2-0-1-1. However, the initial TRC regimen was intended to provide a full immunisation course over a three-month period.In the previous three decades, this has been improved in terms of dose, frequency of vaccination, and length of vaccination to increase acceptance by health professionals and patient compliance without jeopardising vaccine efficacy.

According to the ninth WHO expert consultation, the original Thai Red Cross Regimen has been changed with a one-month schedule, with two doses of vaccine given on days 0, 3, 7, and 28 (“2-2-2-0-2” regimen).The modified Thai Red Cross Regimen significantly improves compliance rates because patients receive the entire course of immunisation within one month. In 2017, the Strategic Advisory Group of Experts (SAGE) Working Group on Rabies Vaccine advocated a one-week ID immunisation schedule based on evidence gathered in Asian countries (WHO, 2018a).A one-week ID schedule was also proposed by the WHO Expert Consultation on Rabies, Third Report 2018 (WHO, 2018b).

There are several alternatives for PEP of already immunised patients. On days 0 and 3, one intradermal dose of 0.1 mL per location is suggested. If time is a constraint, the patient may be offered a single-visit 4-site intradermal regimen consisting of 4 injections of 0.1 mL evenly divided over the left and right deltoids as an alternative to this regimen (Gongal and Sampath, 2019).

Conclusion

Amidst the most agonising death posed by the rabies virus and the pioneering works that formed the cornerstone of successful vaccine development, rabies still remains a neglected tropical zoonosis that kills about 60,000 people annually.It is of no doubt that vaccination remains the holy grail for the therapeutic management and prophylaxis of rabies and is the most effective public health intervention strategy. Despite the fact that rabies is vaccine preventable, the high expense of cell culture vaccinations for intramuscular delivery prevents their widespread usage in many rabies-endemic areas. However, the development of a low-cost multi-site intradermal (ID) immunisation approach prompted high-burden countries to phase out the production and use of rabies vaccine derived from nerve tissue in the subsequent years.

FUTURE PROSPECTS

The concept of One Health remains relevant to vaccine development also where both the human and animal vaccine companies can collaborate to tackle the shared obstacles. The need for improved programmatic delivery, demonstrating non-inferiority of new rabies vaccine regimens, immunising people who have had multiple rabies virus exposures, efficacy and clinical outcomes of abbreviated PEP and PrEP schedules, novel vaccine delivery technologies, and the use of RIG are all new avenues for research that will aid in the formulation of strategic interventions to eradicate this disease.

Vaccines that are both feasible and cost-effective to administer for community programmes are still in high demand. Better dosage fractionation, vaccines labelled for ID use, and developments in ID vaccine delivery technology (e.g., microneedles) could make ID rabies PEP and PrEP easier to use and more widely adopted.

Rabies vaccines that can be stored and transported outside of the traditional 2–8 cold chain have the potential to revolutionise vaccine delivery by boosting the cost-effectiveness, efficiency, and reach of immunisation programmes. This is especially significant in underserved rural areas where access to vaccines may be prevented by the cold chain.

Clinicians may turn to the ID route of immunisation and customised equipment to administer vaccines in order to improve patient compliance. Traditional rabies vaccine production is likely to be dominated by bioreactor-based mass cell cultivation, which is essentially free of animal and human raw materials. These novel products would inturnbe subjected to new regulatory criteria in terms of advanced molecular techniques for vaccine strain authentication for licensure and ELISA-based potency testing for batch releases.

Changes in wildlife rabies management programmes may occur in terms of authentication of viral strains used for vaccination, designing novel baits that facilitate better absorption by target hosts, and improved monitoring of oral rabies vaccine by assessment of serological responses using approved procedures and enhanced sample collection. Direct rabies vaccine inoculation into rabid animal bite wounds could be utilised as part of a PEP.

Rabies vaccines using novel and adaptable adjuvants or without adjuvants may serve as a low-cost, safe, and effective method of prevention in endemic areas around the world.

In comparison to domestic animal vaccination, which will continue to range from modified-live to inactivated products and recombinant vaccines, human vaccination will remain conservative. Regardless of whether any of these predictions come true, all of the tools necessary to prevent human deaths, eliminate canine rabies, and control disease in mesocarnivore populations are now available.

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Association of temperature humidity index during summer with haematological parameters in native and crossbred goats of Kerala*

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Abstract

The study was conducted to investigate the association of temperature humidity index (THI) during summer with haematological responses in Malabari, crossbred and Attappady goats of Kerala. The research work was conducted at University Goat and Sheep Farm, KVASU, Mannuthy, Thrissur district in Kerala from March to May, 2020. In-house temperature and in-house relative humidity were measured daily at 7.00 AM, 10.00 AM, 2.00 PM and 5.00 PM. Haematological parameters such as total erythrocyte count (TEC), total leucocyte count (TLC), haemoglobin concentration (Hb), volume of packed red cells (VPRC), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were analysed during 2nd, 32nd day and 60th day of the study. There was no significant change in TEC, VPRC levels in between the breeds and within the observed days. However, TLC were significantly increased for Malabari goats and MCHC were significantly increased in all the breeds at 32nd day of study period. The Hb concentration were significantly increased in crossbred and Attappady black at 32nd day of study period. There was a significant decrease in MCV values in 2nd and 32nd day for all the breeds. The study demonstrated certain altered hematological features in all the breeds under study indicating the adaptive ability of these animals during heat stress.

Keywords: *Goats, thermal stress, Temperature Humidity Index, haematological parameters*

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India's average temperature has increased by 0.7°C during 1901–2018. According to Intergovernmental Panel of Climate Change (IPCC) 2018, global warming is likely to reach 1.5°C in between 2030 and 2052; 1.5°C increase in global surface temperature will have devastating consequences on both human and animal population. The climate change has lately turned to be one of the most serious and long-standing term of considerable challenges experienced by livestock owners and farmers. In these circumstances, goat rearing has been identified as a sustainable livestock rearing system and has gained economic importance in almost all countries facing harsh climatic events frequently. Goats has the ability to cope with climate change by expressing adaptive strategies as compared to other ruminants (Silanikove, 2000) because of their lower body mass, lower metabolic requirements, ability to reduce metabolism, skillful grazing behavior and effective urea cycling (Silanikove and Koluman, 2015). The main climatic variables that impose impact on goats are high ambient temperature, high direct and indirect solar radiation, wind speed and humidity (Silanikove, 2000). Temperature humidity index (THI), a reliable indicator of stressful thermal environment could be used for measuring the heat load in animals. Goats are known for their tolerance to heat stress but they suffer from thermal stress beyond their comfort zone and environmental temperatures for goats fall in the range between 13-27°C (Mishra, 2009). Goat subjected to heat, would experience a transition from being ideal in its internal state and thus experience a degree of stress.

There are several phenotypic and genotypic adaptive abilities that provide goats to counter thermal stress. Identification of these adaptive capabilities in various goat breeds of Kerala would help to select the most appropriate goat breed for the future and also to identify the various management strategies to be adopted for sustainable goat farming.

Assessment of haematological parameters gives an evaluation of the health status of the animals. During periods of thermal stress there will be alteration in physiological features for maintenance of homeostasis. Assessing the severity of these alterations would give an idea about the adaptive capacity of these animals to a great extent and would also help to identify the most climate resilient goat breed suitable for hot, humid tropical climate of Kerala. Hence, this study was undertaken to assess the association of THI with haematological parameters in Malabari, crossbred and Attappady goats of Kerala.

Materials and methods

The research work was conducted at University Goat and Sheep Farm, Kerala Veterinary and Animal Sciences University (KVASU), Mannuthy, Thrissur district in Kerala state. Farm is located at 10° 56" N and 76° 26" E at an altitude of 2.83m. The period of study was 60 days from March 2020 to May 2020 during which high thermal stress was experienced. Six animals each from Attappady black, Malabari and crossbred (Malabari X Saanen) female goats of eight to 12 months of age were selected and divided into three groups. All animals were apparently healthy and free from any physical abnormalities. Animals were housed in the elevated platform with corrugated roof sheet and were fed as per Indian Council of Agricultural Research (ICAR) feeding standards and provided with *ad lib.* water (ICAR, 2013).

Ambient temperature (°C) and ambient relative humidity (%) inside the shed were recorded by electronic digital logger (HOBO pro V2, Onset Computer Corporation, USA) on every alternate day of study period at 7.00 A.M, 10.00 A.M, 2.00 P.M and 5.00 P.M. Temperature Humidity Index was calculated as per livestock and poultry heat stress indices **(**LPHSI, 1990**)**

$$THI = T_{(db)} - \{(0.55-0.55RH)\ (T_{(db)} - 58)\}$$

Where,

$T_{(db)}$ = Dry bulb temperature (°F)

RH = Relative humidity (%)

Blood samples were collected into vacutainers through venipuncture on days 2, 32 and 60 between 11.00 AM to 12 noon. Whole blood was collected in vacutainer containing 3.6 mg potassium ethylene diamine tetra acetic acid salt (EDTA) for immediate haematological

analyses. Haematological parameters like total erythrocyte count (TEC), haemoglobin (Hb), volume of packed red blood cells (VPRC) and total leucocyte count (TLC) were estimated in whole blood using haematological analyser (Mythic 18 vet-blood analyser). The erythrocyte indices such as mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated as described by Schalm *et al.* (1986).

Statistical analysis

Results were expressed as means (±SE). The data obtained on various parameters were statistically analysed as per the method of Snedecor and Cochran (1994) using analysis of variance (ANOVA), repeated measures of ANOVA and Pearson's correlation method. The whole data were analysed using computerized software programme SPSS V. 24.0.

Results and discussion

In-house ambient temperature (IT)

The mean in-house temperatures were highest in the month of March (39.54°C) and the lowest during month of April (23.91°C). Harikumar (2017) recorded the maximum interior temperature during March (39.86°C) in Kerala. The in-house temperature recorded by Zarina (2016) in Thrissur was maximum during 2nd half of March (36.03°C). Jisha *et al.* (2021) also found that highest mean of average temperature over a period from 2011-2016 was in March in Thrissur, Kerala.

The diurnal in-house temperature when observed during the different times, the lowest was observed at 7.00 AM (24.61°C) and the highest was at 2.00 PM (37.54°C). Harikumar (2017), could observe highest between 2.00-3.00 PM in Thrissur and Jisha *et al.* (2021) found highest in-house temperature in the afternoon (around 3.00 PM) during the period of March to May at Mannuthy, Thrissur, Kerala.

In-house relative humidity (IRH)

The highest monthly mean in-house relative humidity was noticed in the month of May (98.69%) and lowest in the month of March (37.83%). Similar results were also recorded by Zarina (2016) and Harikumar (2017), where the interior relative humidity was maximum in May and lowest in April (when compared for a period from March – May) and explained that the higher humidity in May was due to summer rain. The relatively lesser summer rain received and higher ambient temperature in the month of March, 2020 might be the reason for the lowest relative humidity observed in this study during the month.

The mean diurnal in-house relative humidity (recorded at four times) was the highest at the time of 7.00 AM (98.69%) and the lowest at 2.00 PM (37.83%). The lowest value recorded at 2.00 PM might be due to the highest ambient temperature at 2.00 PM.

Temperature Humidity Index (THI) (LPHSI, 1990)

Highest THI of 88.63 was recorded in April while the lowest THI of 74.45 was noticed in March. This study was in accordance with Jisha *et al.* (2021), where the highest mean THI was recorded in April (81.74) during the period from 2011-2016 in Thrissur. The maximum THI in April (86.70) was also noted by Harikumar (2017) in Thrissur.

In the present study highest mean THI of 89.65 was observed at 2.00 PM and lowest THI of 75.69 at 7.00 AM. This study was in accordance with Jisha *et al.* (2021) who found lowest THI in the morning and highest in afternoon during the period of March to May at Mannuthy, Thrissur, Kerala.

Total Erythrocyte Count (TEC)

In the present study, the IT and THI had no effect in TEC values of breeds during the entire study period and the TEC values were within normal range. This study was similar with Hassan *et al*. (2013), Bhatta *et al*. (2014). In the latter study they found no changes in TEC levels in Beetal goats during post monsoon (34.6°C) and pre monsoon (42.6°C). Whereas, Alam *et al*. (2011), Okoruwa (2014) and Habibu *et al.* (2017) found increase in TEC levels in goats when ambient temperature and THI were high.

Total Leucocyte Count (TLC)

The total leucocyte count showed an increasing trend for Malabari goats during the study. However, no significant difference in TLC levels were observed for crossbred goats and Attappady black goats breeds. The result of the present study was in agreement with Alam *et al*. (2011), Banerjee *et al*. (2015), Olayemi *et*

Table 1. Inside the animal house during March 2020 to May 2020

Period	In-house temperature (°C)			In-house relative humidity (%)			In-house THI		
	Mean ± SE	Lowest	Highest	Mean ± SE	Lowest	Highest	Mean ± SE	Lowest	Highest
March	30.88 ± 0.08	24.29	**39.54**	67.39 ± 3.34	**37.83**	92.40	82.64 ± 0.82	**74.45**	87.34
April	30.76 ± 0.12	**23.91**	37.51	70.97 ± 1.51	52.97	97.84	83.71 ± 0.46	75.69	**88.63**
May	29.56 ± 0.14	24.56	36.01	78.15 ± 1.61	58.19	**98.69**	82.94 ± 0.40	76.40	87.81

Table 2. Inside the animal house during different times from March 2020 to May 2020

Time	In-house temperature (°C)			In-house relative humidity (%)			In-house THI		
	Mean ± SE	Lowest	Highest	Mean ± SE	Lowest	Highest	Mean ± SE	Lowest	Highest
7.00 AM	27.45 ± 0.21	**24.61**	29.43	89.94 ± 1.15	74.16	**98.69**	80.08 ± 0.31	**75.69**	83.28
10.00 AM	32.18 ± 0.26	28.15	34.51	73.30 ± 1.32	57.17	95.68	85.16 ± 0.31	81.53	87.88
2.00 PM	34.99 ± 0.36	28.30	**37.54**	63.10 ± 1.58	**37.83**	84.74	87.33 ± 0.34	80.84	**89.65**
5.00 PM	33.56 ± 0.27	28.69	35.88	67.57 ± 1.39	44.54	83.74	86.18 ± 0.27	81.35	88.59

Table 3. Mean ± SE of TEC, TLC and Hb of Malabari, crossbred and Attappady black goats

Day of study	THI	TEC (X 10^6/µL)			TLC(X 10^3/µL)			Hb (g/dL)		
		Malabari	Crossbred	Attappady black	Malabari	Crossbred	Attappady black	Malabari	Crossbred	Attappady black
2nd day	82.64	11.87 ± 0.66	11.82 ± 0.88	12.16 ± 0.36	9.87^{aA} ± 0.65	13.58^{abA} ± 2.07	14.78^{bA} ± 1.60	7.87^{A} ± 0.33	7.72^{A} ± 0.12	8.03^{A} ± 0.23
32nd day	83.71	10.56 ± 0.80	11.55 ± 0.58	11.06 ± 0.96	11.73^{aB} ± 0.82	13.12^{aA} ± 1.27	13.77^{aA} ± 1.33	9.37^{A} ± 0.42	9.83^{B} ± 0.53	9.27^{B} ± 0.54
60th day	82.94	11.06 ± 0.85	11.45 ± 0.42	12.02 ± 0.86	13.97^{aB} ± 0.94	14.58^{aA} ± 0.71	11.28^{aA} ± 0.67	8.83^{A} ± 0.90	8.06^{A} ± 0.21	7.17^{A} ± 0.24

Means bearing same superscript within a row (a-c) and columns (A-C) do not differ significantly ($p<0.05$) (n=6)

Table 4. Mean ± SE of VPRC, MCV and MCHC of Malabari, crossbred and Attappady black goats

Day of study	THI	VPRC (%)			MCV (fl)			MCHC (g/dL)		
		Malabari	Crossbred	Attappady black	Malabari	Crossbred	Attappady black	Malabari	Crossbred	Attappady black
2nd day	82.64	26.00 ± 1.32	26.45 ± 1.66	26.48 ± 0.89	22.20^{A} ± 0.22	22.70^{A} ± 0.93	21.77^{A} ± 0.61	30.33^{A} ± 1.71	29.56^{A} ± 1.27	30.45^{B} ± 0.99
32nd day	83.71	25.26 ± 1.63	26.75 ± 1.76	25.38 ± 2.04	24.02^{A} ± 0.44	24.08^{A} ± 0.31	23.80^{B} ± 0.28	37.60^{B} ± 2.27	37.25^{B} ± 2.23	35.58^{C} ± 0.94
60th day	82.94	30.21 ± 2.02	28.58 ± 1.40	27.78 ± 0.67	34.27^{B} ± 1.63	34.96^{B} ± 0.99	34.67^{C} ± 0.39	29.10^{A} ± 1.77	28.45^{A} ± 1.02	25.80^{A} ± 0.59

Means bearing same superscript within a columns (A-C) do not differ significantly ($p<0.05$) (n=6)

al. (2015) and Habibu *et al.* (2017), where they found higher TLC levels with an elevated THI. As leucocytes are engaged in immune system, under thermal stress the immune system becomes activated (Okoruwa, 2014), thereby increasing the TLC.

In the present study, in Attappady black goats, mean TLC levels on 2nd day were increased and then steadily decreased when THI was high suggesting a partial adaptation to the heat stressed conditions. Whereas, crossbred goat breeds maintained a steady TLC level during entire study period, showing that the stress had not affected the immune functions. In the case of Malabari goats there was a drastic change in TLC indicating heat stress induced immune system activation.

Haemoglobin (Hb)

Significant difference in Hb concentration was not observed between breeds. However, mean Hb level was significantly increased in Crossbred goats and Attappady black goats when IT and THI were at

peak. At the same time Malabari goats showed statistically non significant difference in Hb concentration which might be due to relatively decreased number of RBCs in Malabari goats on the 32nd day. The observation was in accordance with Alam *et al*. (2011), Bhatta *et al*. (2014), Okoruwa (2014) and Habibu *et al.* (2017) who found an elevated Hb in goats in the afternoon than in the morning which could be due to high demand for oxygen which in turn necessitated high concentration of red blood cells to support respiratory activity under heat stress condition.

Volume of Packed Red Cells (VPRC)

No significant difference was noticed among the VPRC levels in breeds in the experimental period. A lower VPRC levels were recorded in breeds during high IT and THI. Observations of Abdelatif *et al*. (2009) supported the present study who recorded the decrease in VPRC in Nubian goats during summer which might be due to the lowered thyroid hormone levels in summer and that would be affecting the synthesis of RBCs as thyroid hormone level was crucial for erythropoiesis.

Mean Corpuscular Volume (MCV)

Significant difference was not observed between the breeds. A steady increase in MCV value was noticed in Attappady black goats from 2nd day whereas in Malabari goats and crossbred goats from 32nd day onwards. This emphasised the fact that adaptational alterations in volume of RBC were taking place most effectively in Attappady black goats compared to other two breeds to counteract the numerical changes in the RBC or to increase the O_2 carrying capacity. This was in agreement with the findings of Abdelatif *et al*. (2009) who found a decreased MCV values during summer which could be related to the inverse relationship of number and volume of erythrocytes.

Mean corpuscular haemoglobin concentrations (MCHC)

No significant difference was noticed in MCHC levels between breeds. A higher MCHC level was recorded in all breeds during high THI. The MCHC had increased during peak summer to meet the increased O_2 demand of the body. Similarly, Olayemi *et al*. (2015) found an increased level of MCHC in goats during summer. The results of the study indicated that the average erythrocyte cell size and haemoglobin content per erythrocyte were influenced by the THI and the amount of Hb relative to the size of the cell increased with increase in THI.

Conclusion

The mean TLC showed an increasing trend for Malabari goats during the entire experimental period. The mean Hb levels were significantly increased in all the breeds when IT and THI were at peak on 32nd day indicating increased oxygen demand due to thermal stress. The MCV values were significantly lowered in Attappady black and MCHC values were increased in all the animals from 2nd to 32nd day of study indicating adaptive alterations in the haemogram suggestive of the impact of impact of thermal stress

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Conflict of interest

The authors declare that they have no conflict of interest.

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Detection of *Salmonella* spp. in exotic pigeons of North Kerala and its antibiogram

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Abstract

Pigeon breeding has transformed from being a mere hobby to becoming established as an industry. The increased trade of pigeons inadvertently invites the risk of dissemination of infections including zoonoses like salmonellosis. Pigeons once infected remain carriers for life. This coupled with the ability of the organism to acquire antimicrobial resistance makes salmonellosis, particularly from pigeons an important, public health risk for pigeon handlers. Cloacal swabs from a total of 200 exotic pigeons belonging to 24 lofts from Northern districts of Kerala were collected and attempted to isolate Salmonella and understand its antimicrobial resistance profile. Five isolates of salmonella could be obtained from four of the lofts studied. A prevalence of 2.5 per cent was identified for salmonellosis with 16.67 per cent of the lofts affected. Antimicrobial sensitivity based on disk diffusion assay revealed that all the five isolates were sensitive to amoxicillin-clavulanate and all were resistant to tetracycline and streptomycin. Sixty per cent of the isolates were sensitive to co-trimoxazole, chloramphenicol, ampicillin, cefoperazone, amikacin and gentamicin.

Keywords: *Salmonellosis, exotic pigeons, antibiogram*

Pigeon breeding requires minimal investment for housing, feed cost and veterinary care and its reproduction management is comparatively easy. These factors along with high sales return makes pigeon breeding a lucrative business and a sustainable entrepreneurship. Increasing volume of pigeon trade has resulted in increased trading of birds across the countries, which inadvertently invites the risk of dissemination of infections including zoonoses. Salmonellosis is one such common disease that gets traded along with pigeons. Pigeons once infected could remain as lifelong carriers. People who handle the infected stock can easily acquire the infection from

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pigeons. Salmonellosis is a major threat from the public health perspective as the organism is notorious in acquiring antimicrobial resistance (Arcangioli *et al*., 1999). Antimicrobials are extensively used in pigeon breeding industry to improve the performance and health of the birds. Unscrupulous and unsupervised use of antimicrobials can induce a drug induced selection pressure that results in the evolution of multiple drug resistant bacteria. Studies are lacking regarding the prevalence of salmonella among fancy pigeons in India, particularly in Kerala. Lack of systematic studies adds to the knowledge gap that exists regarding the occurrence and prevalence of multiple drug resistant Salmonellae among domestic pigeons in the state.

Materials and methods

A total of 200 cloacal swabs of exotic pigeons were collected as per García *et al*. (2011) from 24 different pigeon lofts from the Northern Districts of Kerala namely, Kannur, Kozhikode, Wayanad and Malappuram. Loft owners were interviewed to understand the antimicrobials used in the respective lofts. Samples were collected aseptically in buffered peptone water and incubated at 37ºC for 24 hours followed by selective enrichment in Rappaport-Vassiliadis (RV) broth at 42ºC for 24 hours. Plating was done on Salmonella Shigella (SS) agar, Xylose Lysine Deoxycholate (XLD) agar, Brilliant Green Agar (BGA) plates at 37ºC. The resultant colonies were analysed after 24 to 48 h.

Biochemical tests of the isolated *Salmonella* spp. were done according to the standard protocols (Edward and Ewing, 1986). Salmonella was identified based on colony morphology, Gram staining characters and biochemical tests *viz.* catalase test, oxidase test, IMViC test, urease test and Triple Sugar Iron (TSI) agar test.

Antibiogram of the salmonella isolates were studied using disc diffusion (HiMedia) technique (Bauer *et al.,* 1966) using amikacin (30µg), amoxicillin-clavulanate (20/10µg), ampicillin (25µg), cefoperazone (75µg), chloramphenicol (30µg), trimethoprim-sulphamethoxazole (1.25/23.75µg), gentamicin (10µg), streptomycin (10µg) and tetracycline (30µg). The zone of inhibition of bacterial growth around each disc including the diameter of the disc was measured and interpreted as sensitive or resistant by comparing the ranges given by the manufacturer. Polymerase chain reaction was done targeting *aadA2*, bla_{CARB-2}, *cmlA*, *sull, tetA* and *tetR* which encodes resistance against streptomycin, ampicillin, chloramphenicol, sulfamethoxazole and tetracycline respectively (Table 1, Table 2).

Table 1: Primers used for the molecular characterization of the isolates

Sl. No	Gene	Primer sequence 5'-3'	Protocol	Product size (bp)	Reference
1	*aadA2*	F- GTACGGCTCCGCAGTGGA TGGCGG	1	522	Briggs and Fratamico (1999)
		R-GCCCAGTCGGCAGCGACA TCCTTC			
2	bla_{CARB-2}	F- CAATGGCAATCAGCGCTTCCCGTT	1	639	
		R- CGCTCTGCCATTGAAGCCTGTGTT			
3	*cmlA*	F- CGC CAC GGT GTT GTTGTT AT	2	394	Chen *et al*., 2003
		R- GCG ACC TGC GTA AAT GTC AC			
4	*sull*	F- TCA CCG AGG ACT CCT TCT TC	2	331	
		R- CAG TCC GCC TCA GCA ATA TC			
5	*tetA*	F- GCG CCT TTC CTT TGG GTT CT	2	831	
		R- CCA CCC GTT CCA CGT TGT TA			
6	*tetR*	F- CGCTCCTTCGATCCCGT	3	260	Yang *et al*.,2001
		R- GCTGCGTTCATCTACAACAGAT			

Results and discussion

Among the 200 samples tested, five isolates produced typical colonies with biochemical properties suggestive of *Salmonella* spp. The isolates formed colourless and transparent colonies with black center in SS agar (Fig. 1A), red colonies with black centre in XLD agar (Fig. 1B) and pink colonies with pink coloration surrounding the media in BGA (Fig. 1C). The findings are in accordance with Rahman *et al.* (2016) and El-Prince *et al*. (2019) who also reported similar colony characteristics exhibited by *Salmonella* spp. in their studies. The biochemical characters of the isolates obtained are in agreement with Rajagopal and Mini (2013) and Rahman *et al*. (2016) who reported that the isolates of salmonella were gram negative, small bacilli with specific IMViC reaction results (-+-+) as all were found to be indole negative, methyl red positive, voges-proskaur negative and utilized citrate (Fig. 2). The findings also agree with Sharma *et al*. (2019) and Ranjbar *et al*. (2020) who reported that salmonella isolates produced acid butt and alkaline slant in TSI agar slants (Fig. 2).

A prevalence of 2.5 per cent was identified for salmonellosis with 16.67 per cent of the lofts affected. Comparable prevalence was reported by Casanovas *et al*. (1995) and Perez-Sancho *et al*. (2020) who reported a prevalence of 1.5 per cent and 4.41 per cent. However, higher prevalence were reported by Hosain *et al*. (2012) who reported a prevalence of 35.71 per cent and Saifullah *et al*. (2016) who reported prevalence of 34 per cent. Santos *et al*. (2020) suggested that samples should be collected every five days to detect the presence of *Salmonella* spp. due to the intermittent shedding nature of the bacteria.

Table 2. PCR cycling conditions used for the study

Protocol No.	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles	Final extension
1	95°C, 5 min	95°C, 1 min	60°C, 1 min	72°C, 1 min	30	72°C, 10 min
2	95°C, 10 min	95°C, 30 sec	55°C, 1 min	72°C, 1 min	30	72°C, 7 min
3	95°C, 5 min	95°C, 1 min	48°C, 30 sec	72°C, 30 sec	40	72°C, 3 min

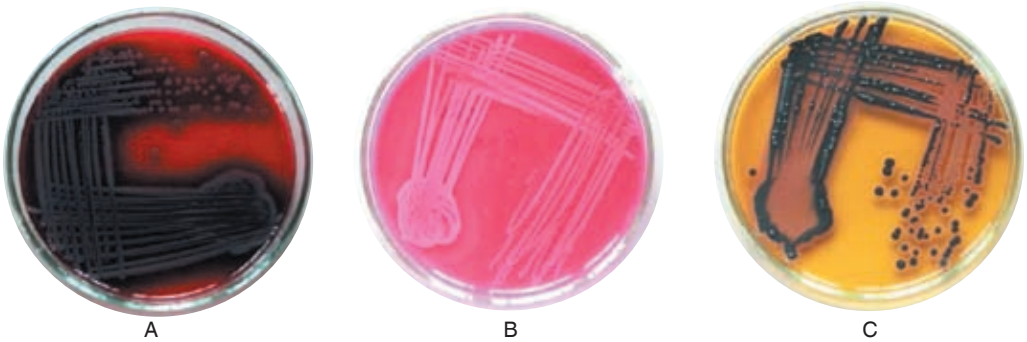
Table 3. Antimicrobial Sensitivity pattern of the isolates

Isolate	C	AMC	AMP	COT	CPZ	TE	S	AK	GEN
C1	R	S	R	R	R	R	R	R	R
C2	R	S	R	R	R	R	R	R	R
W1	S	S	S	S	S	R	R	S	S
W2	S	S	S	S	S	R	R	S	S
W3	S	S	S	S	S	R	R	S	S

C- Chloramphenicol; AMC- Amoxicillin-clavulanate; AMP- Ampicillin; COT- Trimethoprim-sulphamethoxazole; CPZ- Cefoperazone; TE- Tetracycline; S- Streptomycin; AK- Amikacin; GEN- Gentamicin; S - Sensitive; R - Resistant

Table 4. Antimicrobial resistance genes in the *Salmonella* isolates

Genes	ISOLATES				
	C1	C2	W1	W2	W3
aadA2	+	+	-	-	-
bla_{CARB-2}	-	-	-	-	-
cmlA	+	+	-	-	-
sulI	-	-	-	-	-
tetA	+	+	+	+	+
tetR	-	-	-	-	-



A B C

Fig. 1. *Salmonella* isolate on selective Media
A: SS Agar B: XLD Agar C: BGA

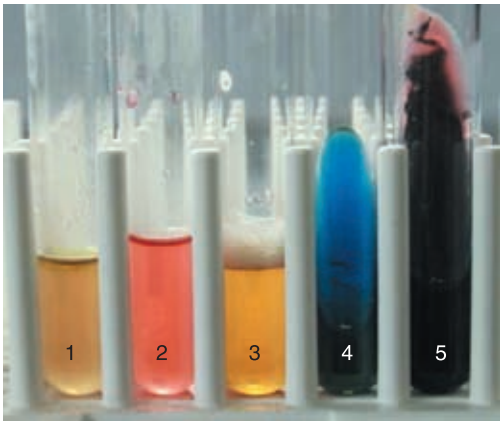


Fig. 2. IMViC test and TSI of Salmonella isolate
1. Indole: Negative 2. Methyl red: Positive
3. Voges- proskauer: Negative
4. Citrate utilisation: Positive
5. Triple Sugar iron with H2S production

Similar findings were also reported by Teske *et al*. (2013). Repeated sampling of all birds in the selected lofts during the present study might have improved the detection rate.

Among the five isolates tested for antimicrobial sensitivity, all (100 per cent) were sensitive to amoxicillin-clavulanate. Three isolates (60 per cent) each was sensitive to chloramphenicol, ampicillin, co-trimoxazole, cefaperazone, amikacin and gentamicin. All isolates were resistant to tetracycline and streptomycin. The details regarding the antimicrobial sensitivity pattern of the isolates are given in the Table 3.

High sensitivity towards amoxicillin-clavulanate was reported by Stenzel *et al*. (2014). Since none of the lofts surveyed for the present work used amoxicillin-clavulanate, absence of resistance to the drug could be explained to be due to poor selection pressure induced by the drug. The findings also agree with that of Jahantigh and Nili (2010) who reported high level of resistance towards tetracycline among salmonella in pigeons. The varying resistance to antimicrobials among different populations of fancy pigeons could be because of different preferences of antimicrobials being used in different localities (Kaczorek-Lukowska *et al*., 2020).

Among the six genes tested for detecting antimicrobial resistance, amplification was obtained in three. All of the isolates were positive for *tetA* gene and produced 831 bp sized amplicons (Fig. 3). The *aadA2* gene (522 bp amplicon) was detected in two isolates (40 per cent) (Fig. 4). The *clmA* gene (394 bp amplicon) was detected in two isolates (40 per cent) (Fig.5). No amplification was obtained for *sulI, tetR and* bla_{CARB-2}genes in any of the isolates (Table 4).

Kaczorek-Lukowska *et al*. (2020) reported that *tetA* gene was among the most common antibiotic resistance genes that were isolated from domestic pigeons. Extensive use of tetracycline could have contributed to the selection of isolates with *tetA* gene. Yousef and Mamdouh (2016) reported the presence of Class I integrons in *Salmonella* Enteritidis isolated from pigeons, which was associated with a variety of resistance genes including the *aadA*. Absence of *sul1* gene even in presence of isolates resistant to co-trimoxazole could be because of the involvement of other genes belonging to the *sul* family (Kozak *et al*., 2009; Xu *et al*., 2020).

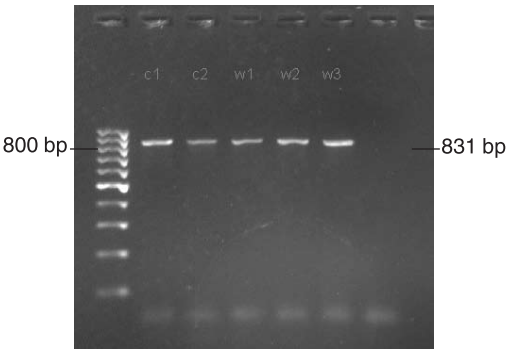


Fig. 3. Agarose gel electrophoresis of *tetA* specific PCR
Lane1: DNA marker 100bp
Lane 2-6: Positive samples (831 bp)

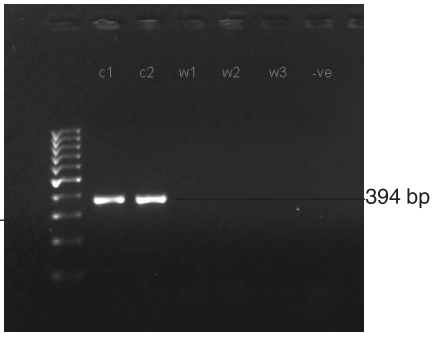


Fig. 5. Agarose gel electrophoresis of *clmA* specific PCR
Lane1: DNA marker 100bp
Lane 2.3: Positive samples (394 bp)
Lane 4-6: Negative samples

Conclusion

Among 200 cloacal samples of exotic pigeons tested for salmonellosis from 24 lofts, five positive isolates could be obtained. The study showed a prevalence of 2.5 per cent of salmonellosis among exotic pigeons with 16.67 per cent of the lofts affected. The antimicrobial sensitivity tests revealed that all the five isolates positive for salmonellosis were sensitive to amoxicillin-clavulanate and all were resistant to tetracycline and streptomycin. The same revealed that 60 per cent of the positive isolates were sensitive to co-trimoxazole, chloramphenicol, ampicillin, cefoperazone, amikacin and gentamicin.

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The authors duly acknowledge the Kerala Veterinary and Animal Sciences University for the facilities provided.

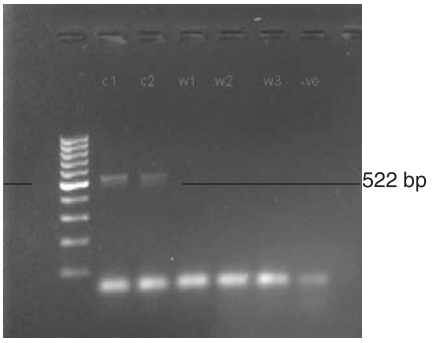

Fig. 4. Agarose gel electrophoresis of *aadA2* specific PCR
Lane1: DNA marker 100bp
Lane 2.3: Positive samples (522 bp)
Lane 4-6: Negative samples

Conflict of interest

The authors declare that they have no conflict of interest.

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Healing of cutaneous wounds using GaAs laser in diabetic rat models

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Abstract

Conventional treatment modalities usually fail to heal wounds in diabetic animals. Photomodulation of the wound bed using low-level laser has proved its efficacy in treating chronic wounds. Considering the fact that the majority of the diabetic wounds end up in amputation of affected part and reduce the quality of life of the patient, the present study was undertaken to find out the efficacy of gallium arsenide (GaAs) laser in the healing of full-thickness cutaneous wounds in type 2 diabetic rat models. The study was conducted in six adult male Wistar rats with an average body weight of 150 g in which hyperglycemia coexisting with hyperlipidemia was induced. Two wounds of 1 cm² area were induced of which left side wounds served as the control and right side wounds were treated with GaAs laser at 4J/cm² area for one minute continuously for four days.

Keywords: *Type 2 diabetes, streptozotocin, GaAs laser*

Changes in the way of life of human beings have led to the development of a variety of lifestyle diseases. Since in modern times pets are like family members, the situation applies to them also. Wounds in diabetic patients are difficult to heal due to diabetic neuropathy and degraded growth factors. Management of such wounds require unconventional or modern treatment modalities so that healing of the wounds can happen fast. Low-level laser therapy (LLLT) involves the use of a laser of wavelengths between 500 and 1100 nm and typically involves the delivery of 1–4 J/cm² to the treatment site having output power between 10 and 90 mW (Hawkins *et al.,* 2005). Photomodulation of the wound bed with low energy laser has been proved efficient in bringing about healing of the chronic wounds. (Varghese, 2002). Irradiation of the wound bed using low level

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laser at appropriate doses cause stimulation of cell function, producing biological, chemical and physical effects without thermal effects (Posten *et al.,* 2005) This in turn promotes wound healing.

In diabetic wounds, infection and inflammation leads to imbalance of protease and reactive oxygen species, degradation of essential growth factors, the ability of angiogenesis is impaired and cell recruitment to the wound sites is inhibited. This in turn is found to delay wound healing. Considering the above, a study was conducted to find out the efficacy of gallium arsenide (GaAs) laser for the management of wounds in type II diabetic rat models.

Materials and methods

The study was approved by the Institutional Animal Ethics Committee of College of Veterinary and Animal Sciences, Pookode of Kerala Veterinary and Animal Sciences University (IAEC/ COVAS/ PKD/16/2019 dtd. 17/01/2019).

Ten adult male Wistar rats with an average body weight of 150 g were randomly selected for the study. Hyperlipidemia was induced by feeding a high-fat diet for three weeks as per Suman *et al.* (2016) in all the rats. The high-fat diet was prepared by mixing standard rat diet, raw cholesterol, and a mixture of vanaspati and coconut oil in the ratio of 2:1. Two per cent of raw cholesterol powder was mixed in coconut oil and was also fed orally at the dose rate of 3 mL/kg body weight. Hyperlipidemia was confirmed by estimating serum total cholesterol levels after three weeks. Diabetes mellitus was induced by a single dose of streptozotocin at the rate of 40mg/kg body weight given intraperitoneally. Diabetes mellitus was confirmed by estimating blood glucose levels after three days. Two wounds of size 1cm^2 area each were created on either side of the body in the thoracolumbar region under general anaesthesia. The wound bed was irradiated with GaAs laser at 4J/cm^2 for 1 minute for four consecutive days followed by dressing with povidone-iodine lotion till the end of the observation period. Control wounds were treated with povidone-iodine lotion until the end of the study. Observations were continued till both the wounds healed completely.

Healing of the wound was assessed by monitoring the factors as suggested by Reddy *et al.* (2012) which included nature of wound edges, presence of necrotic tissue, nature of exudate if present, tissue oedema and induration, amount, colour and texture of granulation tissue and epithelialisation. Wound surface area was estimated by the method put forth by Jothi *et al.* (2007) in which the wound area was plotted on a graph paper and counted. The rate of wound contraction was estimated by the formula put forth by Kirubanandan and Sehgal (2010)

Results and discussion

Hyperlipidemia could be induced with the feeding protocol adopted in this experiment. The total cholesterol value ranged from 123 - 278 mg/dL with a mean of 181.57± 20.09. The high cholesterol level was maintained throughout the period of observation. Diabetes mellitus was effectively induced with a single intraperitoneal injection of streptozotocin at the rate of 40mg/kg body weight. The serum glucose levels ranged from 203- 584 mg/dL with a mean of 358.29±18.25. The high glucose level was maintained throughout the period of observation.

According to Grey *et al.* (2006) the edges of a healthy healing wound slops towards the centre whereas that of a non-healing wound will be either punched out or rolled out in nature. In the present study, both the treated and the control wounds showed a sloping wound edge which indicated that the wound was healing.

In the present study, the condition of the wound was assessed by monitoring the colour of the wound bed. As per Grey *et al* (2006 wound could be considered healthy and healing when the wound bed is pink in colour whereas unhealthy if the wound bed is dark red or maroon. All the wounds in the treatment site remained pink in colour indicating that the wounds were healthy and healing whereas the control wounds were dark red during the initial days of observation indicating an unhealthy initial phase.

Grey *et al.,* (2006) suggested that the amount of granulation tissue present in the wound during the healing phase determines the healing rate. It should be moderate to medium in a healing wound. The wound will not heal if the quantity of granulation tissue is minimum and can become a Keloid if it is heavy. In the present study, the amount of granulation tissue in the treated wounds were moderate to medium whereas, it was minimum in control wounds. Similar findings were observed by Lazovic *et al.* (2005).

Epithelialisation and generation of epithelium over a wound is the final stage of healing. Epithelial tissue which is light pink in colour, migrates inwards from the wound edges or may appear as small islands of tissue over the surface of the wound (Fraser, 2020). The treated wounds in the present study started epithelisation by day five but was minimal except in rat 8 in which it was moderate. In rats 21, 17 and 18 maximum epithelisation could be noticed by day 9. In control wounds, epithelisation started by day seven, which was minimum. Similar observations were made by Lavozic *et al.* (2005), Mitra *et al.* (2015), and Karri *et al.* (2016).

The wounds were mapped onto a sterile transparent sheet and the area was estimated by graphical method. The wound surface area (cm^2) is presented in table 1. Wound surface area was statistically analysed using one-way ANOVA The wound surface area did not differ significantly between the control and treatment groups on day zero. The surface area showed significant ($p<0.05$) difference between the control and treatment groups during the subsequent days of observation and it was highly significant ($p<0.01$) on days five and seven.

The rate of wound contraction was estimated using the formula $((A_0-A_n)/A_0) \times 100$ where A_0 is the wound area in day zero and A_n is the wound area in n^{th} day.

On day three, the mean rate of wound contraction (%) on control side was 17.34±5.07,

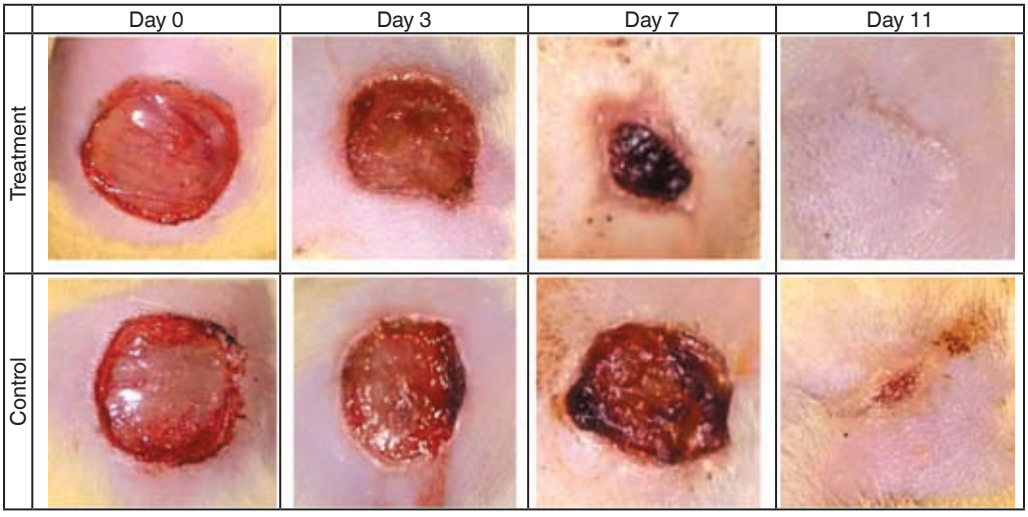

Fig.1. Gross morphology of wounds

Table 1. Wound Surface area (cm^2) n= 10

	Day 0	Day 3	Day 5	Day 7	Day 9
Control Wound	0.97±0.38	0.79±0.03	0.48±0.06	0.20±0.04	0.05±0.01
Treatment Wound	1.04±0.02	0.68±0.09	0.31±0.06	0.13±0.03	0.01±0
T value	1.39^{ns}	1.21^{ns}	2.28^{*}	2.90^{*}	4.34^{**}
P value	0.22	0.31	0.09	0.03	0.007

*ns-non-significant at 0.05 level; * - significant at 0.05 level;** - significant at 0.01 level*

and on the treatment side it was 24.22±8.05. On day five, the rate of wound contraction was 49.37±7.67, 55.43±5.11, in control and treated wounds, respectively. The rate of wound contraction on day seven was 78.55±5.59 for the control wounds and 86.69±3.79 for the treated wounds. The rate of wound contraction (%) on day nine of control wounds was 94.17±2.03 and that of treated wounds was 98.19±1.25.

The average time of complete healing of treated wounds was 8.8 ± 0.33 days and on control wounds, it was 10.4 ± 0.40 days ($p<0.01$).

Conclusion

Based on the present study the wound treated with GaAs laser took lesser time to heal compared to that of the control wound. Wounds treated with GaAs laser healed comparatively faster than the control wound. The size of the treatment wound significantly reduced from the day 5. Laser treated wound completely healed on day 9. However, the control wound healed completely only after 12 days.

Conflict of interest

The authors declare that they have no conflict of interest.

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Multi-locus sequence typing for species/ serovar identification of clinical isolates of *Leptospira* spp.*

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Abstract

Leptospirosis is an emerging zoonotic disease endemic in Kerala and close monitoring of emerging serovars is essential to adopt appropriate control strategies. Multi-Locus Sequence Typing (MLST) was reported to be less expensive compared to other cumbersome methods like whole genome sequencing. The present study was conducted to obtain isolates of Leptospira from infected animals and rats and for the identification of serovars using MLST. A total of 205 blood samples (dog, cat, cattle, goat), 43 urine samples (dog, cattle) and post-mortem kidney samples from various animals such as dog (n=12), cattle (n=2) and rat (n=25) were collected and subjected to polymerase chain reaction (PCR) using G1/G2 primers to identify the pathogenic Leptospira. Fifteen samples were found to be positive, these samples when inoculated in the Ellinghausen-McCullough-Johnson-Harris (EMJH) semi-solid medium to obtain ten isolates. These ten isolates were further subjected to secY, icdA and GyraseB PCR and sequenced. The obtained sequences were analysed using BLAST and were fed into specified MLST database of Leptospira scheme-3, the allelic profile and sequence type were generated. The MLST results obtained in the study indicated that the isolates S24 and S33 belonged to serovar Canicola, S40 and 47 were Sejroe and S19, S27, S55, S69 and S71 were Bataviae, Autumnalis, Pomona, Icterohaemorraghiae and Australis, respectively. It was concluded that MLST is a convenient method for identifying leptospiral serovars.

Keywords: *Leptospirosis, isolation, PCR, MLST.*

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Leptospirosis, a widespread zoonotic disease, is significant in both developed and developing countries particularly in tropical regions. It is caused by the pathogenic bacteria of the genus *Leptospira*. The disease affects a variety of mammals including dogs, cattle, sheep, goats, pigs, horses and humans. Rodents spread the disease directly through contaminated urine, water, feed and soil (Harskeerl and Terpstra, 1996). Although vaccination has been carried out in dogs in Kerala, it has been observed that even vaccinated dogs are often diagnosed with leptospirosis with non vaccinal serovars (Abhinay *et al*., 2012). Hence, monitoring the emergence of serovars is of great importance in adopting appropriate control strategies for controlling the disease. Methods for monitoring different serovars include Microscopic Agglutination Test (MAT), the gold standard serological test for identification leptospiral serovars. However, for performing MAT, live leptospiral culture should be maintained for use as antigen and technically cumbersome. Hence, alternative DNA based methods such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Pulsed Field Gel Electrophoresis (PFGE), Variable Number Tandem Repeat (VNTR), and Multi-Locus Sequence Typing (MLST) were used for identification of leptospiral serovars (Ahmed *et al.,* 2006). When compared to all these DNA based methods, MLST was preferred because of its high reproducibility and discriminatory power to differentiate leptospiral serovars (Ahmed *et al*., 2011). Considering these facts, the present study was conducted to isolate leptospires from domestic animals and identify the serovars of the pathogenic *Leptospira* using MLST.

Materials and methods

Sample collection

A total of 205 blood samples were collected from dogs (n=139), cats (n=11), cattle (n=29) and goats (n=26) presented to Teaching Veterinary Clinical Complex, College of Veterinary Animal Sciences, Mannuthy with clinical signs indicating to acute leptospirosis, during the study period of March 2019 to March 2020. Post-mortem kidney tissue from dogs (n=12) and cattle (n=2) received from Department of Veterinary Pathology, CVAS, Mannuthy and those from rats (n=25), trapped from college premises, hostels and farms in Mannuthy campus were used in the study. Urine samples were also collected from dogs (n-40) and cattle (n-3). Whole blood samples collected in heparin and ethylene diamine tetra-acetic acid (EDTA) vials were used for isolation of *Leptospira* and to perform PCR, respectively.

Polymerase Chain Reaction

Genomic DNA was extracted from whole blood, urine and kidney samples using Qiagen DNA extraction kit. The concentration and purity was checked by NanoDrop spectrophotometer and DNA was stored at -20°C. The genomic DNA extracted from samples were subjected to PCR using specific primers G1 (5'-CTGAATCGCTGTATAAAAGT-3') and G2 (5'-GGAAAACAAATGGTCGGAAG-3') for an expected amplified product of 285 bp (Gravekamp *et al*., 1993). A 25 µL reaction containing 12.5 µL 2X PCR master mix, 1 µL of 10 p*M* of each forward and reverse primers, 5 µL of template DNA and 5.5 µL nuclease free water was prepared. The amplification protocol used was: initial denaturation at 94°C for 5 mints followed by 25 cycles of denaturation (94°C for 1 min.), annealing (55°C for 1 min.) and extension (72°C for 45 sec.), final extension was done for 10 min. at 72°C followed by hold at 4°C for infinity.

Isolation

Whole blood collected in heparinised vials, urine and homogenised kidney tissues were inoculated aseptically into Ellinghausen McCullough Johnson Harris (EMJH) semi-solid medium containing 5-Fluorouracil (400 µg/mL) to prevent contamination. The samples were incubated at 30°C for a time period of three months (Ellis and Thiermann, 1986). The growth of leptospires were examined at weekly intervals using dark field microscopy (DFM) and Polymerase Chain Reaction (PCR) using G1/G2 primers.

MLST

Housekeeping genes namely *secY, icdA* and *GyraseB* were selected for MLST analysis. The primers used for amplification of these three genes are listed in table 1.

The PCR amplification was carried out in a volume of 25 µL reaction in 200 µL capacity PCR tubes containing, 12.5 µL 2X PCR master mix, 1 µL of 10 p*M* of each forward and reverse primers, 5 µL of template DNA and 5.5 µL of molecular biology grade nuclease free water. The optimised PCR conditions for different genes are listed (Table 2).

The PCR products were identified by agarose gel electrophoresis followed by visualisation in gel documentation system, for recording the results.

The *secY, icdA* and *GyraseB* gene amplicons from the isolates were sequenced using commercial sequencing service provided by M/s Scigenome Labs, Kochi.

MLST data analysis

MLST alleles were assigned using the optimized *Leptospira* MLST scheme 3 from pubMLST database (https://pubmlst.org/leptospira/). The sequence of each locus was checked in *Leptospira* MLST database for the determination of the allele and to generate the allelic profile / Sequence Type (ST) numbers. Based on the ST numbers, the serovar differentiation of each isolate was done.

Results and discussion

Polymerase chain reaction was an efficient and accurate tool for diagnosis of leptospirosis (Merien *et al*., 1995). Out of the 205 samples collected in the present study, 15 were found to be positive in PCR using G1/G2 primers. Among the 15 PCR positive samples, 10 samples demonstrated a distinct subsurface white ring like discrete zone (Dinger's ring) growth in EMJH semi-solid medium in three weeks to three months of incubation (Fig. 1).

Table 1. Primer sequence used for MLST analysis

Sl. No.	Primers	Sequence	Amplicon size	Reference
1	*secY* F *secY* R	5'-ATGCCGATCATTTTTGCTTC-3' 5'CCGTCCCTTAATTTTAGACTTCTTC-3'	549 bp	Ahmed *et al.* (2006)
2	*icdA* F *icdA* R	5'-GGGACGAGATGACCAGGAT-3' 5'-TTTTTTGAGATCCGCAGCTTT-3'	674 bp	Ahmed *et al.* (2006)
3	*GyraseB F* *GyraseB* R	5'- ACATCCCATGCACAAAGTGA-3' 5'- CGGAAAGACCTGTTGGATGT -3'	236 bp	Slack *et al.* (2006)

Table 2. PCR protocol for the amplification

Step		Temperature	Time
Initial denaturation		94°C	5 min.
35 cycles	Denaturation	94°C	1 min.
	Annealing	58°C♦ 58.5°C♦♦ 62.5°C♦♦♦	1 min.
	Extension	72°C	45 sec.
Final extension		72°C	10 min.

♦ Annealing temperature of *secY* gene

♦♦ Annealing temperature of *icdA* gene

♦♦♦ Annealing temperature of *GyraseB* gene

Table 3. MLST results

Sl. No.	Sample No.	Possible STs	MLST (Possible Serovar)
1	S07	-	-
2	S19	70	Bataviae
3	S24	34	Canicola
4	S27	53	Autumnalis
5	S33	38	Canicola
6	S40	60	Sejroe
7	S47	74	Sejroe
8	S55	58	Pomona
9	S69	2	Icterohaemorrhagiae
10	S71	35	Australis

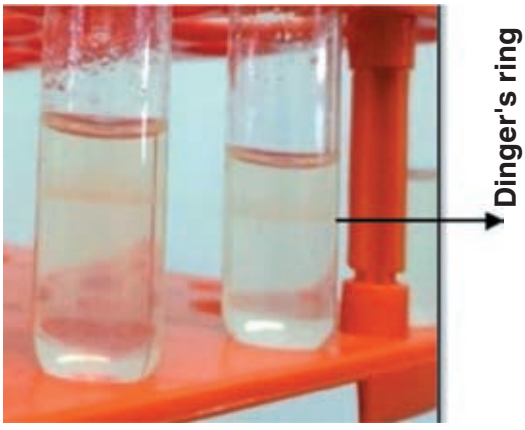

Fig. 1. Growth of leptospires in EMJH semi-solid medium

The isolates were designated as S07, S19, S24, S27, S33, S40, S47, S55, S69 and S71. Among the ten isolates six were from dogs, two from cattle and one each from goats and rats, respectively. Though the percentage of isolation (4.88 per cent) was low in the present study, similar observation was made by Chandran (2017). Reason for low isolation rate could be the smaller number of bacteria in clinical samples owing to the treatment before the sample collection and also sampling which was done to early/late stage of infection.

The isolates obtained were subjected to PCR using G1/G2 primers and observed and all of them yielded 285 bp amplicons (Fig. 2) thus confirming that all were pathogenic *Leptospira*. Similar work has been reported

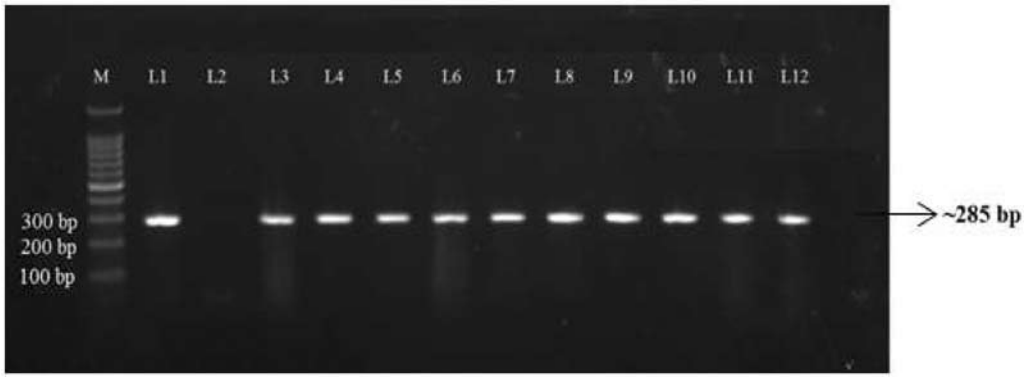

Fig. 2. PCR amplicons from *Leptospira* with G1/G2 primers

Molecular marker : 100 bp ladder Lane1 : Positive control
Lane 2 : Negative control Lanes 3-12 : DNA extracted from the isolates

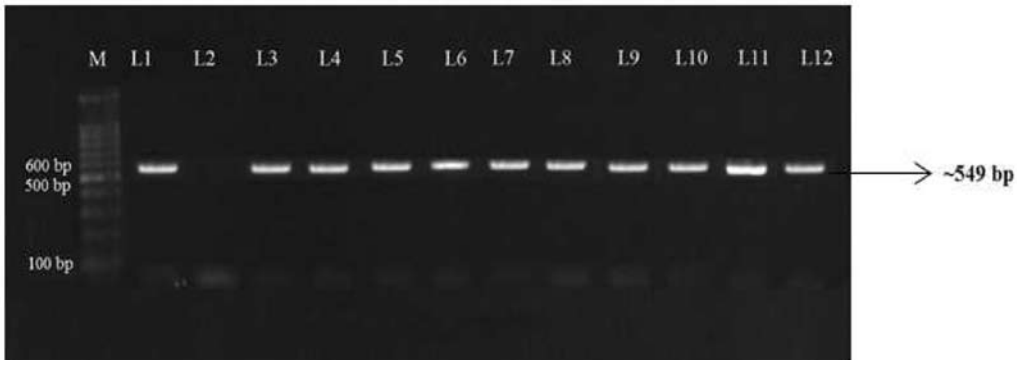

Fig. 3. PCR amplicons of *secY* gene of *Leptospira*

Molecular marker : 100 bp ladder Lane 1 : Positive control
Lane 2 : Negative control Lanes 3-12 : DNA extracted from isolates

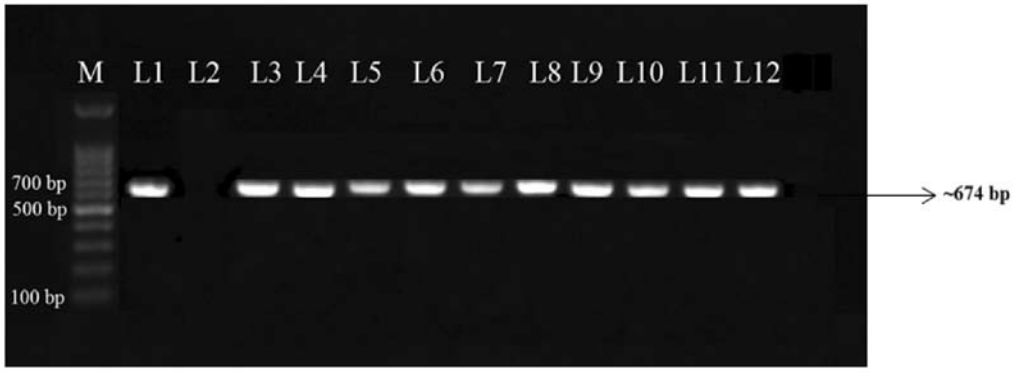

Fig. 4. PCR amplicons of *icdA* gene of *Leptospira*

Molecular marker : 100 bp ladder Lane 1 : Positive control
Lane 2 : Negative control Lanes 3-12 : DNA extracted from isolates

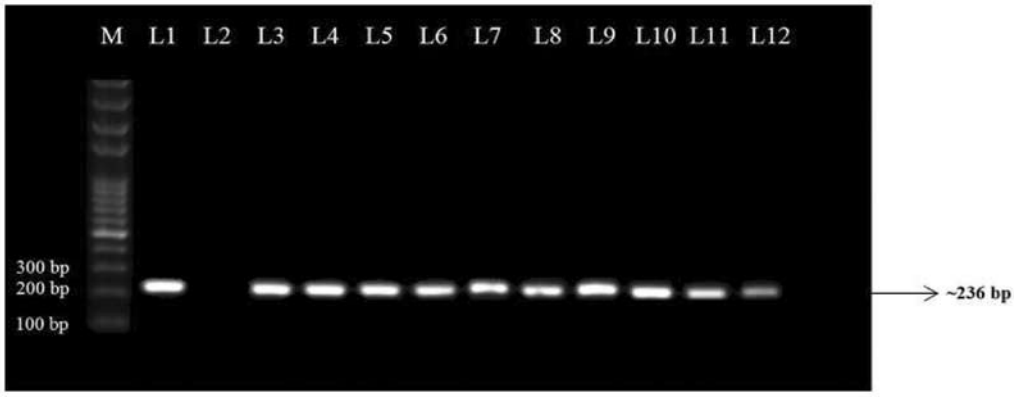

Fig. 5. PCR amplicons of *GyraseB* gene of *Leptospira*

Molecular marker : 100 bp ladder Lane 1 : Positive control
Lane 2 : Negative control Lanes 3-12 : DNA extracted from isolates

by Natarajaseenivasan *et al*. (2011). After confirming the isolates as pathogenic *Leptospira,* they were further subjected to PCR for amplifying *secY, icdA* and *GyraseB* genes, the isolates could successfully yield amplicon size of 549 bp, 674 bp and 236 bp, respectively.

Positive amplicons are depicted in Fig. 3, 4 and 5.

Using optimised *Leptospira* MLST scheme 3 from pubMLST database (https://pubmlst.org/leptospira/), sequence types were obtained as given in table 3. Due to limitations of the study, the present study's focus is on identification of isolates up to the serovar level and not on measuring genetic diversity. Based on Sequence Type number, nine out of ten isolates could be identified at the serovar level (Table 3). The results indicate that the samples S24 and S33 belong to serovar Canicola (Varni *et al*., 2018), S40 and S47 were Sejroe (Bourhy *et al*., 2012), S19, S27, S55, S69 and S71 were Bataviae, Autumnalis, Pomona, Icterohaemorrhagiae and Australis, respectively. Sera samples from the same animals were also subjected to Microscopic Agglutination Test (MAT) and the results were found to be in agreement with the serovars identified using MLST. Remaining one isolate identified by MAT as belonging to serovar Icteroharmorrhagiae failed to generate products for the three genes in the database. The isolate may be further confirmed by advanced typing methods such as PFGE or Whole Genome Sequencing (WGS). A similar issue in identifying the serovar had been reported by Romero *et al*. (2011).

Coclusion

Microscopic agglutination test is routinely performed in reference laboratories for diagnosis and serovar identification. A major drawback is the significant occurrence of cross-reactions between different serovars. Based on the above results, the present study concludes that MLST is a robust method for a more accurate serovar level identification of *Leptospira* which include less cumbersome procedures compared to the more advanced molecular techniques including whole genome sequencing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Isolation and pathotyping of Newcastle disease virus isolated from birds in Kerala*

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Abstract

Newcastle disease (ND) is a pandemic viral disease of poultry. It is highly contagious and causes high morbidity and mortality in affected flocks. The disease is caused by Avian orthoavulavirus 1, commonly known as Newcastle disease virus (NDV) belongs to the family Paramyxoviridae. The virus affects almost 241 species of birds. Based on the pathogenicity, the virus is classified into five pathotypes viz., viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric NDV. The severity of the disease varies with the viral pathotype. Isolation and identification along with pathotyping of the virus provides a basis for understanding the type of virus circulating in the region. In the present study, tissue samples from dead/ ailing birds showing lesions/clinical signs suggestive of ND were collected. They were subjected to virus isolation in embryonated chicken eggs and identified by haemagglutination test and confirmed by haemagglutination inhibition test. Eight NDV isolates were obtained out of 55 tissue samples and were classified into pathotypes by intracerebral pathogenicity index (ICPI) and mean death time (MDT). The ICPI values varied from 0.75 to 1.53 and MDT from 54 h. to 79.2 h. Out of eight isolates, three belonged to velogenic group and five were of mesogenic pathotype. The study revealed the circulation of virulent NDV in Kerala. The pathogenicity tests provide a basis for understanding the epidemiology of ND.

Keywords: *Haemagglutination test, intracerebral pathogenicity index, mean death time*

Newcastle disease (ND), caused by *Newcastle disease virus* (NDV), is an affliction which causes severe losses in both commercial and backyard poultry production (Alexander, 1997). It is one of the 'Word Organization for Animal Health' listed notifiable disease, which affects international trade and is responsible for major constraint in world economy. According to International Committee on Taxonomy of Viruses report of 2018, NDV is officially known as *Avian orthoavulavirus* 1 (AOAV-1) and belongs to the genus *Orthoavulavirus* of the family *Paramyxoviridae*. It is known to infect almost 27 out of 50 orders of avian species, but the severity of the disease varies with the strain of the

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virus and the host affected. Even low virulence strains of the virus may produce severe disease when co-infected with other organisms or due to adverse environmental conditions (OIE, 2018).

The infection can occur by inhalation, ingestion of contaminated material and by direct contact. The isolates show variation in their pathogenicity and as well as in the organs they affect and have considerable influence on mode of spread of infection between the birds (Alexander, 1988). Based on the pathogenicity, the virus is classified into five pathotypes *viz.,* viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric NDV (Beard and Hanson, 1984). The severity of disease varies from mild local infection to lethal systemic infection. The clinical signs of the disease depend on the virulence of the infecting virus, immune status of the host, age of the host, the species of bird and environmental conditions (Seal *et al.,* 2000).

Proper diagnosis is essential for controlling the disease. Even though virus isolation is the gold standard test for diagnosis of ND, due to the wide use of live vaccine and its interference during the isolation, pathogenicity tests or nucleotide sequencing is required for viral characterisation. *In vivo* pathogenicity tests like mean death time (MDT) in embryonated chicken eggs (ECE), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) are commonly employed for identification of pathotypes of the virus (Alexander, 2000).

Several outbreaks of ND were reported in Kerala for the past several years even in vaccinated flocks. Hence, this study was conducted to isolate the NDV prevalent in Kerala and subsequently, pathotype them by ICPI and MDT tests.

Materials and methods

Sample collection and processing

Tissue samples (lungs, liver, spleen, kidney, brain, heart and intestine) from the dead and ailing birds showing lesions/signs suggestive of ND presented to Departments of Veterinary Pathology and Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy were collected in phosphate buffer saline (PBS). Brain and intestine were collected separately and all other organs from a single bird were pooled. The tissue samples were homogenised and the 20 per cent (w/v) suspension was subjected to centrifugation at 3000 rpm for 10-15 min. The supernatant obtained was filtered through membrane syringe filter (0.22 µm) and antibiotic-antimycotic solution containing penicillin (1,00,000 Units/µL), streptomycin (100 mg/µL) and amphotericin B (200 µg/µL) was added, incubated at 37°C for 30 min. and was used as inoculum for virus isolation.

Isolation of virus using embryonated chicken eggs

It was performed according to OIE (2018). Tissue suspension treated with antibiotic-antimycotic solution (0.1 mL) was inoculated into nine to eleven day-old ECE intra-allantoically. Three eggs were inoculated per sample and one control egg was inoculated with 0.1 mL of PBS. They were incubated at 37°C for five days and candled twice daily. The death of the embryo within 24 h post inoculation (PI) was considered as non-specific and discarded, while embryos died after 24 h PI were chilled at 4°C overnight and viable embryos after five days PI were terminated by chilling at 4°C overnight. Next day, the amnio-allantoic fluid (AAF) was harvested and spot haemagglutination (HA) test was conducted with 10 per cent chicken erythrocytes. The embryos were observed for lesions. The samples were considered as negative only after three blind passages.

Confirmation of the virus isolates by haemagglutination inhibition (HI) test

The spot HA positive AAF samples were subjected for plate HA test using one per cent chicken RBC. They were confirmed by HI test using NDV specific antiserum maintained in the department. Four HA units were calculated and AAFs were diluted accordingly and used for HI test. The HA and HI tests (β-method) were done as per FAO (2002).

Pathotyping of NDV isolates

In vivo tests namely ICPI and MDT were performed for biological characterisation of the confirmed isolates. The experimental work on animals were carried out after obtaining approval from Institutional Animal Ethics

Committee (IAEC).

ICPI test

Day-old chicks were used for the test according to OIE (2018). The AAF was diluted (1:10) in normal saline (NS) and 0.05 mL was inoculated intracranially into chicks. Ten birds were inoculated per isolate and control birds (N=10) were inoculated with 0.05 mL of NS. These birds were examined every 24 h. for eight days and scored accordingly. The score 0 was given to normal birds, 1 to sick birds and 2 to dead birds. Birds with paralysis and unable to drink and feed were killed humanely and taken dead for next observations. Mean score per bird per observation was made over a period of eight days is the ICPI value.

MDT test

The test was performed in nine to eleven day-old ECE. Ten-fold dilution of AAF was made from 10^{-1} to 10^{-9} in NS. Dilutions from 10^{-6} to 10^{-9} at the rate of 0.1 mL was inoculated into ECE intra-allantoically as per the protocol of FAO (2002). Five eggs were inoculated per dilution. The remaining part of the dilutions (10^{-6} to 10^{-9}) made were retained at 4°C and inoculated into five eggs per dilution after 8 h. All the eggs were incubated at 37°C for seven days. The embryos were observed for death twice daily. After seven days, the highest dilution of the virus which kills all the inoculated embryo was taken as minimum lethal dose (MLD). The mean time in hours, taken for MLD to kill all the embryos was taken as MDT.

Results and discussion

The samples were collected from a total of 55 birds (Table 1). Out of which, 45 tissue samples were collected from ailing birds. These birds showed clinical signs like diarrhoea, oedema of head and respiratory distress. Torticollis and paralysis were noticed in some of the birds. Similar observations were made by Chowdhary *et al.* (2020) in the birds from which NDV was isolated. Tissue samples were collected from ten recently dead birds. Pinpoint haemorrhages in the proventriculus, caecal tonsils, congestion of the lung and liver were the predominantly observed post-mortem lesions (Fig. 1). Even though the clinical symptoms and post-mortem lesions aids in diagnosis of ND in field conditions, the definitive diagnosis could not be arrived solely on these findings. Hence, virus isolation and characterisation is necessary (Hines and Miller, 2012).

On virus isolation, AAF of eight samples revealed HA on spot test (Fig. 2) and produced lesions in embryo. All the eight samples were from chicken. Among the eight samples, five were from tissue samples collected from ailing birds and three were from dead birds. The embryos inoculated with these samples died within three to five days PI. and lesions like generalised congestion, pinpoint haemorrhages in occipital region and congestion of chorio-allantoic membrane were observed in first passage and these lesions were prominent in third passage (Fig. 3). These results are in agreement with the findings of Balachandran *et al.* (2014) and Qosimah *et al.* (2018).

Plate HA test for the AAF which gave positive spot HA was carried out. The HA titre of the eight samples varied from 4 to 10 $\log_2$ (Table 2). All these samples were confirmed as NDV by HI test and the HI titres were found to be 4 $\log_2$. The HA might also be due to other

Fig. 1. Pinpoint haemorrhages in the summit of papillae of proventriculus

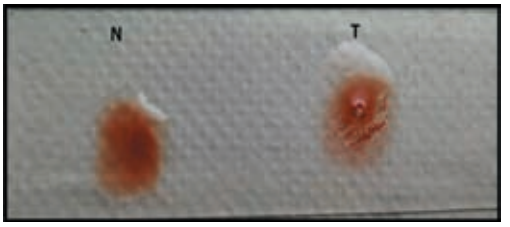

Fig. 2. Spot haemagglutination test

N: Negative control
T: Harvested AAF - Positive

serotypes of NDV or due to Influenza A viruses, hence HI tests has to be carried out using NDV specific antiserum. Inhibition of HA property of the viruses in HI test confirms the presence of NDV (Alexander, 2000).

The pathogenicity of the virus is mainly due to fusion (F) protein hence, along with virus isolation the evaluation of virulence of the viral isolates by ICPI test or amino acid sequencing at fusion protein cleavage site (FPCS) of F protein is necessary (OIE, 2018). The ICPI and MDT tests were conducted for the NDV viral isolates obtained in this study for the assessment of virulence. The ICPI values for eight isolates varied from 0.75 to 1.53. During the ICPI test, birds showed clinical signs like torticollis, paralysis, depression and huddling behaviour. The MDT was found to be between 54 h. to 79.2 h.

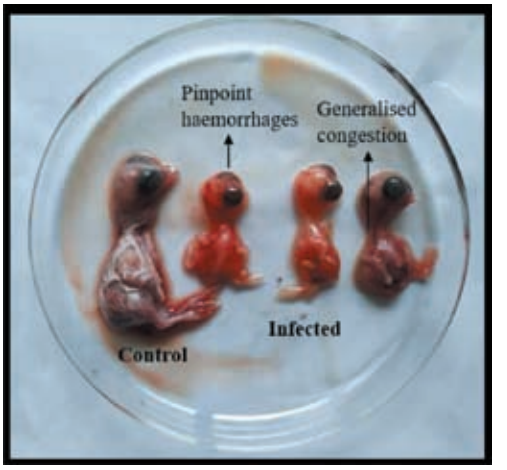

Fig. 3. Characteristic post-inoculation changes of the embryo (9-11 day-old)

Alexander (1998) reported that velogenic and mesogenic NDV showed ICPI values of 0.7 to 1.5 and >1.5, respectively. Hanson and Brandly (1955) classified NDV based on MDT. The viruses with MDT 60 h., 60 to 90 h. and >90 h. were grouped as velogenic, mesogenic and lentogenic viruses. In the present study, three of the eight isolates were grouped under velogenic and the remaining five belonged to mesogenic group (Table 2). Velogenic and mesogenic group of viruses show similar amino acid sequence at FPCS region *i.e.,* polybasic amino acid sequence. Based on molecular characterisation of F gene

Table 1. Details of the samples collected

Sl. No.	Condition of bird	Species				
		Chicken	Quail	Duck	African love bird	Total
1	Dead birds	10	-	-	-	10
2	Ailing birds	33	8	3	1	45
Total		43	8	3	1	55

Table 2. HA, HI titre and pathogenicity indicies of NDV isolates

Sl. No.	Isolates	HA titre (Log_2)	HI titre (Log_2)	ICPI	MDT (h.)	Pathotype	Species
1	NDV-S5	9	4	1.5	60	Velogenic	Chicken
2	NDV-S6	10	4	0.98	62.4	Mesogenic	Chicken
3	NDV-P2	8	4	0.75	75.6	Mesogenic	Chicken
4	NDV-S9	6	4	1.35	62.4	Mesogenic	Chicken
5	NDV-P4	5	4	0.9	73.2	Mesogenic	Chicken
6	NDV-S17	4	4	1.51	58.8	Velogenic	Chicken
7	NDV-S18	4	4	1.53	54	Velogenic	Chicken
8	NDV-P6	4	4	1.03	79.2	Mesogenic	Chicken

the virulent viruses cannot be distinguished into velogenic and mesogenic pathotypes hence, biological characterisation of the virus by pathogenicity tests like MDT, ICPI and IVPI tests is required (Balachandran *et al.,* 2014).

Coclusion

In the present study, it was found that virulent type of NDV (both mesogenic and velogenic) is prevalent in Kerala. The pathotype of the virus could be identified in *in vivo* pathogenicity tests and is useful during the surveillance of the disease. Strict biosecurity measures and vaccination regimen are to be followed for prevention and control of ND. For the success of vaccination programme, characterisation of the virus is necessary for the selection of a proper vaccine candidate.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Myeloid to Erythroid (M: E) ratio in the evaluation of bone marrow cytology of Porcine Circovirus type 2 affected pigs

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Abstract

Porcine circovirus associated diseases (PCVAD) caused by porcine circovirus type-2 (PCV-2) are emerging viral diseases with unfavourable effects on animal health and swine economy. We have a lot of information regarding the changes in the lymphoid organs and spleen in PCV-2 infected pigs whereas the reason for anaemic changes in the carcasses and the pathological effects of PCV-2 in bone marrow are still not well studied. Hence, an extensive study to identify the changes in myeloid and erythroid cells of bone marrow in PCV-2 infected pigs was carried out. Myeloid and erythroid series of cells were counted and analysed from the freshly collected bone marrow cytological smears from the PCV-2 suspected samples. Later, PCV-2 infection was confirmed by polymerase chain reaction (PCR) and characteristic histopathological findings. The PCR yielded an amplicon of ~ 481 bp product and those positive cases were selected for determining the Myeloid to Erythroid ratio (M : E ratio). However, values did not significantly differ in any of the cellular components between PCV-2 positive animals and PCV-2 negative animals which indicated that the bone marrow was not the specific target organ for PCV-2 viral infections. However, increased lympho-histiocytic and plasmacytic infiltration was noticed in both lymphoid and non-lymphoid organs. These characteristic features of PCV-2 infection could be considered as a major reason for increased proliferation of myeloid cells.

Keywords: *PCV-2, polymerase chain reaction, bone marrow M: E ratio*

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PCV-2 infection was first reported thirty years back in Canada. Later it has been reported globally in association with many disease manifestations. They are PCV-2-systemic disease (PCV-2-SD), PCV-2-reproductive disease (PCV-2-RD) and porcine dermatitis and nephropathy syndrome (PDNS), which are now recognised as porcine circovirus diseases (PCVD) or porcine circovirus-associated diseases (PCVAD) (Segalés, 2012; Correa-Fiz *et al.,* 2020). The PCV-2 infection is considered as an immunosuppressive disease with devastating effects on global swine farming (Saikumar and Das, 2019). The infected animals expressed clinical signs such as wasting, diarrhoea, unthriftiness, pallor, respiratory distress and icterus (Rossell *et al*., 1999). Lymphoid organs mainly lymph nodes and spleen are found to be extensively affected in PCV-2 infection (Segales *et al.*, 2000; Sharma *et al.,* 2010). Profound alteration of the immune system is characteristic of for PCV-2 infections. Microscopically, there was lymphoid depletion in T and B cell areas and histiocytic infiltration in lymphoid organs and presence of multinucleated giant cells (Keerthana *et al.*, 2017; Sairam *et al.,* 2019). These changes in the lymphoid organs are well documented. However, there is limited information on the changes in bone marrow in PCV-2 infection. Bone marrow is widely studied for haematopoietic and mesenchymal cells morphologies (Tadjalli *et al.,* 2013). Alteration in the cytology of myeloid and erythroid progenitor cells usually happens in viral infections. Early detection of these changes could help in management of the disease as well as its prevention and control. Hence, the present study was aimed with the objectives of evaluating bone marrow of PCV-2 infected pigs. This study can improve our current understanding of the effects of PCV-2 on bone marrow of pigs.

Materials and methods

Sample collection

A total of 39 pig carcasses suspected of PCV-2 infection were submitted to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences Mannuthy, Kerala during the period from March 2019 to November 2020 for postmortem examination. The pooled tissue samples from mesenteric lymph nodes, tonsils, lungs and bone marrow (Keerthana *et al.*, 2017) were collected and investigated. Lymphoid organs and non-lymphoid organs were collected in 10% neutral buffered formalin for histopathological evaluation (Suvarna *et al*., 2019). The PCV-2 was detected by PCR from pooled tissue samples using primers designed for the nucleocapsid gene (*ORF-2*) specific for PCV-2 (Ellis *et al.,* 1999).

Bone marrow smear examination

The bone marrow smears were collected from the epicondyle portion of the femur by paint brush technique as soon as possible after death (Reagan *et al.*, 2011). The smears were fixed using methanol and were stained by Wright-Giemsa stain. The stained smears were examined under oil immersion objective. The myeloid and erythroid ratio was estimated by counting 500 cells in total (Ryan, 2001). The data were expressed as Mean ± SEM. All results were processed using SPSS (Version 24.0 for windows, SPSS Inc., Chicago, IL, USA). The results were analysed using Student's *t*-test for comparison between PCV-2 positive and negative animals. The bone marrow smears from the apparently healthy slaughtered pigs (PCV-2 negative animals) were treated as the control group for our study. Statistical significance was considered at $p < 0.05$.

Results and discussion

The primers were selected for PCR for the region specific to the nucleocapsid gene (*ORF-2*) as per previously published reports (Ellis *et al.,* 1999; Sairam *et al.,* 2019). In PCR, the amplified products were obtained at 481 bp amplicons. In the current study, 39 samples were processed for the detection of PCV-2 by PCR, among them seven were positive for PCV-2 **(**Fig.1).

Affected animals were seen emaciated with enlarged lymph nodes. Gross lesions noticed were hydropericardium, diffusely non-collapsing lungs, pale friable liver, multifocal white areas on mucosa of tonsil or congestion and ulcerative lesions of soft palate, splenic

infarcts, swollen kidneys with foci of pallor and haemorrhages (Fig. 2 and 3) Lymphocytic depletion and histiocytic infiltration were noticed in all the lymphoid organs of PCV-2 positive animals (Fig. 5). Histiocytosis was noticed in histological sections of bone marrow (Fig. 4). Kidney revealed cloudy swelling in the renal tubules (Fig. 6). Although a significant statistical difference could not be observed in haematopoietic precursors between PCV-2 positive and negative animals (Table 1); an increase in mean myeloid and erythroid counts was observed in PCV-2 positive animals **(**Fig. 7 and 8). The mean cellular percentage of myeloid and erythroid precursors of bone marrow of pig are listed in Table 1.

The present study employed the PCR technique as a diagnostic tool to detect PCV-2 virus by using the viral nucleocapsid gene (*ORF-2*) specific PCR. Bone marrow suppression is a general manifestation in viral infection, especially in immunosuppressive infections like PCV-2 (Hansen *et al*., 2013; Pascutti *et al.,* 2016*)*. Even though immunosuppression is a feature of porcine circovirus associated diseases (Segalés, 2012), the mechanism by which PCV-2 produces immunosuppression is not clear. Here, lymphoid depletion and histiocytic infiltration were noticed in all the cases of PCV-2 affected pigs in lymphoid organs which were in agreement with the

Table 1. The mean cellular percentage of myeloid and erythroid precursors of bone marrow of pigs

Myeloid cells	Mean percentage in PCV-2 negative samples	Mean percentage in PCV-2 positive samples	Erythroid cells	Mean percentage in PCV-2 negative samples	Mean percentage in PCV-2 positive samples
Myeloblast	7.53±0.13	7.830±0.17	Rubriblast	1.10±0.17	1.40±0.24
Promyelocyte	10.75±0.30	11.25±0.65	Rubricyte	2.5±0.27	2.50±0.27
Myelocyte	12.25±0.52	9.30±0.15	Metarubricyte	6.00±0.69	2.75±0.27
Metamyelocyte	17.50±0.55	22.41±0.46	Polychromatic normoblast	21.38±0.53	13.86±0.22
			Reticulocyte	10.00±1.09	13.00±0.79
			RBC	8.07±0.81	7.486±0.25
Total	48.03±0.32	50.79±0.46	Total	49.05±0.59	41.58±0.34

The mean percentage values of the myeloid and erythroid cells in PCV-2 positive animals were 50.79 and 41.58 respectively, where as in PCV-2 negative animals were 48.03 and 49.05 respectively. We observed an increase in myeloid to erythroid ratio (M: E) in PCV-2 affected pigs. When compared with PCV-2 negative animals, the PCV-2 positive animals had reduced percentage of haematopoietic tissue in the bone marrow (Fig. 7 and 8). A numerical decrease of erythroid cells and mild increase of myeloid cells were noticed on bone marrow cytology. The M: E ratio in PCV-2 positive animals was 1.2: 1 whereas that in apparently healthy pigs was 0.9:1.

previous reports of Keerthana *et al.* (2017) and Sairam *et al*. (2019). In the present study, cases suggestive by gross and histopathological lesions were confirmed by PCR.

In the present study, carcasses of PCV-2 infected pigs were blanched, anaemic and also had an increased myeloid series of cells. The characteristic features of myeloid series of cells have been described (Cowell *et al.,* 2007). Myeloblast cells are morphologically undifferentiated cells having prominent nucleoli in the nucleus and agranular cytoplasm. Nucleoli are also visible in promyelocytes but the cytoplasm mostly has non-specific

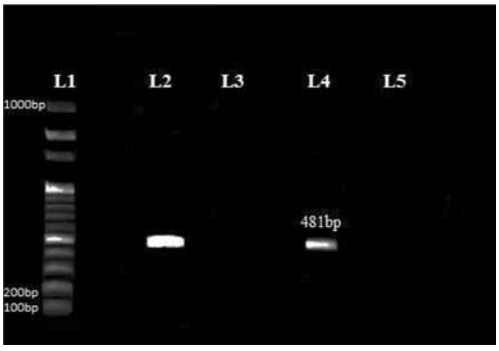

Fig. 1. Agarose gel electrophoresis picture showing 481 bp PCR amplified product of PCV-2 (lane 1-DNA ladder; lane 2-positive control; lane 3-negative control; lane 4 –positive sample; Lane 5 – negative sample)

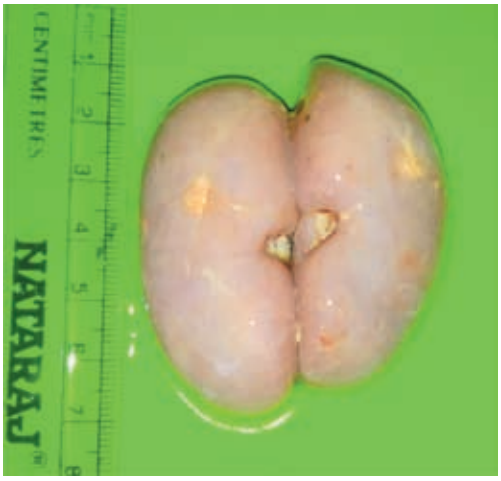

Fig. 2. Kidney showing few white areas with multi-focal pinpoint haemorrhages

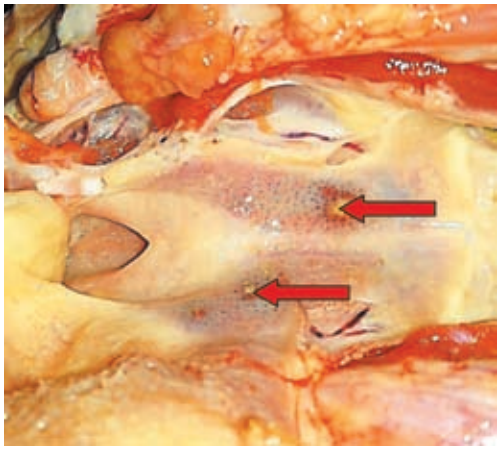

Fig. 3. Soft palate tonsil showing congestion and multi-focal ulcerative lesions (arrows)

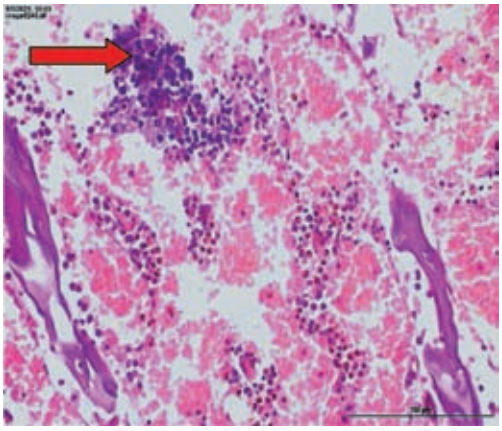

Fig. 4. Hypocellular bone marrow with areas of histiocytosis (arrow) (H&E X 100)

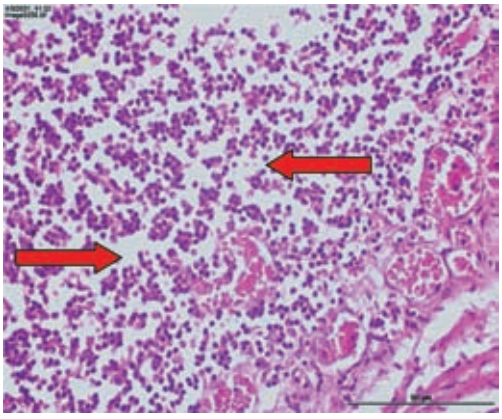

Fig. 5. Lymph node showing severe histiocytic infiltration (arrows) (H&E X 400)

granules. Promyelocyte matures and later differentiate into myelocyte. From the myelocyte stage onwards, the cells start differentiating

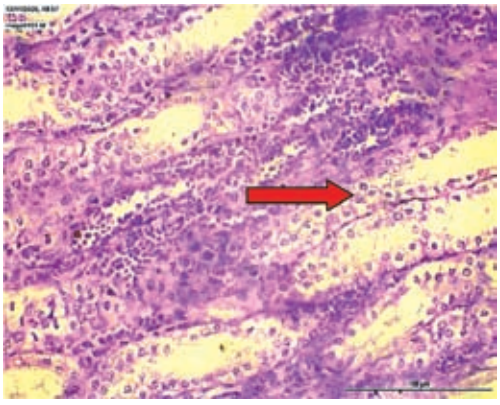

Fig. 6. Kidney showing cloudy swelling in the renal tubules (arrow) (H&E X 400)

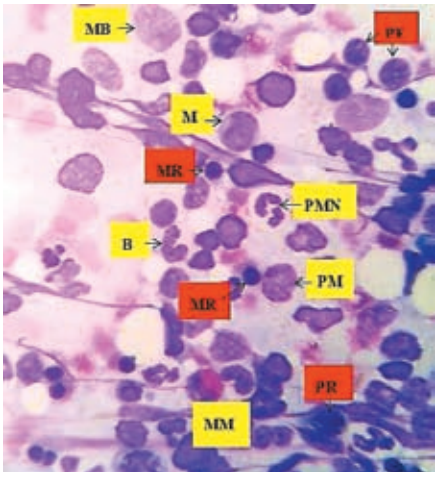

Fig. 7. Bone marrow cytological smear showing different myeloid and erythroid series of cells (Wright-Giemsa stain, 1000x). MB- Myeloblast; PM – Promyelocyte; M – Myelocyte; MM – Metamyelocyte; B- Band cells; PMN- Matured segmented neutrophil; PR – Prorubricyte; PE- Polychromatophilic erythrocyte; MR- Metarubricyte

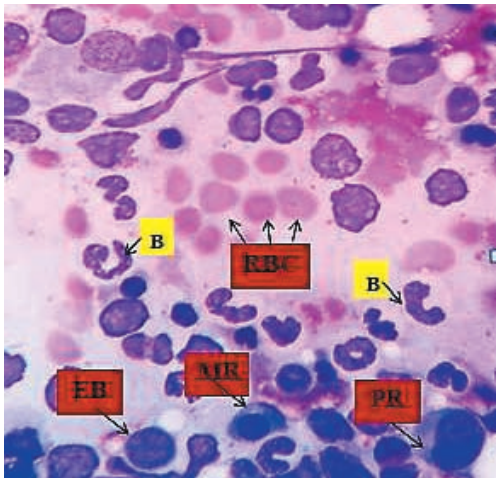

Fig. 8. Bone marrow cytological smear showing different myeloid and erythroid series of cells (Wright-Giemsa stain, 1000x) EB- Erythroblast; B- Band cells, PR – Prorubricyte, MR- Metarubricyte; RBCS-Red Blood Cells

into neutrophilic, basophilic and eosinophilic myelocytes. When the indentation of the nucleus starts to appear in the myelocyte these cells are classified as metamyelocyte. If the indentation of metamyelocyte nucleus becomes greater than half of the cell, it is called a band cell. Matured neutrophils have distinctive lobes inside the cells.

In the present study, reduced erythroid series of cells in PCV-2 infected pigs were also noticed. The characteristic features of erythroid series of cells have been described (Cowell *et al.*, 2007). The rubriblasts are the most immature form of erythroid cells, having higher nuclear cytoplasmic ratio. The cytoplasm is intensely basophilic and forms a narrow rim around the nucleus. Nucleus has clear smooth round border with one or two pale to medium blue nucleoli. In prorubricyte, the nuclear chromatin is slightly coarser, nucleolus is usually not visible and the cytoplasm forms a thick rim around the nucleus. The rubricyte has spokes of wheel appearance with extremely coarse chromatin. The colour of the cytoplasm varies from blue to bluish-red-orange to red-orange. The nucleus of metarubricytes is highly pyknotic and appears black with indistinct nuclear chromatin pattern. Polychromatophillic erythrocytes are the non-nucleated erythrocytes and are larger than the matured erythrocytes. Mature erythrocytic stage is the last stage in erythroid cell series maturation.

The increased M: E ratio could be due to decline in erythroid activity and increase in myeloid activity. An increased M: E ratio in the current study was due to reduction of erythroid cells and increase in myeloid cells in the bone marrow. Since PCV-2 has the potency to produce lesions in kidney (Rossell *et al.,* 1999), it is unable to affect erythropoiesis in infected animals. Erythroid hypoplasia of bone marrow could be related to the fact that anaemia is a common finding in PCV-2 affected cases which was in agreement with Segales *et al*. (2000).

The increase in myeloid cells might be due to their demand to encounter the PCV-2 viral infection. Increased mononuclear infiltration in the thickened alveolar septa of pneumonic lung, histiocytic infiltration in lymphoid and non-lymphoid organs also accounted for

the increased demand of myeloid cells. Bone marrow histological examination also suggested focal histiocytosis which evinced an increase in proliferation of monocyte/ macrophage lineage cells which might be due to PCV-2 infection. This fact could be related to the increase of macrophages (as histiocytic and plasmacytic cells) infiltrating in target tissues such as lymphoid and non-lymphoid organs which is characteristic of PCV-2 infection. This also could be stated as a reason for increased proliferation of myeloid cells observed in cases of PCV-2 infection (Rosell *et al*., 1999).

Conclusion

A detailed analysis to recognize the effects of PCV-2 on the myeloid and erythroid series of bone marrow based on cytological smears in pigs was carried out. The results indicated that there is an increase in M: E ratio in PCV-2 infected pigs which might be due to hypoplasia in erythroid cells and hyperplasia of myeloid cells. Hence, this study has improved the current understanding of pathological effects on bone marrow in PCV-2 infection. However, further studies are required with a greater number of samples to validate the preliminary results of our study as PCV-2 has the highest mutation rates among DNA viruses.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Effect of storage on biochemical parameters of packed red blood cells of goats in citrate phosphate dextrose adenine/ saline adenine glucose mannitol*

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Abstract

A study was conducted to assess the suitability of citrate phosphate dextrose adenine / saline adenine glucose mannitol as a storage media for packed RBCs of goats. Samples collected from ten apparently healthy goats were utilized for the study. Biochemical studies were carried out on day 0, 14, 28 and 42 days of storage using the parameters, viz. pH, glucose, potassium, malondialdehyde and reduced glutathione. The pH was stable throughout the study, whereas glucose showed significant reduction. Rest of the parameters increased significantly from 0th day to 42nd day. Based on the results, the storage media can be considered to be suitable for storing caprine packed RBCs.

Keywords: *Biochemical parameters, blood transfusion, goat, packed RBCs, storage studies*

With advances in veterinary transfusion medicine, blood component therapy is being increasingly practiced. Packed red blood cell (pRBC) transfusion is one of the most common component transfusion practiced in animal transfusions. As the need outweighs availability, storage of blood and its components is required for emergency transfusion procedures. Suitable storage media is a necessity with regard to storing of blood, as any storage lesion can adversely affect the viability of RBCs. Biochemical studies on caprine pRBCs stored in citrate phosphate dextrose adenine/ saline adenine glucose mannitol (CPDA/SAGM) are scarce. Hence, the present study was envisaged to assess the suitability of CPDA/SAGM as a storage media for caprine pRBCs.

**Part of M.V.Sc. thesis submitted by the first author to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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Materials and methods

Ten apparently healthy goats weighing 35 to 60 kg within an age range of 4 to 8 years were selected from University Goat and Sheep Farm, Mannuthy and other organized farms in Thrissur district. All the goats were dewormed and the health status of each animal was evaluated by physical examination and laboratory analysis including complete blood count, examination of blood smear and faecal sample. Whole blood units were collected from the selected animals using commercially available CPDA-SAGM blood bags under aseptic conditions. Each of these units was centrifuged at 5,000 × *g* for 7minutes, at 4°C using FTBC- 6100R blood bank refrigerated centrifuge. After centrifugation, plasma was extracted in empty bag using plasma extractor from primary blood bag and SAGM was added from the satellite bag using the valves. After separation, pRBCs with SAGM was stored at 4 ± 2°C in a dedicated refrigerator for 42 days and biochemical parameters analysed every two weeks from day 0 to day 42. Blood pH was estimated with portable Oakton waterproof pH meter. The level of GSH in erythrocyte suspension was determined as per Bain *et al*. (2016). This method is based on the development of a yellow colour when 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) is added to sulphydryl compounds. Level of lipid peroxides in erythrocyte suspension was determined by estimating MDA using the method of Okhawa *et al*. (1979) as modified by Al-Azzawie and Alhamdani (2006). The supernatant glucose estimation of packed RBC stored in SAGM was carried out using glucose estimation kit- GenX GLUCOSE-ML (GOD-PAP Trinders method). Potassium was estimated from the supernatant of packed red blood cells stored in CPDA/ SAGM blood bag using ion selective electrode technology.

Results and discussion

The biochemical parameters assessed in this study were pH, glucose, potassium, MDA and GSH. Statistical analysis using repeated measures ANOVA was carried out to find out the changes in the variables at different period of time. In the cases where F-value was found significant, pair wise comparison was done by using least significant difference test. The mean values of biochemical parameters are as in table 1.

In this study, mean pH value decreased from day 0 to 42 during storage, though it was not significant. The mean value was in the range 7.18 ± 0.03 to 7.13 ± 0.03. Hess and Greenwalt (2002), opined that pH value above 7.2 will favor 2, 3-bisphoshoglycerate production and might affect the ATP synthesis. The permissible lower pH limit for the stored blood was 6.65. Reduction in pH values occur as RBCs remain metabolically active, with glucose breakdown and anaerobic metabolism leading to production of lactate and hydrogen ions (Mudge *et al*., 2004; Hess *et al.,* 2009). However, as the decline of pH was not found to be significant and was within the limits, the stored blood could be used for transfusion.

Table 1. Variation of bio-chemical parameters during storage

Variables	Day 0	Day 14	Day 28	Day 42	F-value (P-value)
pH	7.18 ± 0.0389	7.18 ± 0.0327	7.14 ± 0.0371	7.13 ± 0.0367	1.317^{ns} (0.289)
Glucose (mg/dL)	650.4 ± 18.45^{a}	607.5 ± 18.92^{b}	573.2 ± 22.09^{c}	535.5 ± 16.92^{d}	20.707** (<0.001)
Potassium (mmol/L)	4.02 ± 0.22^{d}	6.96 ± 0.27^{c}	9.61 ± 0.39^{b}	13.15 ± 0.57^{a}	160.61** (<0.001)
MDA (µmol/L)	10.47 ± 1.13^{d}	15.15 ± 1.36^{c}	20.59 ± 1.60^{b}	28.16 ± 1.76^{a}	33.342** (<0.001)
GSH (µmol/g of Hb)	0.02 ± 0.01^{d}	0.05 ± 0.01^{c}	0.08 ± 0.02^{b}	0.10 ± 0.02^{a}	46.62** (<0.001)

** Significant at 0.01 level (P<0.01); ns - Non-significant (P>0.05)

Means having different superscripts differ significantly within a row

The mean value of glucose in supernatant reduced as the storage period increased. This is in agreement with Tavares (2013), who observed decrease in glucose values during storage from day 0 to 42. This reduction might be due to the consumption of glucose by the metabolically active erythrocytes as an energy source. However, it has been recorded that glucose consumption of caprine erythrocytes is low in comparison to other species. Higher ATP reserves of caprine erythrocytes may also result in non-decline of glucose levels (Kaneko *et al*., 2008). Even at the end of storage period, significant reduction in glucose was noticed in the mean value from 573.2 ± 22.09 mg/dL on 28th day to 535.5 ± 16.92 mg/dL on 42nd day. This suggests that RBCs remained metabolically active and fit for transfusion. The excess glucose in the nutrient media at the end of storage period was easily balanced post transfusion by the liver (Fonesca *et al*., 2018).

A significant increase was recorded in the supernatant potassium level during the study. On the 42nd day of storage, the supernatant mean potassium value was 13.15 ± 0.57 mmol/L, *vis-à-vis* day 0 value of 4.02 ± 0.22 mmol/L. During blood storage, a slow and continuous leakage of intracellular potassium to the plasma has been recorded. This has been considered to be associated with failure of Na^+- K^+ ATPase pump (Opoku-Okrah *et al*., 2015). Species wise difference in intracellular potassium level has been reported (Sousa *et al*., 2013). Opoku-Okrah *et al*. (2015), stated that plasma potassium level of blood stored in CPD solution can increase by 0.5-1.0 mmol/L per day. Extracellular potassium value can be used as a marker for assessing the quality of stored erythrocytes as the ATP depletion and improper refrigeration may result in elevation of potassium in ECF as suggested by Fonesca *et al*. (2018). Perusal of literature did not reveal any similar study for caprine pRBC storage with regard to potassium levels. The increase in potassium in stored blood of goat was slow and constant when compared with the increased potassium levels of stored canine and human blood. Further, the values of the present study can be used as reference values for caprine pRBC storage studies.

The mean MDA values increased significantly throughout the storage from 10.47 ± 1.13 µmol/L (day 0) to 28.16 ± 1.76 µmol/L (day 42). As MDA is an end product of lipid peroxidation, quantifying the MDA in stored blood was a useful marker to determine the extent of lipid peroxidation (Simsek *et al*., 2006; Nazifi *et al*., 2009; Pandey and Rizvi, 2011). Malondialdehyde formation indicated the loss of phospholipid from RBC membrane. Proportionating the MDA values, oxidative damage and resultant haemolysis can be assessed and viability of the cells in the storage media can be predicted (Fonesca *et al*., 2018). Increase in MDA values on storage of pRBCs in SAGM of human blood has been reported (Chaudhary and Katharia, 2012). However in a similar study of human pRBCs, increase was found to be insignificant (Mustafa *et al*., 2016). Similar studies with regard to stored caprine pRBCs are lacking. It has been suggested by Antosik *et al*. (2018) that addition of membrane interacting antioxidants like vitamin E analogue has beneficial effects in reducing lipid peroxidation level of RBCs stored in SAGM.

A significant increase in GSH was recorded with storage. Huyut *et al*. (2016) reported that MDA value and GSH value were negatively correlated. Reduced glutathione studies with respect to caprine pRBC stored in SAGM are lacking. Increase in GSH during the study period suggests antioxidant activity to manage the oxidative stress. According to Roback *et al*. (2011), the oxidised form of GSH is GSSG, which is not an antioxidant but it can be converted back to GSH by using NADPH derived from pentose phosphate pathway. It was also reported that supplementation of amino acid precursors can also stimulate GSH synthesis. Two other antioxidants reported were α- tocoferol and ergothioneine, which are obtained from the diet or some unknown mechanisms and not synthesized by the erythrocytes. These were relatively stable during the storage. Additive used in the study, SAGM contains mannitol which acts as an antioxidant by scavenging hydroxyl radical in various systems (Antosik *et al*., 2018). Therefore, several factors influence the GSH values and no specific conclusion could be drawn with regard to the increase GSH values recorded in the present study.

Conclusion

The suitability of CPDA/SAGM as a storage media for caprine pRBCs in the present study was assessed using biochemical parameters. Based on biochemical studies, the storage media could be considered suitable for storing caprine pRBCs for 42 days.

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Conflicts of interest

There were no conflicts of interest reported by the authors.

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Diagnosis and therapeutic management of malasseziosis in dogs

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Abstract

Malassezia spp. are commensals of the normal cutaneous microbiota of humans and animals. These yeasts may become opportunistic pathogens under certain conditions and cause dermatitis and otitis externa in dogs. Malassezia pachydermatis is the most common cause of malasseziosis in dogs. In this study skin and ear swabs from suspected cases were cultured on Modified Dixon's Agar (MDA). The isolates obtained were initially characterized on the basis of colony characteristics, result of Gram staining and microscopic morphology. Total DNA was extracted from the pure cultures of the isolates and subjected to confirmation by polymerase chain reaction (PCR) targeting large subunit ribosomal RNA gene. Positive cases were treated with oral itraconazole at 5 mg/kg bodyweight, orally once daily for 28 days.

Keywords: *Malassezia pachydermatis, MDA, PCR*

Members of the genus Malasseziaare lipophilic basidiomycetous yeasts, which are part of the normal cutaneous microbiota of humans and other warm blooded animals. Malasseziosisis recognized as a secondary complication to other conditions like keratinization defects, hypersensitivity disorders, staphylococcal pyoderma and endocrinopathies. Itraconazole is a keratinophilic and lipophilic triazole antifungal agent used to treat malassezia infection (Pinchbeck *et al*., 2002). The paper reports the isolation of *Malassezia* species from clinical cases in dogs and its successful management.

** Part of M.V.Sc. thesis submitted by the first author to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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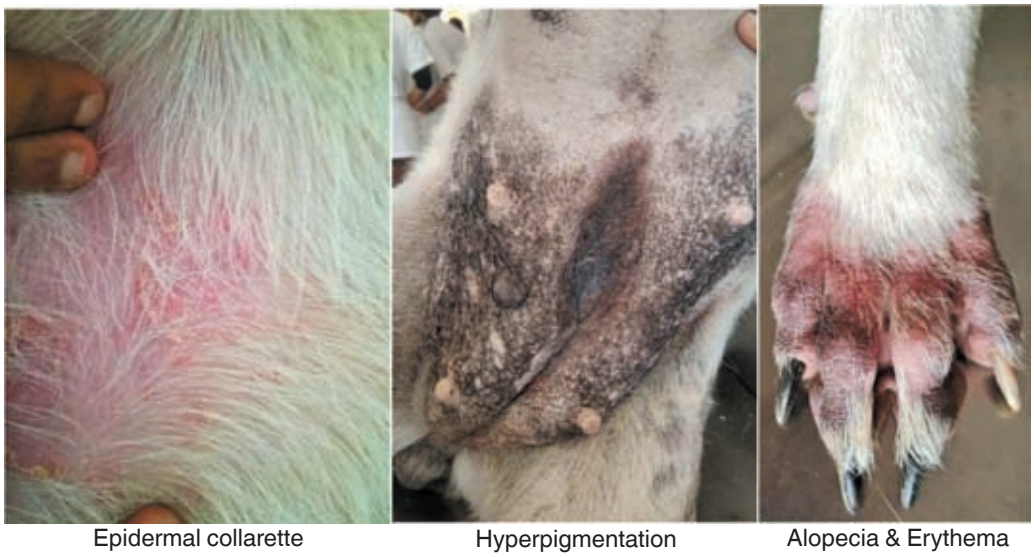
Materials and methods

Ten dogs of different breed, age and gender that were presented to Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Pookode, with clinical signs of alopecia, pruritus, erythema, hyperpigmentation, lichenification, foul odour and otitis externa suggestive of malasseziosis were subjected to detailed clinical examination. Cytological examination of skin and ear swabs were carried out by Methylene blue staining. On cytology, at least one body site having a mean of ≥ 1 yeast organism per oil immersion field (OIF) during microscopic examination of 10 consecutive OIF with corneocytes considered positive for malasseziosis (Pinchbeck, 2002). Cultural examination was done by inoculating the samples onto Modified Dixon's Agar and incubating at 37˚C for up to seven days (Rathnapriya*et al*., 2016). Total DNA was extracted from the pure cultures of the isolates using conventional phenol – chloroform method and were subjected to polymerase chain reaction targeting large subunit ribosomal RNA gene of *Malassezia* spp. (Guillot*et al*., 2000). Dogs with malasseziosis were treated with oral itraconazole at 5 mg/kg bodyweight, orally once daily for 28 days along with supportive therapy, which included essential amino acids and herbalimmuno-stimulants. Six apparently healthy dogs were selected for the control group.Haematological and serum biochemical studies were performed.Comparison between study and control animals was done by using independent t-test.

Results and discussion

Clinical parameters were found to be within the normal range upon general clinical examination with mean respiratory rate of 27.22 ± 1.02/ min, pulse rate of 92 ± 0.6/ min and temperature of 102.14 ± 0.15 °F. Haematological and serum biochemical study revealed leucocytosis and mild non regenerative anaemia in affected dogs. Significantlyless Haemoglobin (Hb), Volume of Packed Red Cells (VPRC) and Mean Corpuscular Haemoglobin (MCH) ($p < 0.05$) values were obtained in dogs with malasseziosis when compared to control group (Table 1).Takahira (2009) reported that mild to moderate non regenerative anaemia is the most common anaemia in dogs with fungal, bacterial, viral and protozoal diseases. Further, it was stated that reduced erythrocyte production and erythrocyte removal by immunologic mechanisms or oxidative damage could lead to development of anaemia in the above conditions.Significantly highvalues of TLC ($p < 0.01$) were observed in affected dogs when compared to control group (Table 1). Hromada*etal*. (2005) also observed a significant increase in total leukocyte count in dogs infected with malassezia yeasts and,whichmight be due to inflammatory changes in the skin. There is



Epidermal collarette | Hyperpigmentation | Alopecia & Erythema

Fig. 1. Clinical manifestations of malasseziosis in dogs

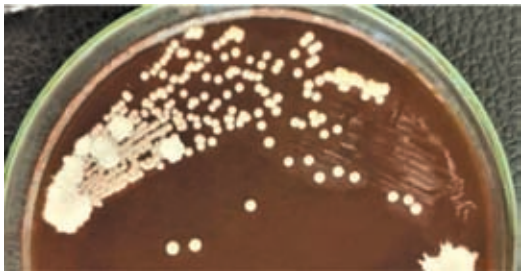


Fig. 2. Colonies of *Malassezia* spp. on MDA

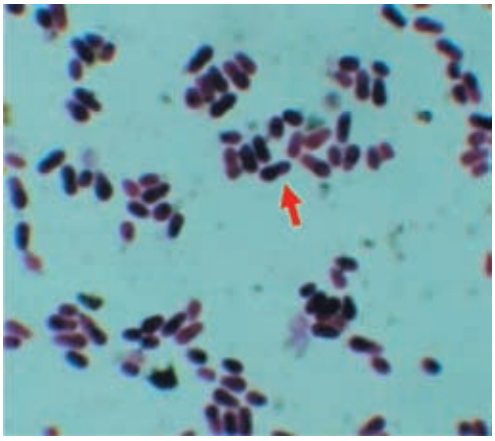


Fig. 3. Microscopic examination of colonies of *Malassezia* spp. using Gram's staining

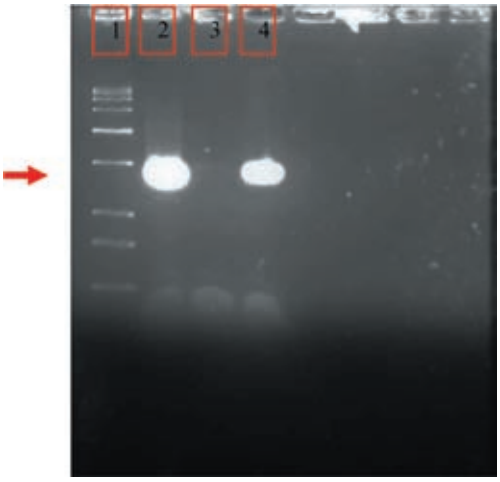


Fig. 4. Molecular detection of *Malassezia spp.* (Lane 1 – 100 bp ladder**;** Lane 2, 4 **–** Isolates of *Malassezia* spp**.;** Lane 3 **–** Negative control; Amplicon size between 541 bp and 579 bp**)**

Fig. 5a.1st day

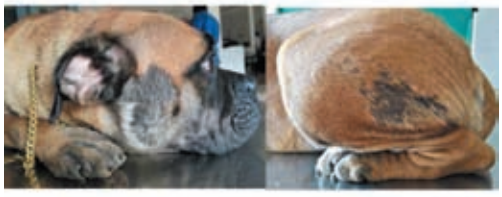


Fig. 5b. 14th day

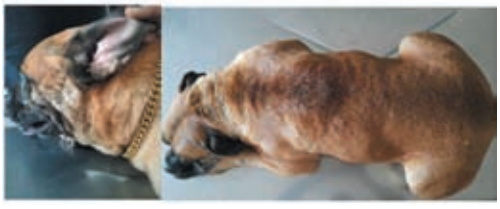


Fig. 5c. 28th day

Fig. 5. Clinical response to treatment with Itraconazole

no statistically significant difference in ALT, ALP, total protein, albumin and globulin values in dogs with malasseziosisin comparison with control group (Table 2). Detailed examination of the skin revealed alopecia, pruritus, erythema, hyperpigmentation, lichenification, epidermal collarettes, pustules, vesicles and nodules (Fig. 1). These findings were in accordance with Bond *etal*. (2020) who reported that pruritus, erythema, hyperpigmentation, malodour and traumatic alopecia were the major clinical signs in dogs with malasseziosis. Microscopic examination of acetate tape impression smears revealed the presence of Gram positive budding yeast cells. Jatavath and Kumar (2014) identified malassezia yeasts by cytology using the tape-strip technique. Impression and swab smear examinations of ten diseased dogs revealed the presence of cocci in six dogs suggestive of bacterial pyoderma. Dogs in association with concurrent bacterial infections were treated with antimicrobial therapy.Ten isolates suggestive of *Malassezia*spp. were obtained on MDA. The colonies were cream to buff coloured with smooth, convex surface (Fig. 2). Of these, seven were isolated from skin lesions of dogs and the rest were obtained from cases of otitis externa.

Table 1. Comparison of haematology of dogs with malasseziosis and control animals

Sl. No.	Parameter	Mean ± SE		t-value	P-value
		Study Group	Control Group		
1	Hb(g/dL)	13.63 ± 0.73	16.03 ± 0.70	2.2*	0.046
2	PCV (%)	40.12 ± 2.36	48.18 ± 1.89	2.36*	0.033
3	TEC (x 10^6/cmm)	6.40 ± 0.3	6.87 ± 0.26	1.09^{ns}	0.292
4	TLC (x 10^3/cmm)	23.9 ± 2.9	11.85 ± 0.66	3.06**	0.009
5	Platlet count (x10^3/µL)	339.1 ± 28.9	274.67 ± 25.28	1.52^{ns}	0.151
6	MCV (fL)	63.05 ± 2.6	70.2 ± 0.6	2.08^{ns}	0.056
7	MCH (g/dL)	21.3 ± 0.59	23.23 ± 0.22	2.48*	0.026
8	MCHC (pg)	34.1 ± 0.76	33.2 ± 0.2	0.873^{ns}	0.398
9	N (%)	79.63 ± 2.63	76.75 ± 1.66	0.79^{ns}	0.445
10	L (%)	15.1 ± 2.42	16.97 ± 1.79	0.54^{ns}	0.595
11	M (%)	3.2 ± 0.32	4.55 ± 0.44	2.54^{ns}	0.23
12	E (%)	2.1 ± 0.3	1.73 ± 0.38	0.78^{ns}	0.446

*** Significant at 0.01 level (P<0.01); * Significant at 0.05 level (P<0.05); ns Non-significant (P>0.05)*

Table 2. Comparison of serum biochemical values of dogs with malasseziosis and control animals

Sl. No.	Parameters	Mean ± SE		t-value	P-value
		Diseased Group	Control Group		
1	ALT (U/L)	30.6 ± 8.22	25.16 ± 2.13	0.5^{ns}	0.626
2	ALP (U/L)	106.8 ± 14.21	77.16 ± 7.19	1.53^{ns}	0.148
3	TP (g/dL)	5.5 ± 0.24	6.11 ± 0.12	1.85^{ns}	0.085
4	Albumin (g/dL)	2.4 ± 0.17	2.88 ± 0.08	2.15^{ns}	0.05
5	Globulin (g/dL)	3.12 ± 0.25	3.23 ± 0.05	0.34^{ns}	0.738

ns Non-significant (P>0.05)

Rathnapriya*etal*. (2016) and Marin *et al*. (2018) also successfully isolated malassezia yeasts on Modified Dixon's Agar at 37 ^{0}C for five days with a higher isolation rate compared to Sabouraud Dextrose Agar with olive oil overlay. Microscopic examination of colonies revealed dark blue coloured footprint shaped organism on Gram's staining. Microscopically, budding yeast cells with buds attached to the mother cell by a broad base were observed (Fig. 3). All the ten isolates were positive in the PCR targeting LSU region of *Malassezia* spp. (Fig. 4). Affes*et al*. (2009) reported that the use of PCR method provides a sensitive and rapid identification system for *Malassezia* species, which can be applied in epidemiological surveys and routine practice. Clinical response to itraconazole therapy was monitored at 14th and 28th day of treatment. An excellent clinical response along with quick recovery (by 14th day itself) was noticed in all the ten dogs treated with oral itraconazole. By 28th day of treatment, all the animals showed complete recovery from all the clinical signs. Clinical response to treatment with itraconazole were shown in Fig. 5a, 5b and 5c. Haimbach (2019) reported that itraconazole given at 5 mg/kg bodyweight daily for three weeks had good efficacy against malasseziainfection in dogs.

Conclusion

The present study concluded that cytological, cultural and molecular examination of clinical samples can be effectively used for diagnosis of Malassezia species. Orally

itraconazole was found to be effective for the treatment of canine malasseziosis with excellent clinical response and quick recovery.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Molecular test for detection of *Mycoplasma ovipneumoniae* associated with respiratory tract infection from goats in north and central parts of Kerala*

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Abstract

Mycoplasmal pneumonia is an important contagious disease that significantly affects the economy of small ruminant farming worldwide and Mycoplasma ovipneumoniae (M. ovipneumoniae) is one of the major aetiological agents associated with pleuropneumonia in goats. It is considered as a serious epidemic disease of goats due to its huge economic impact and hence, rapid and early diagnosis of the disease is warranted. Clinical mycoplasmosis often lacks pathognomonic signs, so definitive diagnosis of the disease is quite burdensome. Polymerase chain reaction (PCR) test has been proven to be a specific and sensitive technique for the early diagnosis of mycoplasmosis. The present study highlights the detection of M. ovipneumoniae employing PCR test in 150 nasal swab samples collected from goats with symptoms of respiratory tract infection from five districts of Kerala. Results revealed that, out of 150 samples, 83 (55.33 per cent) were positive in 16S rRNA Mycoplasma genus specific PCR test. Among the 83 genus positive samples, 68 samples (45.33 per cent of total 150 samples) were positive in M. ovipneumoniae specific PCR test.

Keywords: *Goats, polymerase chain reaction (PCR), Mycoplasma ovipneumoniae*

In India, goat farming is one of the major sources of income for small scale farmers. Among the various infectious diseases, the one caused by *Mycoplasma* causes significant economic losses to goat industry. It is one of the *Office International des Epizooties* (OIE) listed notifiable diseases, which affects international trade and is responsible for major constraint in world economy (OIE, 2008). *Mycoplasma* produces various disease manifestations such as pneumonia,

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conjunctivitis, arthritis and mastitis (Nicholas, 2002). Several *Mycoplasma* species are identified to be of pathogenic significance in goats worldwide (Nicholas *et al.,* 2008). Among these, *M. ovipneumoniae* is well thought-out as the cause of non-progressive pneumonia and was first identified in Australia in 1972 (Ongor *et al.,* 2011). In goats, primary infection with *M. ovipneumoniae* may facilitates invasion by other bacteria such as *Mannheimia haemolytica*, which may enhance the pathological process (McAuliffe *et al.,* 2003). Along with other pathogenic mycoplasmas in the same animal, *M. ovipneumoniae* might cause variation in the morbidity and mortality of the disease in the same animal (Halium *et al.,* 2019).

Diagnosis of *M. ovipneumoniae* based on clinical and post-mortem lesions will not give confirmatory diagnosis because symptoms can be shared by other clinically important infections. *Mycoplasma* is a highly fastidious organism and is very difficult to isolate on artificial medium. Nowadays, molecular techniques such as PCR test employing specific oligonucleotide primers offer the rapid and specific detection of *M. ovipneumoniae*.

In India, studies on caprine mycoplasmosis mainly focused on contagious caprine pleuropneumonia and contagious agalactia (Kumar *et al.,* 2011; Reji *et al.,* 2018). On the other hand, only few studies concerning *M. ovipneumoniae* have been conducted (Jana *et al.,* 2005; Reji, 2018) and therefore, there is paucity of information regarding the infections caused by this organism.

In the present study, we report the molecular detection of *M. ovipneumoniae* from goats with respiratory tract infection during a period of 14 months from central and north parts of Kerala.

Materials and methods

Collection of samples

A total of 150 nasal swabs were collected from goats with respiratory infection from different parts of Kerala. Sterile cotton swabs were pre-wetted in phosphate buffered saline (PBS) and inserted deep into the nasal passage. The swabs were then placed back in PBS and snapped off the handle. The samples collected were transported immediately to the laboratory under cold conditions and subjected to direct detection of *M. ovipneumoniae* employing PCR.

DNA extraction

Deoxyribonucleic acid (DNA) was extracted from the nasal swabs using Hi Pura multi sample DNA extraction kit (HiMedia, India) and stored in elution buffer at -20˚C till use. Concentration and 260/280 OD value of the extracted DNA were checked by Nanodrop 2000 (Thermo Scientific).

Polymerase chain reaction (PCR) assay

Two different PCR assays were used to identify the organism up to the species level. Initially all the DNA samples were subjected to their reactivity with *16S rRNA* genus specific primers and later with *M. ovipneumoniae* specific primers.

The PCR amplification was carried out in a volume of 12.5 µL reactions in 200 µL capacity PCR tubes containing 1.25 µL molecular biology grade nuclease free water, 6.25 µL 2X PCR master mix, one microlitre (10 p*M*) of each of the forward and reverse primers and three microlitre of DNA (10 ng/µL). One negative control without template DNA was included to monitor any contamination. The contents of the tubes were mixed gently, spun briefly and the tubes were placed in an automatic thermal cycler for amplification.

Identification of 16S rRNA gene specific to genus Mycoplasma

Mycoplasma genus specific PCR was performed using the *16S rRNA* of *Mycoplasma* specific primers GPO3F (5' TGG GGA GCA AAC AGG ATT AGA TAC C3') and MGSO (5' TGC ACC ATC TGT CAC TCT GTT AAC CTC3') for an expected amplified product of 280 bp (Botes *et al.,* 2005). The conditions used in the PCR test are given in table 1.

Table 1. PCR conditions for amplification of *16S rRNA* gene specific to genus *Mycoplasma*

Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	2 min.	1
Denaturation	94°C	15 sec.	35
Annealing	59.3°C	15 sec.	
Extension	72°C	15 sec.	
Final extension	72°C	5 min.	1

Identification of M. ovipneumoniae

The PCR was performed using the *M. ovipneumoniae* specific primers MOVPF (5' GTT GGT GGC AAA AGT CAC TAG 3') and MOVPR (5' CTT GAC ATC ACT GTT TCG CTG 3') for an expected amplified product of 418 bp (Halium *et al.,* 2019). The conditions used in the PCR test are given in table 2.

Submarine agarose gel electrophoresis

Amplified PCR products were resolved in one per cent agarose gel in 1X TBE buffer. Five microlitre of the PCR product was loaded into the wells. A 100 base pair DNA ladder (SRL) was also run alongside the samples to ascertain the size of the amplified products. Electrophoresis was carried out at 50 V and 16 mA until the dye migrated two-third of length of the gel. The gel was visualised under UV transilluminator and the results were documented in a gel documentation system (Bio-Rad).

Results and discussion

As per OIE (2008), nasal swab containing clinical material from live goats showing respiratory signs was the sample of choice for the diagnosis of *Mycoplasma*. Hence, the same was collected from ailing goats with clinical signs suggestive of mycoplasmosis. Similar procedure was also followed by Reji *et al.* (2018).

The nasal swabs were collected from a total of 150 goats showing respiratory tract infection. Out of which, 83 were positive for *16S rRNA* genus specific PCR (Fig. 1). The genus positive samples were then subjected to a gradient PCR with *M. ovipnuemoniae* specific primers to find out the optimised annealing temperature for further PCR. A nasal swab collected from Thrissur produced an amplicon size of approximately 418 bp at the annealing temperatures of 59.9°C, 61.5°C and 62.4°C. Of these, 61.5C for one minute was selected as the optimum. The above mentioned sample was taken as the positive control. Among 83 samples that are positive for *Mycoplasma* genus, 68 found to be positive for *M. ovipneumoniae* (Fig. 2). Similar observations were made by Ongor *et al.* (2011) in goats, who detected *M. ovipneumoniae* directly from nasal swabs employing PCR.

Clinical symptoms and post-mortem lesions even though aid in the diagnosis of mycoplasmosis in field conditions, the definitive diagnosis cannot be made solely on these findings because symptoms and lesions can be shared by other clinically similar infections. Sampling requires expertise and culture, isolation and identification by biochemical tests

Table 2. PCR conditions for amplification of *M. ovipneumoniae* specific PCR

Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	1 min.	1
Denaturation	94°C	1 min.	35
Annealing	61.5°C	1 min.	
Extension	72°C	2 min.	
Final extension	72°C	5 min.	1

Fig. 1. Agarose gel electrophoresis of *Mycoplasma* genus specific PCR products

Lane M: 100 bp DNA ladder
Lane 1: Positive control
Lane 2: Negative control
Lane 3, 4, 5, 6, 7, 8 and 9: Positive samples
Lane 10 and 11: Negative samples

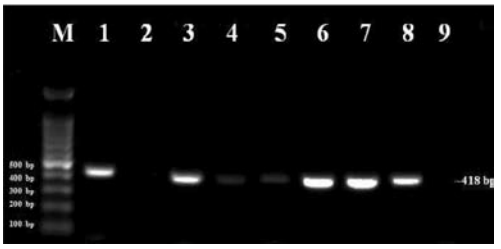

Fig. 2. Agarose gel electrophoresis of *Mycoplasma ovipneumoniae* specific PCR products

Lane M: 100bp DNA ladder
Lane 1: Positive control
Lane 2: Negative control
Lane 3, 4, 5, 6, 7 and 8: Positive samples
Lane 9: Negative sample

require specialised laboratory, infrastructure and costly media, hence rarely practiced (Thiaucourt and Bolske, 1996). Meanwhile, it was reported that reduced viability and fastidious nature of *Mycoplasma* in clinical samples could also be affecting the culture results (Bolske *et al.,* 1996). As per Woubit *et al.* (2004), cross reactions with other *Mycoplasma* species were the main limitation of the serological techniques. Molecular techniques like polymerase chain reaction (PCR) being highly specific and sensitive, enables the rapid detection of *M. ovipneumoniae* in samples containing multiple *Mycoplasma* species. Hence, PCR test proved effective both at field and laboratory level diagnosis (OIE, 2008; Halium *et al.,* 2019).

The results of the present study suggested that *M. ovipneumoniae* is the main organism associated with respiratory tract infection in goats in Kerala. In another study carried out in the same region by Reji (2018), documented that *M. ovipneumoniae* was the predominant organism associated with respiratory infection in goats, followed by *M. conjunctivae* and *M. agalactiae.* Respiratory disease due to *M. ovipneumoniae* has been reported in India in a few studies (Sikdar and Uppal, 1986; Jana *et al.,* 2005). In a recent study carried out in Egypt, a high frequency of *M. ovipneumoniae* was detected using PCR in goats showing respiratory symptoms such as coughing and nasal discharge (Halium *et al.,* 2019). Apart from domesticated goats and sheep, *M. ovipneumoniae* also affects wild ruminants. In Washington, Highland *et al.* (2018), detected *M. ovipneumoniae* in nasal swab taken from mule deer employing PCR.

Conclusion

More number of samples needs to be tested from wide geographical area by molecular epidemiological studies to study the role of *M. ovipneumoniae* in pleuropneumonic cases. Nucleotide sequencing and phylogenetic analysis are required to identify the prevalent strains so as to develop suitable protocol for effective control and prevention of the disease in the state.

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Conflict of interest

The authors declare that they have no conflict of interest.

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The impact of flood in 2018 on the socio-economic conditions of the dairy farmers in Pariyaram panchayat of Thrissur district of Kerala

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Abstract

Climate change poses existential threat on all forms of life on earth. Flood is one of the major fallout of it. Tropical humid zones experiences worst form of rain related disasters in the recent times. Kerala, a tropical humid state of India experienced one of the most destructive flood events of the century during August 2018. It had heavy impact on every sector including animal husbandry. Based on this event, a study was conducted to assess the impact of flood in 2018 on Livestock Farming System of Pariyaram Panchayat in Thrissur district of Kerala. The entire Panchayat was delineated into affected and unaffected wards and detailed survey on faming system and socio economic profile of the flood affected area was conducted. Pre and post flood livestock system of the flood affected area differed significantly regarding herd strength, milk production and feeding pattern. Livelihood of farmers who were depending on the agriculture and livestock rearing for their survival suffered due to the event, suggesting need for better preparedness.

Keywords: *Climate change, livestock sector, socio-economic impact of flood*

Climate change threatens all the living features on earth. According to intergovernmental panel on climate change, earth has started suffering the consequences of 1°C increase in average global temperature, and due to disruption of the oceanic current leading to frequent episodes of extreme weather, rising sea level and waning polar ice caps. One of the extreme weather events which occur during climate change is a flood. Floods occurs whenever there is excessive runoff of water in any natural drainage channels (Chaw, 1956). During August 2018, Kerala experienced one of the most destructive floods of the century. This happened due to the excessive rainfall in

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a short span of time, geographical peculiarity of the land pattern and lack of proper drainage system. Pariyaram Panchayat is located in Thrissur district which was affected by both flood and landslides due to its topographic pattern. Majority of the inhabitants were involved in agriculture and animal husbandry. The present study reveals the socio economic profile and livestock farming practices of flood affected farmers in Pariyaram Panchayat in Thrissur district of Kerala**.** This would help in future for better adoption and management during such calamities**.**

Materials and methods

Location of study

Pariyaram panchayat is located at 10.3200°N, 76.3705°E in Thrissur district of Kerala near Chalakkudy taluk with 16.8 km^2 area and with population of 31195 (2011 census). Due to its topographic peculiarity, the panchayat was affected by landslides as well as floods during 2018. Out of 15 wards, Peelarmuzhi ward was not affected (control area) remaining 14 wards were affected at varying severity (study area) was selected for this study.

Methodology

For primary data, survey was conducted among 300 livestock farmers of Panchayat to assess the socioeconomic profile and livestock farming systems of flood affected area. Secondary data was collected from the journals, newspaper and grama panchayat office. Primary and secondary data were analyzed both quantitatively and qualitatively. Microsoft Excel was used for data processing and data analysis.

Results and discussion

Socio economic profile of flood affected people

Educational status of the people of the study area (Fig. 7) i.e. the Pariyaram panchayat was found to be as follows. Most of the respondents surveyed had an educational level of higher secondary level (38 per cent), followed by high school (25.33 per cent), primary (18.6 per cent), graduate (15.4 per cent) and post graduate (2.67 per cent). Das and Dey (2011), reported that among the flood affected people from Barak valley, the majority had education up to higher secondary level of education, so the study areas were similar in terms of education status. Agriculture (39.61 per cent) was the primary and major occupation of the people of the present survey (Fig. 2) and a similar study by Prajisha (2019) in Chathanmangalam panchayat also had reported that majority of the respondents from flood affected area were involved in agricultural activities. So, we have to consider vulnerability of farming community as a major aspect in preparedness regime.

Survey revealed that majority of the people lived in nuclear family system (61.9 per cent) in the study area. This finding was in contrast with that of Kumar *et al*. (2013) who reported that majority of farmers in Gujarat were living in joint families (70.83 per cent) and (29.17 per cent) belonged to nuclear families, that will help for managing farming activities. This is because the current social reality of Kerala forces people to have nuclear family structure which at the same time has a lot of bearing on the lack of resilience of the family to counteract and survive calamities.

Majority of the respondents had an annual income (Fig. 6) of less than (Rs) 50,000 (52.63 per cent), 27.04 per cent had an income between 50,000 to 4, 00,000 and 20.33 per cent was above 4, 00,000. This finding was in accordance with Pradeep and Rajesh (2020) who reported that majority of the flood affected households were below the poverty line (52 per cent) in Pothukallu panchayat. Regarding land holding capacity, most of the people had very small land holding with less than five cents (63.28 per cent), 35.09 per cent had between 5 to 10 cents and 1.95 per cent had above 10 cents. This was in accordance with Rathod *et al*. (2012) who observed that majority of the farmers studied had a landholding less than five cents for their crop cultivation. This may be due to the topographic location and thus it reduces the land capacity and most of them were below poverty line that makes them to have less land for farming.

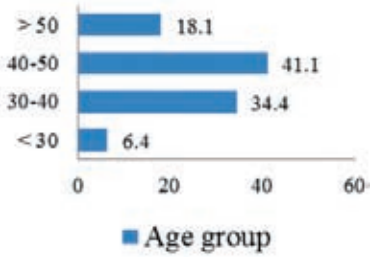

Fig. 1. Age

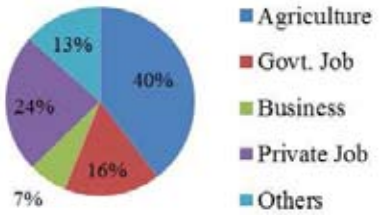

Fig. 2. Occupation

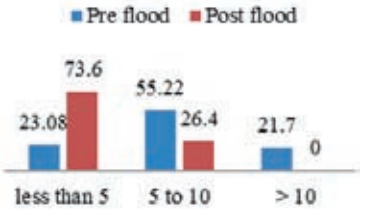

Fig. 3. Herd strength (No. of animals)

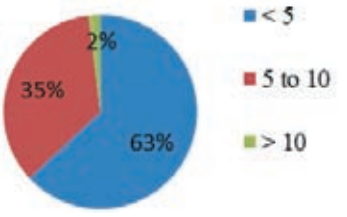

Fig. 4. Land Holding (Cents)

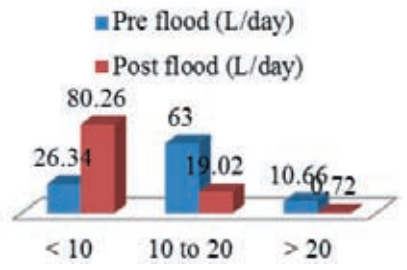

Fig. 5. Milk produced

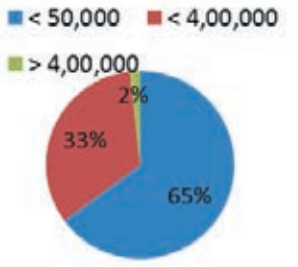

Fig. 6. Annual income (Rs)

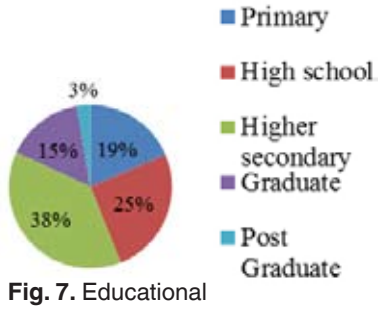

Fig. 7. Educational qualification

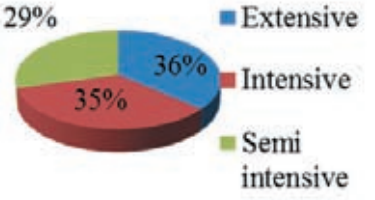

Fig. 8. Animal rearing

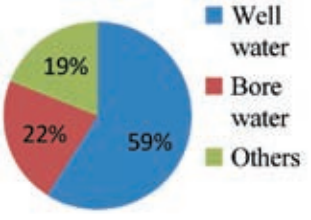

Fig. 9. Water source

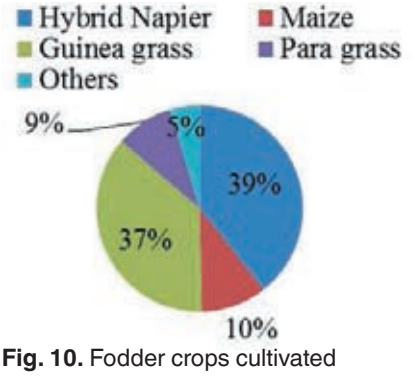

Fig. 10. Fodder crops cultivated

Nearly 93.33 per cent of respondents were directly affected by flood. Rashid and Paul (1987) reported that one third of our country's population was directly affected by flood because of the plain region which gets easily washed off. Regarding the housing pattern, 64.85 per cent of the respondents were having concrete house, followed by tiled (18.06 per cent), and thatched (17.09 per cent). This finding was similar with Pradeep and Rajesh (2020) who revealed that flood affected people from Pothukallu Panchayat had 72 per cent of concrete house, 24 per cent had tiled houses and remaining had thatched houses.

Herd details of livestock farmers

Majority of livestock farmers in Pariyaram had an experience of less than five years (52.73 per cent), followed by five to ten years (37.4 per cent) and more than 10 years (9.87 per cent). This finding was in contrast to that of Rajadurai *et al*. (2018) who reported that majority of livestock farmers had an experience of more than 10 years in rural area where majority of people were involved in animal rearing for their livelihood. There is a shift in the demographic profile of people interested in livestock and in Kerala with more youngsters taking up livestock as a business and at the same time there is a large-scale withdrawal of older people from livestock.

The survey also revealed that majority of farmers had around five to ten animals (55.22 per cent) in pre flood period (Fig. 3). The number of animal units reduced and in post flood situation revealed by the fact that majority had less than five animals (73.16 per cent) post flood. This finding was similar with Kad *et al*. (2014) who studied on the post flood impact in Pune district revealed that majority of farmers had less than eight animals in herd (62 per cent) and also in post flood condition many animals were lost due to drowning and lack of feed for their nutritional support. This was similar with Chauhan and Ghosh (2014) who had reported that majority of livestock were lost after flood due to feed scarcity and wash off in flood water.

Regarding milk production (Fig. 5) majority of farmers produced less than 10 L/d (80.26 per cent) after flood and in pre flood condition majority produced around 10 to 20 L/ day (63 per cent). This was similar with George (2016) who reported that majority of farmers in Kerala produced milk between 10 to 20 L/d from small and medium sized farms. This finding was in accordance with Haile *et al*. (2013) studied 60 per cent of households had negative yield in milk production by reducing two liters per day after 2007.

Feeds and feeding management of livestock farmers

Regarding feeding pattern, majority of farmers fed concentrate to dairy cows at rates between 5 to 10 kg/day (54.68 per cent) and drastically changed after flood, were they fed with less than five kg/day (50.33 per cent). Regarding feeding of roughages, majority of respondents fed less than five kg/day (46.66 per cent). This finding was similar with George (2016) who studied that majority of the dairy farmers provided less than 10 kg/day to their animals in Kerala.

Mostly, people depended on well water as a water source (Fig. 9) to their livestock (59 per cent), followed by bore well (22 per cent) and others (19 per cent). This finding was similar with Riche (2017) who studied that people in Kerala depended on well water for livestock use and human use. So, keeping well water uncontaminated during the time of flood is a major strategy in the climate change adaptation plan for ensuring safe water.

Fodder cultivation Practices of livestock farmers

Major fodder varieties cultivated (Fig.10) by the farmers were hybrid Napier (39.34 per cent), followed by Guinea grass (36.37 per cent), Maize (10.33 per cent), para grass (nine per cent) and other varieties (4.66 per cent). This was in accordance with Rahman (2014) who reported that 40 per cent of people cultivated hybrid Napier for their livestock for feeding. In study area nearly 61 per cent of cultivable land was flooded which was similar with Prajisha (2019) reported that 57 per cent of crops which were cultivated in plantain region were lost in flood that occurred in Kerala in

2018.

Conclusion

Flood is a major fallout of climate change. The present study conducted in a flood affected area reiterates the vulnerability of faming system to flood. Keeping well water uncontaminated during the time of flood is a major strategy in the climate change adaptation plan for ensuring safe water. Study warns long term negative impact on feed, fodder and milk production in affected area. So, we have to consider the vulnerability of farming community as a major aspect in the total preparedness regime.

Conflict of interest

Certified that there is no conflict of interest to be declared in the present work.

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Histopathological study of canine hepatoid gland tumours

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Abstract

Hepatoid gland neoplasms arise due to disorganized and uncontrolled proliferation of cells of hepatoid glands. These are the modified sebaceous glands located mainly in the perianal area. Gross and histological findings of canine hepatoid gland tumours were evaluated. Dogs of different breed, age and sex that were presented to Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Sciences, Mannuthy formed the materials for the present study. Grossly, tumours were solitary or multiple irregular shaped intradermal masses. The excisional biopsy samples were collected in 10 per cent neutral buffered formalin after surgical removal of tumour mass. Histopathologically, the masses were encircled by fibrovascular capsule which extended to the parenchyma as tumour stroma, which separated it into lobules. Two cases of hepatoid gland adenoma one case of hepatoid gland epithelioma and two cases of carcinoma were recognised on histopathological examination. Hepatoid adenoma were characterised by proliferation of hepatocyte like hepatoid gland epithelial cells with extensive sebaceous differentiation and were arranged in cords and anastomosing trabeculae. In hepatoid gland epithelioma, many of the cells were basaloid reserve cells with fewer hepatocyte like cells. Histopathological examination of carcinoma revealed irregular arrangement of the hepatoid cells which showed varying degrees of maturation and marked nuclear pleomorphism. The malignant hepatoid cells had abundant eosinophilic cytoplasm and large nuclei with several prominent nucleoli and mitotic figures.

Keywords: *canine, hepatoid gland adenoma, hepatoid gland epithelioma, hepatoid gland carcinoma*

Neoplasms of perianal glands are most common in male dogs. These non-secretory sebaceous glands are located in the areas of anal orifice, prepuce, tail, hind leg and trunk. This tumour is also known as hepatoid gland tumours due to morphological appearance of cells resembling that of hepatocytes. In canines, these tumours usually appear to be hormone related. Hence it is more common in uncastrated dogs and sometimes spontaneous regression of the tumour

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without excision is also reported. It is reported that Terriers, Mongrels, Cocker Spaniels and Rottweilers are more predisposed to hepatoid gland tumours (Yumusak *et al*., 2016).

The present study was aimed to study the occurrence, and gross and histological findings of canine hepatoid gland tumours. We have also attempted to make a comparative assessment of histomorphological features of canine hepatoid gland tumours.

Materials and methods

The study material comprised of excisional biopsy samples from different dogs that were collected in 10 per cent neutral buffered formalin. Fixed tissue samples were processed routinely, embedded in paraffin, and sectioned at a thickness of 4-5 µm using a manual microtome. Then they were stained with haematoxylin and eosin (H&E) and special staining (Masson's trichrome) as per the standard staining technique (Suvarna *et al*., 2019).

Results and discussion

Of the 5 dogs, three were non-descript, one was Labrador Retriever and one was Dachshund. Furthermore, all the dogs were male with a mean age of 9.8±0.48 years. Clinical history of dogs diagnosed with hepatoid tumour was loss of appetite, polyuria, difficulty in defecation, hair loss, severe itching, haemorrhages and ulcers on the perianal region.

Grossly, hepatoid gland tumours were solitary or multiple irregular shaped lobular structure and 1 to 5 cm diameter located around the anus and in the paraprepucial region. The masses were ulcerated. Cross section of tumour displayed grayish white coloured multilobular structure with haemorrhages and necrosis.

Histologically, two cases were diagnosed as hepatoid gland adenoma, one case was hepatoid gland epithelioma and two were hepatoid gland carcinoma. Hepatoid gland adenoma was characterised by proliferation of hepatocyte like hepatoid gland epithelial cells that were arranged in cords and anastomosing trabeculae with extensive sebaceous differentiation (Fig. 1). The cells were polyhedral in shape with centrally placed, large ovoid, normochromatic and vesicular nuclei. The cytoplasm was eosinophilic with distinct borders. The periphery of the islands had a layer of basaloid reserve cells with a small hyperchromatic nucleus and narrow cytoplasm.

In case of hepatoid gland epithelioma, many of the cells were basaloid reserve cells and fewer hepatocyte like cells with indistinct lobules (Fig. 3). Hepatoid cells were characterised by vacuolated cytoplasm and were organised in nests with a thick basaloid reserve cell layer. The reserve cells showed marked mitotic figures.

Hepatoid gland carcinomas were composed of marked pleomorphic cells with abundant eosinophilic and vacuolated cytoplasm and large nuclei with prominent nucleoli (Fig. 5). Multinucleated giant cells were also observed and some of the neoplastic cells had undergone squamous metaplasia. Mitotic figures were also evident in the carcinoma. The connective tissue stroma dividing the tumour growth into lobules appeared as blue in Masson's trichrome staining (Fig. 2,4 and 6). The special staining helped in differentiating the lobules of neoplastic cells from the surrounding stroma. It also helped in identifying even small nests of invading neoplastic cells, into the stroma a feature seen especially in the epithelioma and carcinoma, compared to adenoma.

All the dogs which were diagnosed with hepatoid gland tumours in the current study were uncastrated males. Pisani *et al*. (2006) reported the higher immunohistochemical expression of androgen receptors in hepatoid gland tumours. Previous research on hepatoid gland tumours has observed that these tumours are mostly reported in Terriers followed by Mongrels. In the present study, out of five cases, three dogs were non-descript, one was Labrador Retreiver and one was Dachshund. This difference in incidence for dog breeds in the current study with that in published literature may be due to the fact that Terriers are not common in Kerala.

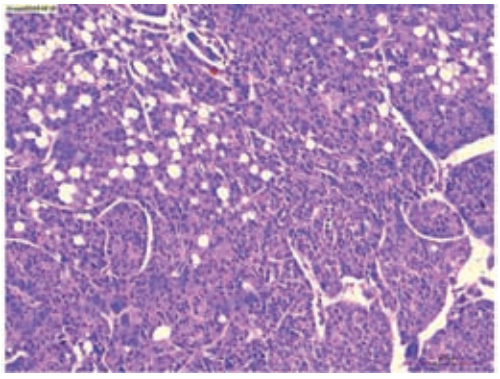

Fig. 1. Hepatoid gland adenoma- Proliferation of hepatocyte like hepatoid cells with extensive sebaceous differentiation (H &E x 100)

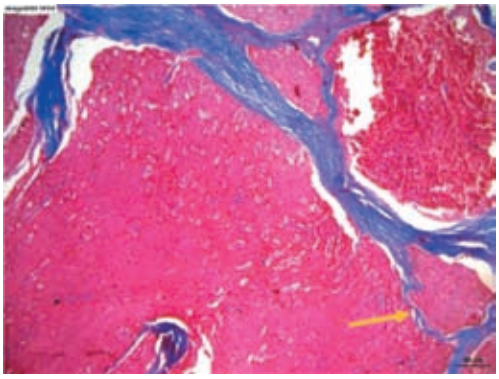

Fig. 4. Hepatoid gland epithelioma- invading neoplastic cells (arrow) into the blue stained connective tissue stroma (Masson's trichrome x 100)

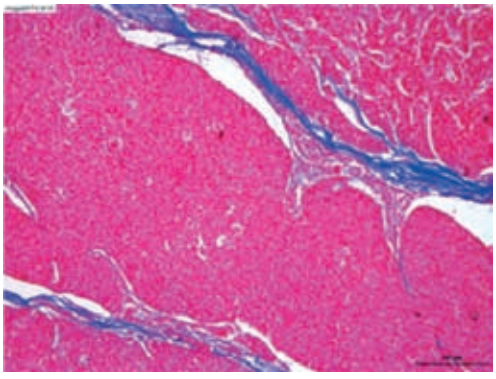

Fig.2. Hepatoid gland adenoma- The connective tissue stroma dividing the tumour growth into lobules appeared as blue (Masson's trichrome x 200) in Masson's trichrome staining

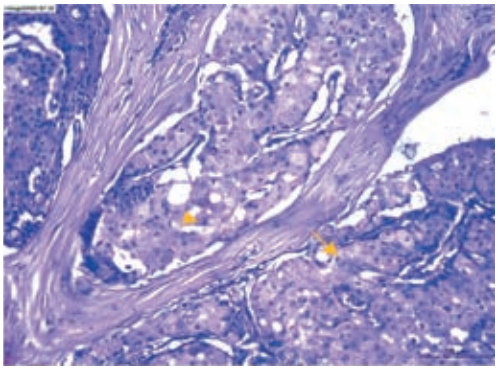

Fig. 5. Hepatoid gland carcinoma- pleomorphic cells with abundant eosinophilic and vacuolated cytoplasm and large nuclei with prominent nucleoli. Multinucleated cell(arrow) and mitotic figure (arrow head) (H&E x 200)

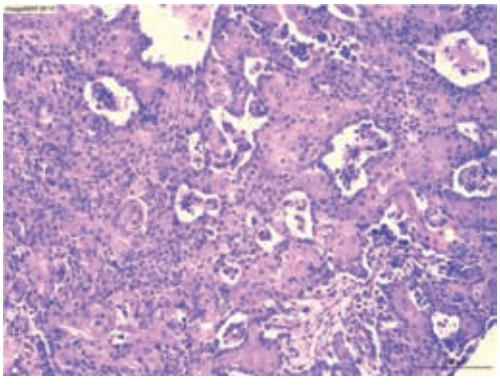

Fig. 3. Hepatoid gland epithelioma- many of cells were basaloid reserve cells and fewer hepatocytes with indistinct lobules (H&E x 200)

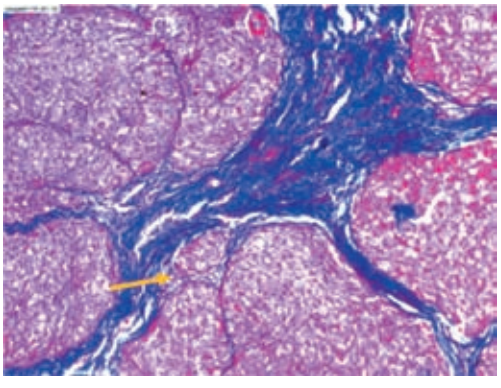

Fig. 6. Hepatoid gland carcinoma- invading neoplastic cells (arrow) into the blue stained connective tissue stroma (Masson's trichrome x 100)

Clinically, the animals affected with hepatoid tumours of diameter larger than 2 cm were showed clinical symptoms like polyuria and difficulty in defecation (Yumusak *et al*., 2016). In the histological examination of hepatoid adenoma, the tumour islets were surrounded by basaloid cells. Hepatoid gland epithelioma had low grade malignancy and characterized by the presence of mainly basaloid cells with few hepatoid cells. These findings were also reported by Goldschmidt and Goldschmidt (2017). Hepatoid gland carcinomas were well differentiated which had a morphology and histological architecture similar to those of hepatoid adenomas in the current study. But, hepatoid cells with vesicular nucleus, hyperchromatic nucleolus and numerous vacoulated cytoplasm are evident in carcinomas. These findings were in agreement with Gross *et al*. (2006) and Yumusak *et al*. (2016).

Conclusion

Metastasis is usually very rare in hepatoid gland tumours and their surgical excision produces good results. No metastasis and post operative complications were observed in the cases documented in the current study. Symptoms disappeared following the excision of tumour mass. Grossly all hepatoid tumours had almost same features and histopathological study helped to diagnose different types of hepatoid gland tumours. The study thus points out the importance of histology and special staining in differentiating the benign and malignant variants and identifying their biological properties important in disease progression.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Haemato-biochemical studies in medically managed open and closed-cervix pyometra in dogs*

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Abstract

Six dogs each with open and closed-cervix pyometra (Group I and II) were medically managed with mifepristone @ 2.5mg/kg body weight orally twice daily for five days, followed by cloprostenol @ 5µg/kg body weight subcutaneously on every alternate day after ensuring cervical patency, till complete evacuation of uterus was assessed by ultrasonography. Haematological and serum biochemical values were estimated on the day of presentation. The treatment response was assessed by reviewing both haematological and serum biochemical values further on days 3, 7, 14 and 21 of treatment. The mean total erythrocyte count (TEC), haemoglobin concentration and volume of packed red cells (VPRC) were significantly lower and mean total leucocyte count (TLC) was significantly higher in all animals of both groups during the day of presentation. The values significantly improved after initiation of treatment. Total thrombocyte count, serum total protein, albumin, BUN and serum creatinine levels were within the normal range. TEC, TLC, Hb concentration, VPRC could be used for prognostic markers of treatment evaluation in canine pyometra.

Keywords: *Pyometra, haemato-biochemistry, medical management*

Cystic endometrial hyperplasia-pyometra (CEH-P) is one of the life threatening uterine pathological conditions affecting mostly the aged, intact female dogs, characterised by accumulation of purulent material inside the uterine lumen. Under the influence of progesterone there will be enhanced endometrial glandular proliferation and increased glandular secretion. This will favour the multiplication of commensal bacteria to many folds and result in pyometra. Based on the

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cervical patency pyometra can be either open-cervix pyometra or closed-cervix pyometra. A stable pyometra at any time can be changed to an unstable one, hence the development of better prognostic markers are necessary. The markers which are considered regularly include clinical and haemato-biochemical parameters like anaemia, leucocyte count, blood urea nitrogen, serum creatinine, C-reactive protein (CRP), serum lactate, serum amyloid-A, serum endotoxin levels and prostaglandin $F_{2\alpha}$ metabolite (PGFM). Thus the laboratorial characterisation of haemato-biochemical parameters are of utmost importance in canine pyometra, as it provides valuable information regarding the clinical status of the animal during the day of presentation and after the initiation of medical treatment. Regular haemato-biochemical monitoring in medically treated pyometra affected dogs will result in much favourable outcome.

Materials and methods

Dogs suffering from pyometra, presented to University Veterinary Hospital, Mannuthy and Kokkalai during the period from February 2019 to January 2020 were utilised for the study. These dogs were subjected to detailed clinico-gynaecological, ultrasonographic and laboratory evaluations for confirmation of the condition. Six dogs exhibiting sanguineous to mucopurulent vaginal discharge of varying consistency, colour, odour and with evidence of definite uterine sacculations on ultrasonography, were selected as animals with open-cervix pyometra (Group I) whereas six dogs which were not exhibiting any vaginal discharge, presented with other symptoms suggestive of pyometra and later confirmed with evidence of definite uterine sacculations on ultrasonography, were selected as animals with closed-cervix pyometra (Group II).

All the animals were administered with mifepristone @ 2.5mg/kg body weight orally twice daily for five days, followed by cloprostenol sodium @ 5µg/kg body weight subcutaneously on every alternate day after ensuring cervical patency, till complete evacuation of uterus was assessed by ultrasonography. Adjunct antibiotics and fluid therapy were also given according to the condition of the animals.

A volume of 2 mL of peripheral blood sample was collected from all the dogs on the day of presentation (day 0) followed by days 3, 7, 14 and 21 of treatment. Blood samples were processed within 3-4 hours of collection and haematological parameters like total erythrocyte count (TEC), total leucocyte count (TLC), thrombocyte count (TC), haemoglobin concentration (Hb) and volume of packed red cells (VPRC) were assessed using automatic analyser (Mythic 18 Vet, Woodley, Switzerland). A volume of 5 mL of peripheral blood samples was collected from all the animals on days 0, 3, 7, 14 and 21 into vacutainers with clot activator. Sera samples were separated, centrifuged, aliquotted and was immediately used for analysis of serum total protein (TP), albumin, blood urea nitrogen (BUN) and creatinine in a semiautomatic analyser (Erba Manheim, Chem-5 Plus V2, USA).

The data obtained were tabulated and analysed statistically (Snedecor and Cochran, 1994) using repeated measures ANOVA and SPSS version 21.

Results and discussion

Haematological parameters in Group I and II dogs are depicted in Table 1.

Mean total erythrocyte count in both the groups up to day 14 was less than the normal physiological value. There was a significant difference ($p<0.05$) in mean erythrocyte count on day 21 in both the groups. The erythrocyte count came to normalcy by day 21 in both the groups. There was no significant difference ($p>0.05$) in mean erythrocyte count between the groups till day 14, but Group II exhibited an increase in the mean erythrocyte count on day 21. Similarly lower values of TEC (4.58±0.04 to 4.99±0.01 $\times 10^6$ /mm^3) were reported by Singh *et al.* (2006) among pyometra cases with acute inflammatory changes. Samantha *et al*. (2018) and Nath *et al.* (2009) suggested that in canine pyometra, the reason behind reduced TEC as reduced erythropoiesis due to the toxic suppression of bone marrow and loss of RBCs into the uterine lumen.

Table 1. Haematological parameters on different days of observation in dogs affected with open and closed-cervix pyometra

Parameter	Gp	Days of observation				
		Day 0	Day 3	Day 7	Day 14	Day 21
TEC ($\times10^6$/mm^3)	I	$3.97^{a,x}$±0.46	$4.05^{a,x}$±0.42	$4.33^{a,x}$±0.42	$5.22^{a,x}$±0.40	$6.30^{b,x}$±0.27
	II	$4.80^{a,x}$±0.23	$4.56^{a,x}$±0.32	$4.38^{a,x}$±0.40	$5.24^{a,x}$±0.43	$7.49^{b,y}$±0.40
TLC ($\times10^3$/mm^3)	I	30.20^{a} ±4.71	25.49^{ac} ±3.42	17.03^{bc} ±2.46	14.10^{b} ±2.46	10.60^{b} ±1.74
	II	40.75^{a} ±5.43	32.90^{a} ±3.24	22.53^{bc} ±3.16	17.76^{bc} ±2.05	9.15^{bd} ±0.90
Hb (g/dL)	I	9.07^{a} ±1.40	9.00^{a}±1.34	10.05^{a}±1.15	11.39^{b}±1.10	13.51^{b}±0.61
	II	10.35^{a} ±0.71	9.48^{a} ±0.72	9.75^{a} ±0.76	11.45^{ac} ±0.51	12.83^{bc} ±0.54
VPRC (%)	I	24.60^{a}±3.50	26.04^{a}±2.68	32.31^{ac} ±3.77	34.25^{ac} ±3.23	40.30^{bc} ±1.57
	II	25.75^{a}±3.31	26.90^{a}±2.30	28.22^{a} ±2.09	33.92^{bc} ±0.75	40.22^{bd}±0.87
TC ($\times10^3$/mm^3)	I	162.17^{a}±26.00	194.17^{ac}±54.97	255.52^{ad}±48.43	335.19^{bcd}±49.82	396.36^{b}±31.60
	II	169.50^{a}±31.70	201.17^{a} ±26.06	249.33^{ac}±27.66	339.17^{bc} ±41.08	364.50^{b}±37.43

abcd Different superscripts in a row differ significantly (p<0.05)
xy Different superscripts in a column between groups differ significantly (p<0.05)

Mean total leucocyte count on the day of presentation was higher than the normal range in both the groups. There was a significant reduction (p<0.05) in total leucocyte count from day 7 of observation in both the groups. A highly significant difference (p<0.01) in mean total leucocyte count could be observed on day 0 and day 21 in both the groups. The results were in accordance with Vidya *et al.* (2020) where the mean TLC ($\times10^3$/mm^3) of pyometra affected dogs were 36.46 ± 4.51, 32.06 ± 4.66, 20.93 ± 2.27 and 17.33 ± 1.45, respectively on days 0, 3, 7 and 14. Fransson *et al.* (1997) and Verstegen *et al*. (2008) reported leucocytosis in pyometra with neutrophilic leucocytosis and shift to left due to severe bacterial infection with resultant release of immature neutrophils from bone marrow to the circulation.

Mean haemoglobin concentration was less than the normal physiological range on all the days except day 21 in both the groups. There was a significant increase (p<0.05) in haemoglobin concentration from day 14 in Group I as well as Group II. There was a significant increase (p<0.05) in the haemoglobin level from day of presentation and day 21 in both the groups. There was no significant (p>0.05) difference in haemoglobin concentration between the treatment groups. Almost similar results were obtained for Yu (2012), in a study conducted in open-cervix and closed-cervix pyometra groups and recorded as 11.73±0.70 and 11.80 ± 0.68 g/dL, respectively. The reason behind the reduced haemoglobin concentration in pyometra affected animals was the iron sequestration inside the myeloid cells in bone marrow which was mediated by lactoferrin and other acute phase proteins (Nelson and Couto, 1992).

The VPRC was subnormal in both the groups except for the last day of observation. In Group I there was no significant difference (p>0.05) in mean VPRC between days 0, 3, 7 and 14, but a significant difference (p<0.05) existed between day 21 and other days of observation. In Group II there was no significant (p>0.05) difference in mean VPRC between days 0, 3 and 7 but a significant difference occurred from day 14 of observation. A highly significant increase (p<0.01) in mean VPRC existed in day 21 in Group II. There was no significant difference (p>0.05) in mean VPRC between days of observation between groups. Similar values were obtained for Vidya (2019), in which the mean per cent of VPRC of pyometra affected dogs were 25.43 ± 1.66, 23.96 ± 1.86, 23.90 ± 1.73 and 25.54 ± 1.64, respectively on days 0, 3, 7 and 14. According to Unnikrishnan *et al.* (2020) low VPRC in the study of pyometra

affected animals was explained as a reflection of reduced TEC, caused by bone marrow suppression by toxins and resultant reduction in erythropoiesis.

Mean thrombocyte count on all the days of observation in both Group I and Group II were within the normal range. A gradual increase in the mean thrombocyte count could be observed in both the groups from the initiation of treatment. A significant increase ($p<0.05$) in mean thrombocyte count could be observed from day 14 in both the groups. There was no significant difference ($p>0.05$) between days of observation between groups could be observed. Similar observations were recorded in the literature by various researchers including Shah *et al.* (2017). According to the author, improvement in thrombocyte count could be considered as a better prognostic factor.

Serum biochemical parameters in Group I and II dogs are depicted in Table 2.

Mean serum total protein levels were observed to be within the normal physiological limit in all the days of observation in both the groups. There was no significant difference ($p>0.05$) in total serum protein concentration in any of the days of observation in Group I. Even though within the normal range, a significant reduction ($p<0.05$) in protein level existed in day 21 in Group I and Group II. There was no significant difference ($p>0.05$) in mean serum protein level between the days of observation between the groups, but the total protein concentration was higher in Group II than in Group I in all the days of observation. In a similar study conducted by Hardy and Osborn (1974) 82.30 per cent of pyometra affected animals showed normal serum protein levels on all the days of observation.

Mean serum albumin concentration in all the days of observation in both the groups were within the normal range. There was no significant difference ($p>0.05$) in mean serum concentration existed in both the groups and between the groups in any day of observation. This finding was in accordance with Lakshmikanth (2016).

Mean serum BUN concentration was within the normal range in both the groups in all the days of observation. There was no significant difference ($p>0.05$) in mean BUN concentration in any of the days of observation in Group I. In Group II there was a significant reduction ($p<0.05$) in mean serum BUN concentration in day 14. There was no significant difference ($p>0.05$) between the groups in the level of mean serum BUN concentration on various days of observation. The results were in accordance with Lakshmikanth (2016). Mean serum creatinine levels in both the groups were within the normal range in all the days of observation. There was no significant ($p>0.05$) difference in levels of serum creatinine in any day of observation within the groups and between the groups. The result was in accordance with Vidya *et al.* (2020) and Unnikrishnan *et al.* (2020).

Table 2. Serum biochemical parameters on different days of observation in dogs affected with open and closed-cervix pyometra

Parameter	Gp.	Days of observation				
		Day 0	Day 3	Day 7	Day 14	Day 21
TP (g/dL)	I	7.36 ± 0.43^{a}	6.99 ± 0.58^{a}	6.85 ± 0.66^{ac}	6.38 ± 0.64^{ac}	5.39 ± 0.25^{bc}
	II	8.10 ± 1.02^{a}	7.43 ± 0.84^{a}	8.13 ± 0.66^{a}	6.99 ± 0.50^{a}	6.73 ± 0.44^{a}
Alb (mg/dL)	I	$2.60^{a}\pm0.33$	$2.40^{a}\pm0.36$	$2.52^{a}\pm0.30$	$2.72^{a}\pm0.34$	$2.32^{a}\pm0.23$
	II	$2.22^{a}\pm0.27$	$2.21^{a}\pm0.28$	$2.35^{a}\pm0.21$	$2.43^{a}\pm0.30$	$2.65^{a}\pm0.33$
BUN (mg/dL)	I	$25.71^{a}\pm7.09$	$18.24^{a}\pm4.35$	$18.29^{a}\pm3.29$	$13.15^{a}\pm2.27$	$13.29^{a}\pm3.09$
	II	$17.56^{a}\pm2.72$	$13.98^{a}\pm1.28$	$15.42^{ac}\pm3.02$	$11.24^{ac}\pm0.92$	$10.09^{bc}\pm1.01$
Creatinine (mg/dL)	I	$1.69^{a}\pm0.36$	$1.59^{a}\pm0.35$	$1.45^{a}\pm0.27$	$1.24^{a}\pm0.18$	$1.04^{a}\pm0.14$
	II	$1.18^{a}\pm0.14$	$1.20^{a}\pm0.16$	$1.16^{a}\pm0.13$	$1.14^{a}\pm0.11$	$1.09^{a}\pm0.12$

abc Different superscripts in a row differ significantly ($p<0.05$)

Results of normal serum biochemical values indicate unaffected or mildly affected hepatic and renal functions Vidya *et al.* (2020).

Conclusion

The study showed that TEC, TLC, Hb concentration, VPRC and thrombocyte count could be used as a prognostic tool for evaluating the response to medical management of pyometra in dogs affected with open and closed cervix pyometra.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Therapeutic study of *Tephrosia purpurea* (linn.) root extract on goat tapeworm with special reference to histopathological parameters

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Abstract

Goat farming forms the backbone of the Indian livestock sector. The prevalence of helminth infection is one of the major problems which affects the milk and meat production of small ruminants. It causes diarrhea, anaemia, oedema, bottle jaw and reduce reproductive performance of animals. Anthelmintic resistance and drug residues in animal products like milk and meat are the results of indiscriminate use of anthelminthics by goat owners for helminth control. Herbal anthelminthics are effective alternatives without any harmful effects. The paper presents the anthelminthic potential of the root extract of Tephrosia purpurea (linn.) plant on common tapeworm of goat, Moniezia. The in vitro study showed that the anticestodal activity of T. purpurea (linn.) at 125mg/ml dilution was comparable to that of albendazole. The histopathological study revealed very prominent shrinkage of scolex and suckers, tegument showed puff formation and was ruptured at the marginal position with irregular folds and crumbling of the segments. Longitudinal section of segment showed more marginal serrations indicating crumbling of the body segment as compared to albendazole, leading to the death of the worm.

Keywords: *Anticestodal activity, in vitro study, histopathology, Moniezia, T. purpurea*

Goat farming is one of the well-established livestock industry in India and different regions of world, and plays an important role in the economy of farmers. Goat products mainly meat and dairy have high consumer demand on account of specific taste, flavour, aroma, leanness and nutritive composition of fats, proteins and fatty acids. This meat is traditionally consumed all over the world (Boyazoglu *et al.*, 2001). Goat products, such as milk and meat have great value in markets (Loewenstein, 1980; Webb, 2014). Goat farming is advantageous to the marginal and landless

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farmers as this vocation does not require costly infra- structure and trained persons (Ghaywat *et al*., 2017). Gastrointestinal parasitic infection is commonly encountered in goat farming across the globe. The prevalence is 59.6 per cent in South Africa (Takalani *et al*., 2020), 75 per cent in Nigeria (Eke *et al*., 2019), 87.25 per cent in Nepal (Ghimire *et al*., 2019) and 96.22 per cent in the Philippines (Rupa *et al*., 2016). In India, the prevalence was around 68.75 percent in Mathura **(**Singh *et al*., 2013), 85.22 per cent in Chhattisgarh (Pathak *et al*., 2008), 92.4 per cent in Shillong and Meghalaya (Bandyopadhyay *et al*., 2010) and 62.75 per cent in Ahmednagar district (Sutar *et al*., 2010).

Gastrointestinal infection is associated with reduced milk and meat production and affects the health of goats by causing diarrhea, anaemia, bottle jaw, oedema and reduced productivity (Sutar, 2010). Infection with the cestode, *Moniezia expansa,* leads to inappetence, diarrhoea and chronic loss of weight (Yadav *et al*., 2019). To control the gastrointestinal parasite goat owners frequently resort to chemical dewormers such as albendazole, a broad spectrum anthelmintic of benzimidazole class. Albendazole causes degenerative alteration in the tegument and endoplasmic reticulum, while in the mitochondria it affects the production of ATP ultimately leading to immobilization and death of the parasite (Albendazole Drug bank -https://go.drugbank.com/drugs/DB00518). Mutations in the target site genes lead to drug resistance and hence alternative control strategies have to be adopted. Some plant bio active compounds are effective against this parasite besides being safe and eco- friendly (Gives *et al.,* 2012). Tribal people use many herbal medicines for themselves and their domestic animals (Iqbal *et al.,* 2003). *Tephrosia purpurea* commonly called *Sarpunkha* or *Unhali* in Marathi is used to cure many diseases of human beings. It is also used in animals to control gastrointestinal parasites (Baranwal *et al.,* 2014). It is a tropical leguminous shrub that grows to a height of 40-80 cm. The plant has pinnate leaves. Flowers are pink or purple in colour, root is a long stout taproot having slender branches. It has a characteristic odor, is yellow to brownish in colour and has an intricate bitter taste. Phytochemicals present in roots are tannins, tephrosin, isotephrosin, phytosterols, purpurin, deguelin, rotenone, glycosides isolonchocarpin **(**Baranwal *et al.,* 2014). In these circumstances, an *invitro* study was done to assess the anticestodal efficacy of the root extract of *Tephrosia purpurea,* the medicinal plant commonly used by the tribal people in Akole and Sangamner talukas of Ahmednagar District of Maharashtra State.

Materials and methods

Collection of plant materials

As per the reference of local goat keepers of Akole Taluka, the plant material was collected from the study area during the month of November. The plant was identified and authenticated by a taxonomist from Botany department of Sangamner College. The roots were collected by uprooting the plants. The aerial part of the root was discarded. Roots were brought to the laboratory, washed with distilled water, cut into small pieces and shade dried. Later it was dried in the oven at 37^{0}C for two days and powdered by grinding in a mixer.

Preparation of plant extract

Tephrosia purpurea powder (50 gm) was used and methanolic extract of the plant root powder was prepared by using Soxhlet apparatus. The extract was then concentrated in Rota evaporator at 40^{0}C in reduced pressure, air dried and extract was used for further treatment.

Collection of parasites

The intestines of slaughtered goats were collected from the local abattoirs in and around Sangamner, in the plastic container and immediately brought to the laboratory. The intestine was further dissected in PBS solution and tapeworms were collected from the lumen of the small intestine. The collected worms were thoroughly washed in PBS (Phosphate buffer solution) and then transferred to a petri plate containing Hank's solution along with two drop of 1 per cent DMSO added as a carrier agent. Samples were identified based on morphologic parameters.

In vitro anticestodal activity evaluation

The worms were collected from the intestine and identified as *Moniezia*. The anticestodal test was performed in the Petri plates of equal volume. Four concentration of the plant extract were used. Five worms were released in each Petri plate in each set of concentration, in triplicate, during experimental setup.

The experimental set up included three groups: Group I with four sets of concentrations (50, 75, 100, 125 mg/ml of Hank's solution) of *Tephrosia purpurea* root methanolic extract; Group II with 10 mg/ml Albendazole as a positive control; Group III with Hank's solution as a negative control. In each case the extract was dissolved in few drops of one per cent DMSO solution as a carrier. The motility of worms was observed frequently at an interval of half an hour till the death of the parasite and the time of paralysis and death was noted. The death was again confirmed by testing the motility of the worm in lukewarm solution for 30 min. Histopathological study was done by observing the effect of the extract on the scolex and integument of normal and treated worms and the results were compared.

Data analysis

Data on paralysis and death was noted and analysed by using SPSS, 2015 version. The data obtained from negative control, positive control and treatment groups were analysed with one – way ANOVA test. Results were considered significant if p value was less than 0.05 at 95 per cent confidence intervals.

Results and discussion

In vitro anticestodal activity

The *invitro* assay showed that the methanolic extract of *T. purpurea* produced anticestodal activity comparable with that of the synthetic drug Albendazole (Albendazole oral suspension containing 25 mg/ml Albendazole IP recommended for sheep, goat and poultry). The effective concentration of albendazole was 10mg/ml demonstrating paralysis and death at minimum period of 1.43 ± 0.14h and 2.3 ± 0.27h, respectively. Similarly, the highest concentration of methanolic extract (125mg/ml) was found to be most effective showing minimum time of 1.29±0.17h and 2.63±0.36h for paralysis and death, respectively. The result revealed that the methanolic extract of *T. purpurea* , at 125mg/ml, showed effective anticestodal activity comparable to albendazole (Table 1). Further the histopathological study was done to see the effect of extract on the various parts of the worm

Histopathological evaluation

Histopathological study of the whole mount and longitudinal section of normal and treated worms were done. The effect of albendazole and herbal extract was observed under microscope. The comparative study

Table 1. Mean mortality time of the control and treated *Moniezia*

Treatment	Mean mortality time of worm (Mean ± SD)		
		Paralysis	Death
Hanks solution + 2 drops of DMSO		17.7± 0.74	26.1± 2.92
Albendazole	1 mg/ml	7.8± 0.51	10.2± 0.57
	5mg/ml	4.8± 0.51	7.7± 0.27
	10mg/ml	1.43± 014	2.3± 0.27
Methanol Extract	10mg/ml	10.5± 0.31	13.5± 1.74
	50mg/ml	7.5± 0.31	11.1± 0.22
	75mg/ml	5.5± 0.8	10.6± 0.65
	100mg/ml	7.9± 1.93	7.9± 0.41
	125mg/ml	1.29± 0.17	2.63± 0.36

*Data represent mean ± SD in hours with 3 experiments

of the effect on normal, albendazole treated and *T. purpurea* root extract treated worms is shown in the Fig. I .When normal PBS solution was used, the scolex with four suckers were seen well in position and structure, tegument did not show any morphological changes at the margin and body surface. In the case of the Albendazole treated group, folds on scolex with shrinkage of the suckers were seen and the tegument showed slight shrinkage while the histological section (L.S) revealed shrinkage of the body segment with serrations on the margins (Fig 2.1 to 2.3). In the case of the group treated with the methanolic extract of *Tephrosia purpurea,* very prominent shrinkage of scolex and suckers, with puff formation of tegument, rupture at the marginal position on body surface, irregular folds and crumbling of the segment were observed. Histological section (L.S) showed more marginal serrations indicating the crumbling of the body segment which was comparable with the changes seen

Normal in Hanks solution	Albendazole10mg/ml	Root extract treatment 125mg/ml

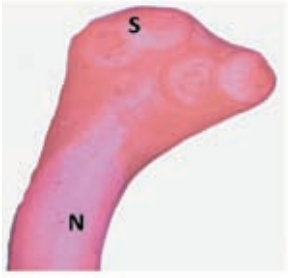
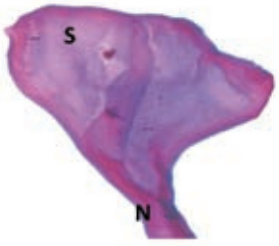
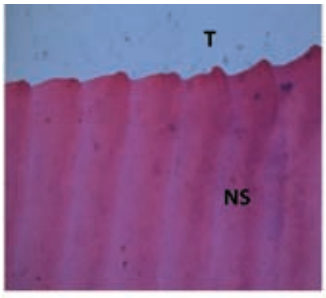
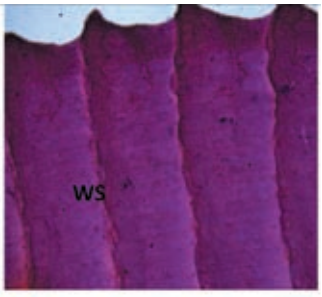
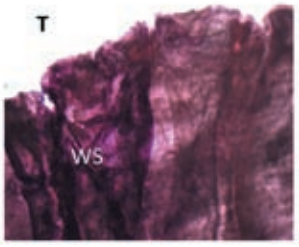

	

Fig -1.1	**Fig -2.1**	**Fig -3.1**
		
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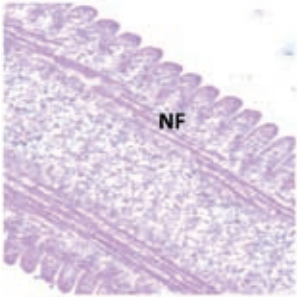	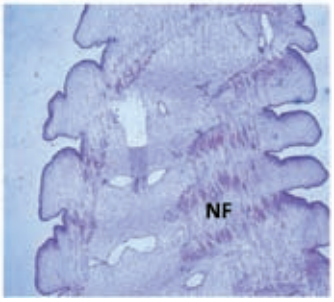	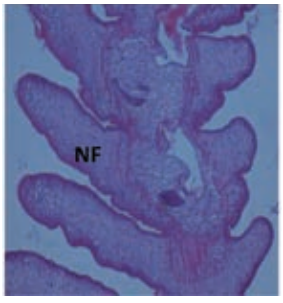
Fig -1.3	**Fig -2.3**	**Fig -3.3**

Fig. 1 – 3. Comparative histopathological features of Moniezia – Scolex and Mature

S-sucker T- tegument W-Wrinkles NS- Normal Segment WS-Wrinkled Segment

P - Parenchyma NF-Nerve Fiber N – Neck

in the group that was exposed to albendazole (Fig. 3.1 to 3.3).

Tapeworm infection is one of the most common problem encountered with respect to the sheep, goat and cattle, affecting the production of milk and meat. Herbal anthelminthics are attractive alternatives in this respect. A medicinal plant, *T. purpurea*, used by tribal communities has been reported to possess antimicrobial, anti-diabetic, antileishmanial and antibacterial activities (Sharma *et al.,* 2013). In this study, the results with the root extract of *T. purpurea* showed effective anticestodal activity. Similar results against earthworm and tapeworm were also seen on using leaf extract of *T. purpurea* by Patel *et al.* (2011). These results are also in agreement with the findings of Manjula *et al.* (2013), who reported the efficacy of leaf extracts against earthworms. The histopathological study was conducted to see the effect of treatment on the scolex and mature segments. It showed that the plant extract caused higher level of destruction of the integument, parenchyma tissue that led to a crumbling of the scolex and segment which may lead to the death of the worm. Phytochemical screening of the root extract, showed that there are some secondary metabolites like phytosterols, glycosides, flavonoids, tannins, phenol etc. (Baranwal *et al.,* 2014), of which . tannin acts as an anthelminthic agent. Further *in vivo* trials are needed to explore the use of these drugs on commercial scale for cestode control.

Conclusion

Medicinal plants are natural sources of metabolites that have antibacterial, antiviral, anti-diuretic properties and can be used as herbal medicines. The use of plants like *T. purpurea* as an anthelmintic drug can be a good alternative for the chemical drugs. The study on root extract of *T. purpurea* to see its *invitro* anticestodal activity would serve as a preliminary work to derive the herbal drug for future use.

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Conflict of interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE
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Life cycle and development rate of *Hemipyrellia ligurriens* (Wiedemann) (Diptera: Calliphoridae) during monsoon season in South India: applications in estimation of postmortem interval

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Abstract

Hemipyrellia ligurriens, considered as one of the forensically important blow fly species, has a wide distribution in many countries including India. To conduct forensic entomological investigations involving deaths of livestock, human beings and wild animals, standard life cycle data should be prepared for the local blow fly species under various weather conditions. Reliable forensic entomological data specific to geographic locations in India are not available presently to assist the post mortem interval assessment. In this study, life cycle and the rate of development of H.ligurriens was determined during monsoon season in Kerala, South India. Survival rate observed from egg to adult emergence was 44.68 %. Total duration of development of the species from oviposition till adult emergence was 462.57 h. Growth curves based on the age, specific length parameter and time taken for development of each larval stage was constructed. This development model would be helpful for the medical, veterinary and law enforcement officials in forensic estimation of post mortem interval by analyzing the length parameters of larvae collected from decomposed dead bodies of humans, cadavers of wild animals and livestock.

Keywords: *Hemipyrellia ligurriens, development stages, postmortem interval, veterinary forensics*

The *Hemipyrellia* genus is represented by four species in the Oriental region. In India, it is represented by two species; *H.pulchra* and *H.ligurriens* (Senior-White *et al*.,1940;Nandi, 2004; Bharti,2011), the latter being reported on decomposing human cadavers in Malaysia (Rajagopal, 2013), Thailand (Moophayak *et al*., 2014)and other regions (Chen *et al*., 2004;Lee *et al*., 2004; Sukontason, 2007) and has significant forensic importance. These speciesare widely distributed

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in Taiwan, Philippines, Singapore, Papua New Guinea, China, Korea, Laos, Sri Lanka, Indonesia, Thailand, Australia and India(Tumrasvin*et al*., 1979;Kurahashi and Chowanadisai, 2001; Kurahashi, 1979).Though studies pertaining to the development of *H.ligurriens* has been done (Sinha and Nandi,2007; Sukontason *et al*., 2008;Sukontason *et al*., 2010; Bunchu *et al*., 2012), association of different larval instars with time since death is yet to be extensively investigated. Estimates of postmortem interval based on the known characteristics of the infesting fauna in the natural conditions of the specific geographical location are very important (Yang *et al*., 2015).Construction of growth curves based on the age specific length parameters of larvae versus time taken for development of each stage would be helpful for the estimation of the post mortem interval. In this study, the developmental rate of *H.ligurriens* was recorded during monsoon season to develop a model for the accurate estimation of postmortem interval in Kerala, South India.

Materials and methods

Rearing of H.ligurriens

The adult flies of *H.ligurriens* were reared in the outdoor open system rearing facility for blow flies in Kolangattukara, Choolissery, Thrissur district, Kerala, India (10° 35′ 34.87″ N, 76° 11′ 22.6″ E). Adult females of *H.ligurriens* were trapped and isolated in the rearing cabinet with decomposing pork (*Sus scrofa*) meat as bait. The adult flies were identified using morphological keys provided by Senior-White *et al*. (1940) with LEICA-S8APO stereomicroscope. Molecular diagnosis of the species was done by sequencing of cytochrome oxidase Subunit I (*COI*) gene which was done at Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India.

The insects were reared in the rearing cabinets (Size: 2ft ×1ft ×1ft) positioned in the outdoor facility. Humidity, rainfall and temperature were monitored in the months of August and September, 2019. Temperature and humidity were monitor edusing hygrometer (HTC-1, Mode No:AP-IS11A056FBA, China). Rainfall data was collected from IMD-Meteorological Centre, Thiruvananthapuram, Kerala. The adult insects were provided with 10 per cent (w/v) sugar solution and 1.5 per cent (v/v) multivitamin syrup solution and water as food and liquid sources. The decomposing pork served as reflex stimuli for the adult female to lay eggs and also served as a food source for the larvae. Wet vermiculite was kept as the bottom layer in the cabinet to assist migration of third instars for pupation.Few of the blow flies trapped were killed and pinned as dry specimens for morphological identification and few were preserved in 70per centethanol for molecular identification.Five numbers each of eggs, three different larvalinstars and pupae were randomly collected every six hours for further studies.

Observations were done regularly on hourly basis to detect the presence of eggs. Once the eggs were found, the bait with the eggs were transferred in to the larval rearing plastic jars. Wet vermiculite was laid at the bottom of the jar to maintain adequate humidity. The jar was covered with a wet cotton cloth to prevent the entry of other insect parasitoids. Fifty grams of fresh pork meat was put in to the jar as larval feed. This was continued until the instars reached the non-feeding stage and started pupal migration. Fresh pupae were collected and transferred to a new rearing jar with moist vermiculite at the bottom and it was kept inside the rearing cabinet for the emergence of the adult fly. Egg, different larval instars (Fig.1) and pupae were collected for studying their morphology and length/width parameters.

Morphological examination of 3rd instar was done to study cephalopharyngeal skeleton and posterior spiracle using potassium hydroxide enabled clearing. The alcohol preserved larvae werecut at the middle of second thoracic segment and at 11thsegment using a sharp blade under LEICA-S8APO stereomicroscope to study the cephalopharyngeal skeleton and posterior spiracle by digesting the anterior and posterior portions as per the methods by Sukontason *et al*. (2004).The cut anterior and posterior portions were treated with 10 percent (w/v) of KOH solution in a watch glass for 48h. After rinsing the parts in four changes of distilled water, the specimen was treated in a mixture of 1per cent glacial acetic acid and 35

per cent ethyl alcohol in a watch glass for 30 min. Serial dehydration of the specimens was done using alcohol gradient(50%, 70%, 80% and 95% and absolute ethanol (99%) by placing the specimen in each for 30 minutes. The specimens were treated in xylene for 60 and mounted on a microscopic slide with a coverslip using DPX mountant. The cephalopharyngeal skeleton and posterior spiracles were observed and photographed using LEICA-S8APO stereomicroscope with camera attachment.

Life table study

Life table studies were conducted to assess the percentage survival and mortality by recording the survival rate of different development stages.

Assessment of developmental rate

The phase from the time of oviposition till the emergence of adult fly from the pupae was considered for the study of developmental rate. The time taken by the eggs for hatching was noted. The freshly hatched larvae were transferred to the new larval rearing chamber and 50 grams of fresh pork meat was provided as food. Ten larvae were collected every six hours and boiled for two minutes at 96-99°C and preserved in 70 % alcohol for the assessment of length and width. The time spent by the species in each life stage was recorded. Based on these observations growth curves were plotted. Effect of temperature, relative humidity and rainfall on larval development was also studied.

Results and discussion

The adults were identified as *H.ligurriens* based on the characteristics provided in standard literature Senior-White *et al*. (1940). The thorax had metallic green to copper colour, the parafrontalia, face and gena were silver grey, the third antennal segment dark brown and palpi had an orange colour. Numerous vertical hairs were present on the supra spiracular convexity. In males, frons was much narrowed. In females, the frons was about the same width as parafrontalia. Molecular identification of species done by sequencing *COI* gene and further analysis by NCBI BLAST confirmed the species identity as *H.ligurriens*. The sequence was submitted in GenBank, NCBI and assigned with accession no.(GenBank Accession No: MN831480).

The study was conducted during August and September, 2019 and the average temperature and relative humidity in the same period were 27.01 ± 1.15 °C and 88.16 ± 4.38 % respectively. The average rain fall recorded during these months were 20.33 ± 10.65 mm.Atotal of 94 eggs were reared in this study. Cephalopharyngeal skeleton of 3rd instar larvae was complete having characteristic features like hooks, parastomal bar, anterior dorsal process , dorsal cornua ,ventral cornua.The posterior spiracle was seen with two well-developed posterior spiracular discs each with a completely separated peritreme, medially oriented 3 slits and very characteristic medially placed button(Fig 2.)

In the life table study conducted, 94 eggs laid during the 24 hour period were considered. Percentage survival and mortality of each development stage were assessed and the details are given in Table. 1. Of the 94 eggs reared, 78 (82.97 %) hatched and reached the first instar stage of which 63(67.02 %) became second instar, 57(60.63 %) reached third instar stage of which 51(54.24 %) became pupae. Fourty two emerged from pupae as adult flies. Total survival rate for*H.ligurriens* during monsoon season was 44.68 %.

The total duration taken by *H.ligurriens* for the development from the egg stage (n=94) till the emergence of adult fly was 462.57 h. Growth curve of different stages of *H.ligurriens* showing the relation between developmental time and length was constructed (Fig.3). Pupation time till emergence of adult fly was 164.85 h (n=47). During larval development the species spent maximum time in third instar stage (215.28 ± 10.22 h). The larvae attained maximum length (7.67 mm) and maximum width (1.46 mm) also during the third instar stage. It was interesting to note that larval length and larval width almost doubled in the second instar stage. The development rate (in hours) and mean length and width for the larval stages of *H.ligurriens* are provided in Table 2.

Table 1. Survival rate of different life stages of *H. ligurriens* in Kerala, India

Sl. No	Stage	No.	Survival rate at each stage	Mortality rate at each stage
1	Egg	94	82.97 %	17.03 %
2	1st Instar	78	67.02 %	32.98 %
3	2nd Instar	63	60.63 %	39.37 %
4	3rd Instar	57	54.24 %	45.76 %
5	Pupa	51	44.68 %	55.32 %
6	Adult fly	42	_	_

Table 2. Length, width and duration of different life stages of *H. ligurriens* in natural conditions in Kerala, India

Stage	Length(Mean±SD)	Width(Mean±SD)	Life span of each stage(Mean±SD)
Egg	1.17 ± 0.03 mm	0.40 ± 0.07 mm	31.71 ± 2.87 h
1st Instar	2.65 ± 0.07 mm	0.52 ± 0.04 mm	18 ± 4.43 h
2nd Instar	5.27 ± 0.43 mm	0.98 ± 0.05 mm	32.71 ± 4.96 h
3rd Instar	7.67 ± 0.33 mm	1.46 ± 0.26 mm	215.28 ± 10.22 h
Pupa	6.1 ± 0.07 mm	2.09 ± 0.01 mm	164.85 ± 6.47 h

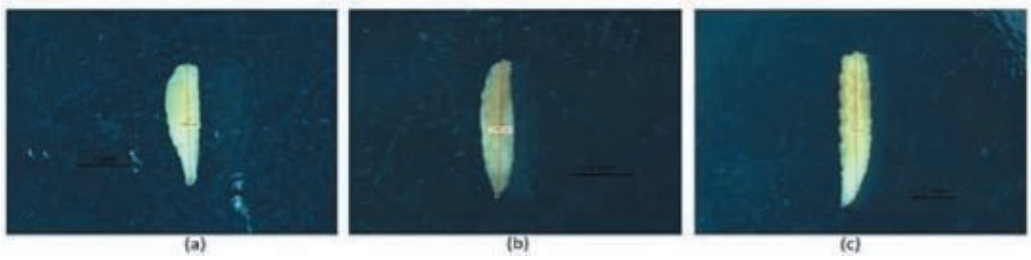

Fig.1. Different larval instars of *H. ligurriens (*a) First Instar (b) Second Instar (c) Third Instar

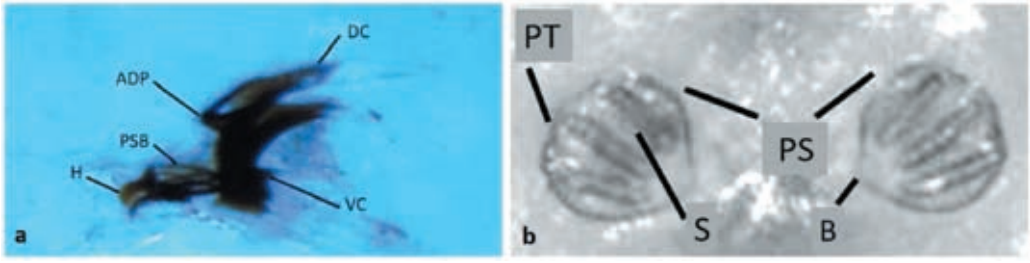

Fig 2.a. Cephalopharyngeal skeleton of 3rd Instar showing hook(H), parastomal bar(PSB), anterior dorsal process(ADP), dorsal cornua(DC),ventral cornua(VC)**b.**Posterior spiracles(PS)of 3rd Instar showing completely separated peritreme(PT), 3 diagonally placed slits(S),medially placed button(B)

The rate of development of *H.ligurriens* in this study (462.57 h) differed from the earlier reports from India (338.54 h) by Sinha and Nandi, (2007). The rate of development of the species in this study was slower by 191.17 h than that reported from Thailand by Bunchu *et.al*. (2012) and 181.27 h slower than that reported from China by Yang *et.al*. (2015).This might be due to the changes in the larval food(fish) and the influence of humidity, rainfall and temperature prevailing in these geographically different areas. The changes in the developmental rate of species also cautions that while performing the assessment of postmortem interval(PMI), the investigators should be very careful about the climatic conditions prevailing in the

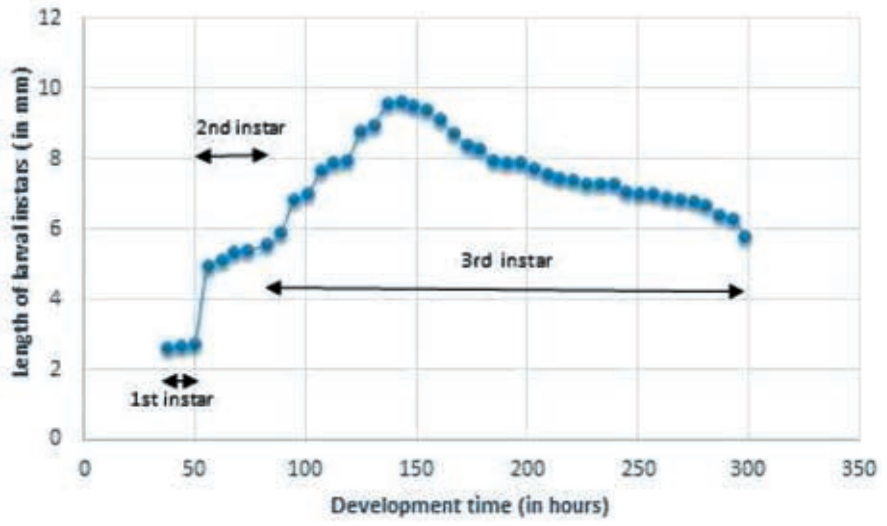

Fig.3 Relation between development time and larval length of *H.ligurriens* under natural conditions in Kerala, India

respective study area(Gallagher *et al*., 2010). This signifies the importance of generating location specific data of forensically important species for accurate assessment of PMI.

Conclusion

The current study on developmental rate of *H. ligurriens* would be useful in the forensic investigations involving decomposed dead bodies of humans, wild animals and livestock as this is the first report of this species from South India. Further studies are needed to find out the rate of development of *H. ligurriens* in other seasons so as to make a comprehensive database for this forensically significant blowfly species from Kerala, South India.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Variants in relation to clinical manifestation of rabies in dogs*

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Abstract

Rabies is one among the most dangerous diseases reported to have a wider range of incubation period and clinical signs. The disease is manifested as furious or paralytic forms in dogs. The combined effects of virulence and pathogenicity of the virus along with host immune factors are the main variables leading to variation in manifestation of the disease. This paper reports the finding of a study conducted in rabies positive canine cases to correlate the role of pathophysiological factors in clinical manifestation of rabies. A total of 23 rabies positive cases were studied and their anamnesis collected. Male dogs less than 5 years of age constituted major proportion of rabies positive cases. Site of bite was observed as a critical factor in disease manifestation. Anti-rabies prophylactic vaccination had significant effect as none of the vaccinated animals developed furious form of rabies. But paralytic form was found in irregularly vaccinated dogs, probably due to effect of vaccination imparting partial immune response in these animals.

Keywords: *Rabies, clinical manifestation, vaccination*

Nervous dysfunction is observed to be a serious condition in dogs which may have multiple etiologies. Rabies is one of the deadliest diseases affecting different species of animals often showing atypical clinical signs. The actual pathogenesis and the factors responsible for different clinical manifestations still remain vague. Different pathophysiological factors of host immune system along with the viral pathogenicity and virulence influence the manifestation of the disease. Single dog at its infectious period have been reported to transmit the disease, which was manifested in different forms in different animals (Hemachudha *et al.*, 2002). Wide variation in incubation period and duration of illness along with absence of any typical signs until death, impart the disease a dangerously vigorous version. Correlating different variants with the clinical manifestation of rabies therefore could help to delineate the role of the same in pathogenesis of disease. Direct fluorescent antibody technique (d-FAT) has been recommended by the WHO as the gold standard diagnostic method for rabies and is found to be highly specific and sensitive test.

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In the present study the animal owners were provided with the preformed questionnaire to collect the details on pre-exposure anti rabies vaccination, bite history, clinical signs, duration of incubation and duration of illness exhibited by the rabid animal. Brain samples from dog carcasses presented for post-mortem examination to Department of Veterinary Pathology during the period of 2019 January to 2020 February, with a clinical history of neurological abnormalities were screened for rabies using d-FAT. Twenty-three brain samples diagnosed rabies positive and six samples absent for rabies viral antigen (control group) were collected and considered for the study. Direct Fluorescent Antibody test was conducted using Fluorescein iso thiocyanate (FITC) conjugated rabies virus nucleocapsid monoclonal antibody (Merck, Germany) on impression smears from cerebrum, cerebellum, hippocampus and brain stem as per the standard protocol for FAT (Dean, 1996) and observed under fluorescent microscope (Zeiss, Progress C5).

The rabies positive cases were segregated as furious and paralytic based on the clinical signs reported. The signs exhibited in rabies were highly variable from individual to individual. Based on the diversity of signs exhibited in patient to patient, the disease was described as ' the abnormal becomes typical...' in this disease (Hanlon *et al*., 2007).

In both the forms, anorexia and weakness was a constant sign. When dropped jaw with salivation (64 per cent) and paraplegia/ quadriplegia (36 per cent) were the predominant signs in paralytic form, excited nature and biting without provocation with vocal changes were the noticeable changes in furious form of rabies (100%). In a comparative study of two forms of rabies conducted by Mitrabhakdi *et al*. (2005), where limbic signs dominated in the furious form, paralysis of lower motor neuron was prominent in the paralytic form of rabies. Hanlon *et al.* (2007) observed that facial asymmetry, rolling tongue, drooling and drooping of lower jaw and severe paralysis were the important notable signs reported in case of dumb/paralytic form of the disease without any signs of aggression. They also reported the remarkable signs of furious form such as hyperesthesia to auditory, visual, tactile stimuli and unprovoked agitation towards animate and inanimate objects in the affected animal. In all the cases of paralytic rabies, generalised weakness progressing from the limbs to the head and culminating in the respiratory distress to the animal was noted by the dog owners which were also observed by Lackay *et al.* (2008).

In general, there were three phases in the course of rabies infection, viz. prodromal phase, furious phase and paralytic phase. Lackay *et al*. (2008) suggested the possibility of overlapping of phases in rabies and occasional bypassing of the furious phase in some of the cases.

An equal proportion of two forms (48% paralytic cases & 52% furious cases) of rabies were encountered during the time period of sample collection inclusive of the stray animal population, otherwise in pet dogs the number of paralytic cases predominated. This finding was contradictory to the observation made by Hemachudha *et al*. (2013) in human rabies, where he found two third of cases to be of furious nature. Some of the variables were analysed and correlated with the variation in clinical manifestation of the disease.

The data regarding anamnesis and clinical history was collected from the dog owners using a preformed questionnaire. Out of the 23 rabies positive cases 61 per cent were male dogs and 39 per cent were female dogs. Widdowson *et al*. (2002) also reported that twice the number male dogs were prone to rabies than female dogs. The physiological behavioural changes in dogs during the breeding season may also be an important factor in this aspect.

Forty eight per cent of the rabies positive dogs were between one to five years of age while only seventeen per cent were below one year of age as reviewed by Widdowson *et al*. (2002); the age of population affected was crucial and followed a cyclic pattern whenever there is a decline in herd immunity and reduced vaccine coverage.

Stray dogs accounted for thirty-five per cent of positive cases, all of which were reportedly showing furious form of the disease and killed by people due to the aggressive behavior. 73 per cent of pet animals showed paralytic form of the disease. Rabies virus was well adapted to host species which manifested behavioural changes favouring transmission of disease, although all the mammalian species are susceptible and are dead end hosts (Hanlon *et al*., 2007). From the available data it was evident that stray dogs played an important role in dissemination of rabies to the pet animal as well as human population (Prasanna, 2012). The other dog breeds positive for rabies in the collected sample included German shepherd (21.7%), Labrador retriever (21.7%), Doberman (4.3%) and non-descript dogs (17.4%),

Among the pet dogs positive for rabies no bite history was reported in 53.3 per cent of animals. Hemachudha *et al*. (2002) suggested that mere absence of a bite history or exposure to a rabid dog, could not exclude the chance for rabies in an endemic area. India as a whole and Kerala is much prone and endemic to rabies (Shyam, 2019). One case among the paralytic group was reported to have the possibility of mongoose bite, since the owner frequently encountered mongoose near the kennel. Sudarshan *et al*. (2007) reported 3.5 percent chance of rabies occurrence through bites from wild animals.

Site of bite was the other important factor in the development of disease and its form. In this study 50 per cent of paralytic group of animals with known history had bite marks on head, neck and dorsum of the body and the other 50 per cent on the hind limbs and tail of the body, while all the known cases of furious form had bite marks on the head, neck and the forelimbs. In a study conducted by Mitrabhakdi *et al*. (2005), 65 per cent of rabies cases with the clinical manifestation of paralytic form had bite marks on the hind limb or distal extremities of the body, while more than 65 per cent of furious form rabies cases were encountered in patients bitten on their face or fore limb by a rabid animal. Hemachudha *et al*. (2002) commented that bite injury from a rabid animal on head, neck, face and hands associated with bleeding were having higher risk and short term of incubation period.

Brown *et al*. (2016) pointed out that the disease was highly communicable when there was periodic shedding of virus through the saliva. Asaye and Getachew (2014) observed that rabies virus travelled at a fairly constant rate from peripheral nerves to brain with an average speed of 8-20mm per day, depending on the site of inoculation of virus. WHO (2005) recommends proper knowledge about the management of dog bite injury as the prime factor to prevent the occurrence of the disease.

Analysis of the vaccination history revealed that most of the dog keepers were ignorant about the importance of prophylactic vaccination for rabies. Out of the 11 paralytic cases only 45.5 percent had undergone regular prophylactic vaccination and in 18 per cent, vaccination was irregular, while none were vaccinated in the furious group suggestive of the effect of vaccination in manifestation of disease. In our study a Labrador of 10 years age which was exposed to stray animal attack developed paralytic form of rabies though it followed all the post-bite vaccination protocol. Tepsumethanon *et al*. (2016) reported that rabies infected dogs which had undergone post-bite vaccination, had a higher chance to develop paralytic form of the disease.

In the paralytic group of animals with known history, the incubation period varied from 3 weeks to 17 weeks, whereas in furious group, most of the cases being stray animal, minimum data about the incubation period could be perceived. From the available data of 2 animals the range was very wide from 1 week to 1.5 years. Hemachudha *et al*. (2013) reported that incubation period for rabies varied widely from seven days to six years or more with an average of one to two months. Brown *et al.* (2016) reviewed that the incubation period for rabies was generally in a range of 3 to 12 weeks, rarely exceeding 6 months.

Duration of illness, reported in paralytic group was ranging from 3 days to one month with an average of 10 days and in furious cases, ranged from 4 days to 10 days with an average of 6 days. In the study conducted by

Gadre *et al*. (2010) average incubation period from animal bite to symptom development was two months and the duration from development of signs to death had a mean time of 11 days. Shuangshoti *et al*. (2016) reported that median survival time of rabid dogs was 4 days, with 25 per cent dying within 48 hours, and usually lapsing into coma 12 hours before death.

Summary

There was an equal chance in rabies infected animal to manifest furious or paralytic form of rabies, as we could encounter equal number of positive cases of both form of rabies during the period of research. Though wide range of clinical signs were exhibited by the animals infected, dropped jaw, salivation occasionally followed by generalised paralysis or quadriplegia were the predominant signs in paralytic form. Aggressive behaviour and vocal changes were notable changes in the furious form of rabies. Young animals, less than 5 years were found more susceptible to the disease whenever there was a decline in immunity due to reduced vaccine coverage in the population. Bite from an infected animal in the regions near to the CNS (face, neck *etc.*) is more prone to be manifested as furious form. Prophylactic vaccination played a significant role in manifestation of the disease as paralytic form in infected animal, suggestive of a partial immune response imparted by the same. Incubation period for paralytic rabies ranged from 21 to 42 days, while 1 week to 17 months in case of furious from. Duration of illness was also seen extending from 3 days up to a month in one of the rabies cases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Occurrence of poultry coccidiosis in different management systems in Thrissur, Kerala*

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Abstract

Coccidiosis is one of the most prevalent and economically important parasitic diseases caused by the infection with Eimeria species contributing to major economic losses of poultry industry worldwide. In this study, occurrence of Eimeria spp. in chicken reared under different management systems was studied. A total of 300 faecal samples from chicken were collected from six organised poultry farms and six backyard poultry units in and around Thrissur, Kerala. Out of this, 167 faecal samples were from organised farms and 133 from backyard poultry units. All the samples were artificially sporulated and examined for studying the oocysts morphology and morphometry. Out of 167 samples from organised farms 52 were found to be positive for Eimeria spp. while 61 out of 133 samples from backyard poultry were positive. The overall occurrence of Eimeria spp. in chicken from 12 different areas in and around Thrissur was 37.66 per cent (113/300). The species of Eimeria identified on morphological examination were E. tenella, E. necatrix and E. maxima. The occurrence rate of E. tenella was found to be significantly higher (46.01 %) compared to E. necatrix (39.82 %) and E. maxima (14.15 %). The rate of occurrence of Eimeria spp. infection was significantly higher in backyard poultry (45.86 %) compared to that in organised farms (31.13 %).

Keywords: *Eimeria species*, *chicken*, *organised farms*, *backyard poultry*.

The role of poultry industry has been significant in the socio-economic development of the country. The economics of poultry industry is often adversely affected by the faulty management of the farms and outbreak of different diseases. Avian coccidiosis caused by apicomplexan parasites of the genus *Eimeria,* is a major menace for poultry industry causing production losses, high mortality

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and morbidity rates. Seven distinct species of the genus *Eimeria* with different degrees of pathogenicity are recognised in chicken *viz., Eimeria tenella*, *E. necatrix*, *E. maxima*, *E. acervulina*, *E. brunetti*, *E. mitis* and *E. praecox*. Some species of *Eimeria* are highly pathogenic causing severe haemorrhagic enteritis with high mortality in young birds, whereas other species are slightly or moderately pathogenic. During an outbreak of coccidiosis, the identification of the infecting species is usually done by considering clinical signs, sporulation time, oocyst morphology and morphometry. The identification of *Eimeria* species infecting chicken has important implications in studying the epidemiology as well as in disease management. Hence, the present study was undertaken to study the occurrence of *Eimeria* spp. in chicken reared under different systems of management.

A total of 300 faecal samples were collected from chicken reared in organised poultry farms and backyard poultry units in and around Thrissur district of Kerala during the period from June 2019 to December 2020. Out of this, 167 samples were from organised farms and 133 from backyard poultry units. In organised farms, the birds were maintained in deep litter system or cage system, fed with commercial feed and routinely vaccinated against viral diseases. The birds in backyard poultry units were maintained in free range system and were not routinely vaccinated.

The faecal samples were examined by sedimentation and floatation techniques following standard protocols (Soulsby, 1982). Morphological identification of the different *Eimeria* spp. in chicken was done by a combination of morphological features of the oocysts (shape, presence or absence of the micropyle) and morphometry as per Soulsby (1982). The oocysts were artificially sporulated in 2.5 per cent potassium dichromate and the sporulation time was recorded in each case. The occurrence rate of *Eimeria* spp. in chicken and the influence of different systems of management on the occurrence were statistically analysed by Chi square test using SPSS version 24.0.

The overall occurrence of *Eimeria* spp. in chicken from 12 different areas in and around Thrissur was 37.66 per cent (113/300). On statistical analysis, the occurrence rate of coccidiosis in different areas was found to be significantly different ($p<0.01$) (Table 1). The three species of *Eimeria* were identified based on morphological features, *viz., E. tenella*, *E. necatrix* and *E. maxima* (Fig. 1). The oocysts of *E. tenella* and *E. necatrix* were ovoid in shape whereas that of *E. maxima* were broadly ovoid. On morphometry, *E. tenella* oocysts measured 21 X 18.25 µ with shape index of 1.15, while *E. necatrix* oocysts measured 23.77 X 22.05 µ and had a shape index of 1.07 and *E. maxima* oocysts was 28 X 22.5 µ in size with shape index of 1.24 (Table 2). This concurs with the observations of Khaier *et al*. (2015) who reported the mean size of *E. tenella* oocyst to be 19.63 X 17.02 µ with a shape index of 1.15. Jadhav *et al*. (2011) observed the mean values of *E. necatrix* oocyst measurement to be 13.2 to 22.5 µ length and 11.0 to 18.7 µ width. Al-Gawad *et al*. (2012) observed the mean size of *E. maxima* oocyst to be 29.9 X 23.8 µ with a shape index of 1.25. Similarly, in a study conducted in Tamil Nadu by Rao *et al*. (2013), the mean morphometric values recorded were 22 X 18 µ for *E. tenella* oocysts, 20.8 X 17.5 µ for *E. necatrix* and 31.19 X 18.5 µ for *E. maxima*. The average sporulation time of *E. tenella*, *E. necatrix* and *E. maxima* oocysts was 48-72 h which was in accordance with Soulsby (1982).

On morphological identification *E. tenella* was found to be the most predominant species with an occurrence rate of 46.01 per cent followed by *E. necatrix* (39.82 per cent) and *E. maxima* (14.15 per cent) (Table 3). On statistical analysis using Chi square test, the occurrence rate of *E. tenella* was found to be significantly higher compared to the other two species ($p<0.01$). The findings agree with many of the previous reports. *Eimeria tenella* was found to be the most prevalent species of *Eimeria* in poultry in a previous study conducted by Gigi George (1997) in Kerala. Similarly, Bhaskaran *et al*. (2010) also reported the incidence of *E. tenella* to be higher when compared to *E. necatrix* and *E. praecox* in Tamil Nadu. In the studies conducted in different farms

Table 1. Occurrence of *Eimeria* spp. in chicken in and around Thrissur

Area	No. of samples examined	No. positive	% positive	p value
Organised Farms				
Poomala	22	-	-	**0.0036**
Pattikkad	20	12	60	
University Poultry Farm, Mannuthy	32	-	-	
Viruppaka	40	22	55	
Pazhuvil	28	07	25	
Changarakkulam	25	11	44	
Total	**167**	**52**	**31.13**	
Backyard Poultry Units				
Kunnamkulam	30	18	60	
Arangali	14	-	-	
Thalikkulam	20	17	85	
Madakkathra	20	-	-	
Kuttattukulam	23	15	65.21	
Amballur	26	11	42.30	
Total	**133**	**61**	**45.86**	
Overall Total	**300**	**113**	**37.66**	

Table 2. Morphology, morphometry and average sporulation time of *Eimeria* spp. in chicken

Species	Shape of the oocyst	Micropyle	Mean size of oocyst (µm)	Shape index	Avg. sporulation time (hrs)
E. tenella	Ovoid	Absent	21 X 18.25	1.15	48-72
E. necatrix	Ovoid	Absent	23.77 X 22.05	1.07	48-72
E. maxima	Broadly ovoid	Absent	28 X 22.5	1.24	48-72

Table 3. Occurrence of *Eimeria* species in chicken

Species	No. of samples examined	No. positive	% positive	p-value
E. tenella	113	52	46.01	**< 0.0001***
E. necatrix		45	39.82	
E. maxima		16	14.15	

*Highly significant (p<0.01)

Table 4. Occurrence rate of *Eimeria* spp. in chicken in different management systems

Management system	No. of samples examined	No. positive	% positive	p-value
Organised farms	167	52	31.13	**0.0002***
Backyard	133	61	45.86	
Total	**300**	**113**	**37.66**	

* Highly significant (p<0.01)

in Ethiopia, Amare *et al*. (2012) as well as Dinka and Tolossa (2012) reported a prevalence of *E. tenella* to be higher compared to *E. necatrix*, *E. acervulina* and *E. brunetti*.

In the study, the rate of occurrence of *Eimeria* infection was found to be higher in backyard poultry (45.86 per cent) compared to that in organised farms (31.13 per cent) (Table

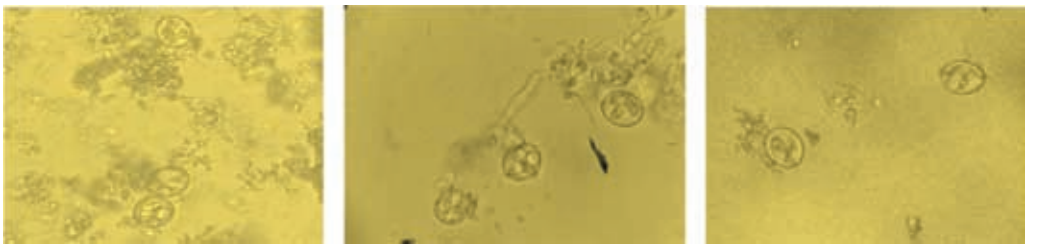

Fig.1. Sporulated oocysts of *Eimeria* spp.

A. Sporulated oocysts of *E. tenella* X400

B. Sporulated oocysts of *E. necatrix* X400

C. Sporulated oocysts of *E. maxima* X400

4). Statistical analysis revealed significant association between the occurrence of coccidiosis and the type of management ($p<0.01$). The findings were in accordance with Sharma *et al.* (2013) who recorded a higher prevalence rate for coccidiosis in backyard poultry (53.61 %) when compared to organised farms (25.55 %) in Jammu which was attributed to poor managemental practices and non-use of anticoccidiostats. Similarly, Garbi *et al*. (2015) reported the higher rate of infection in backyard chicken (27.6 %) compared to chicken under intensive management system (11.45 %) in Ethiopia. The higher occurrence rate observed in backyard poultry in this study could be attributed to poor management practices and lesser use of anticoccidials in these birds. Moreover, the humid climatic conditions prevailing in the state favour rapid sporulation of oocysts and quick transmission of disease. However, Ketema and Fasil (2019) reported higher occurrence rate of coccidiosis in intensive management system (20.6 %) compared to that in backyard poultry (17.9%) in Ethiopia and attributed it to the rearing of chicken in deep litter system, which provides optimal temperature and humidity for the sporulation of oocysts. Factors like overcrowding and water leakage also contributed to higher occurrence rate.

Summary

From the present study it could be summarised that the overall occurrence of *Eimeria* spp. in chicken was 37.66 per cent in and around Thrissur. The rate of infection was significantly higher in backyard poultry and the occurrence rate of *E. tenella* was found to be significantly higher compared to *E. necatrix* and *E. maxima*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Occurrence of repeat breeding in crossbred dairy cattle

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Abstract

The present research work was undertaken to evaluate the occurrence of repeat breeding (RB) among crossbred dairy cattle for three years from January 2017 to December 2019. Crossbred cows having apparently normal genitalia and showing regular oestrous cycle but failing to conceive even after three consecutive inseminations were selected as RB cows based on data collected from breeding registers. The occurrence of RB in the year 2017, 2018 and 2019 were 29.20, 24.12 and 19.52 per cent, respectively with an overall occurrence of 23.60 per cent. Detailed clinico-gynaecological and ultrasonographic examinations of 41 RB cows were carried out for the identification of various etiological factors during the study period (September 2019 to August 2020). Samples for endometrial cytology were collected during oestrus by cytobrush technique to rule out cytological endometritis (sub clinical endometritis). The occurrence of various identified causes of RB were cytological endometritis (17.07 %), fibrosis of cervix (7.31 %), endometritis (7.31 %), follicular cyst (4.88 %), kinked cervix (2.44 %), uterine unicornis (2.44 %) and other reasons (58.54 %). Repeat breeding is a major cause of infertility in crossbred dairy cattle and early diagnosis is essential for the effective management and to enhance production.

Keywords: *Cytological endometritis, occurrence, repeat breeding*

Repeat breeding (RB) is one of the most serious reproductive problems in dairy cattle causing considerable economic loss to farmers due to more number of inseminations, prolonged inter calving interval, lowering of calves production and increased culling rates. Roberts (1971) defined a repeat breeder cow as one that has a nearly normal oestrous cycle with apparently no palpable abnormalities of the genital tract, but has failed to conceive on three or more consecutive artificial inseminations (AI) with good quality semen. About 36.0 per cent of infertility conditions among crossbred cattle have been reported to be contributed by RB (Azeez *et al.,* 2017). Subclinical endometritis (SCE) is known as cytological endometritis on the basis of elevated ratio of polymorphonuclear leukocyte (PMN) cells in the endometrial cytology sample (Baranski *et al*., 2013). About 12.50 per cent RB cow syndrome among bovines was due to SCE and post-partum

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complications such as dystocia and retention of foetal membrane, which predisposed to SCE in cattle (Noakes *et al*., 2012).

The present study was conducted at Livestock Research Station (LRS), Thiruvazhamkunnu, Kerala Veterinary and Animal Sciences University during the period September 2019 to August 2020. Forty one RB cows with a history of not conceiving even after three consecutive inseminations and with apparently normal genitalia were selected for the study based on data collected from breeding registers maintained on the farm. The selected animals were closely observed for any external signs of oestrus. Detailed clinico-gynaecological and ultrasonographic examination of the reproductive tract was conducted. Uterine size, consistency and structures in the ovaries were assessed to confirm oestrus or to rule out any anatomical or other causes of RB. The samples for endometrial cytology were obtained by cytobrush technique as per Kasimanickam *et al*. (2004) to rule out cytological endometritis in RB cattle. Cows with more than one per cent PMN cells in endometrial cytology smears during oestrus were considered as positive for cytological endometritis (Pascottini *et al*., 2017).

The stock density of the cattle herd in the year 2017 was 137, out of which 40 cows were found to be repeat breeders and the occurrence of RB in the herd was 29.20 per cent. There was a progressive decrease in the occurrence of RB from the year 2017 to 2019. In the year 2018, 41 out of 170 cattle were RB (24.12 %), while in 2019, it further decreased to 19.52 per cent (41 out of 210). The overall occurrence of RB in the herd from January 2017 to December 2019 was 23.60 per cent. Out of the 41 RB cows examined in detail during the study, the various etiologies of RB diagnosed were seven cows with cytological endometritis (17.07 %), three cases of cervicitis (7.31 %), three cases of endometritis (7.31 %), two cases of follicular cysts (4.88 %) confirmed later with ultrasonography, one case of kinked cervix (2.44 %) and a case of uterus unicornis (2.44 %) and the remaining 58.54 per cent of cows were found to be repeaters due to unknown reasons.

Arun *et al.* (2020) reported 25.96 per cent occurrence of RB, whereas, Azeez *et al.* (2017) reported a high prevalence of RB (36 %) among crossbred cattle of Kerala. The various reasons attributed were reproductive tract infections (63 %), ovulatory defects (15 %) and reproductive tract infection with ovulatory defects (22 %). Kutty and Ramachandran, (2003) also observed a high prevalence of RB (35 %) among crossbred cattle of Kerala. However, Harichandan *et al*. (2018) reported the highest (51.94%) incidence of RB in dairy cows, in India. The incidence of RB varied with the season, number of calvings, body condition score, average days in milk etc. The probable reasons for an increased occurrence of RB among post-partum cows included a negative energy balance, post-partum complications and lactation stress (Mesafint and Guesh, 2014).

Kasimanickam *et al*. (2004) effectively used cytobrush technique for the first time to identify SCE in clinically normal post-partum dairy cattle. They used a PMN threshold value of 18 per cent at 20 to 33 days in milk (DIM) and 10 per cent at 34 to 47 DIM to detect SCE. In the present study, 17.07 per cent of RB was contributed to by cytological endometritis. Singh *et al*. (2016) reported a higher prevalence (29.40%) of cytological endometritis among RB crossbred cattle at their spontaneous oestrus using cytobrush technique. While Pascottini *et al*. (2017) observed a lower prevalence (7.86%) of cytological endometritis in nulliparous dairy heifers and the threshold level of PMN cells for diagnosing SCE at oestrus was considered as one per cent with cytotape technique.

The incidence of follicular cyst in dairy cattle was 5 to 25 per cent and it varied with age, body condition score, milk production status etc. (Bors *et al*., 2018). About 54.15 and 12.80 per cent of dairy cows were repeat breeders due to endometritis and various types of acquired cervical problems (Thakur *et al*., 2006).

According to Maurer and Echternkamp (1985), about 40.10 per cent of RB problems in cows were caused by hormonal insufficiency and dysfunctions. Singh *et al*. (2005) observed that delayed ovulation associated with extended

follicular phase was one of the causes of RB in cattle. Cenariu and Jospe (2017) stated that hormonal effect as well as timing of AI had a significant influence on the occurrence of RB in cows and they could achieve 70 per cent conception rate by treatment with Ovsynch protocol. The highest proportion of unknown causes of RB in the present study (58.54%) might be due to endocrine dysfunctions which could be attributed to micro-nutrient deficiencies (Singh *et al.*, 2000).

Summary

The present study conducted in an organised dairy farm for three years revealed 23.60 per cent overall occurrence of RB in cows. Various identified causes of RB were cytological endometritis, fibrosis of cervix, endometritis, follicular cysts, kinked cervix and uterine unicornis. Early diagnosis is essential for the effective management of RB syndrome in cattle.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Gastrointestinal parasites of captive Asian elephants in Kerala*

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Abstract

Gastrointestinal parasitic infections invariably affect the health status of elephants and can cause disease and death in elephants. This study was conducted to assess the incidence of gastrointestinal (GI)parasitesof elephants in Kerala and to relate it to the deworming status. A total of 31 Asian elephants presented to the Teaching Veterinary Clinical Complex, Mannuthy, those under private ownership (Thrissur) and forest department formed the basis of the study.It was observed that 32.2 per cent of elephants in the study were affected with GI parasites. High percentage of strongyle ova was observed followed by the mixed infection of strongyle and Strongyloides ova. Statistical analysis of haematological parameters were done by using student t test in SPSS version 24.0. The haematological studies of elephants infected with GI parasites revealed anaemia and eosinophilia.

Keywords: *Elephant, gastrointestinal parasites, Kerala*

Elephants are enlisted as endangered species by the World Conservation Union. Many factors like poaching, habitat loss, fragmentation and disease outbreaks were considered as threatening factors (Riddle *et al*. 2010). Apart from that,parasitism affects the elephant's health,

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fertility, behaviour and which facilitates parasite transmission.Several factors which determine the parasitic transmission include environmental conditions affecting the viability and behaviour of parasite ova, feeding, movementand defecation patterns of the host (Vidya and Sukumar, 2002). Helminthic infections were considered to be a major problem as it can cause mortality in captive animals (Borghare*et al*., 2009). Damp unhygienic conditions and small enclosures for captive and semi-captive elephants predispose them to gastrointestinal parasitic infection (Chandrasekharan*et al*. 1995). The risk of parasitic infection in both wild and captive animals can be reduced by quantitatively assessing the parasitic load. This study reports the prevalent GI parasites among elephants in Kerala and attempts to relate the incidence with the deworming status.

Dung samples were collected from 31 Asian elephants in the age group ranging from three months to 70 yearsof both sexes directly from the ground during November 2019 to December 2020.After procurement, the faecal samples were stored in a zip lock cover, kept at 4^0C and examined within 24 h. Dung samples were examined by direct, sedimentation and floatation techniques in the department of Veterinary Epidemiology and Preventive Medicine as per Soulsby(1982). About 2 ml of whole blood was collected in an EDTA vial and analysedusingAutomatic Haematology Analyzer (Orphee, Mythic Vet 18) in the Teaching Veterinary Clinical Complex (TVCC).

Microscopical examination of dung samples from 31 Asian elephants revealed the presence of strongyle ova in seven (22.6 per cent) cases and*Strongyloides*ova in three (9.7 per cent) cases. The strongyle ova were observed as thin shelled with embryonated egg inside (Fig. 1). *Strongyloides* ova wasobserved as thin shelled ova with larva inside (Fig. 2&3). All the elephants were regularly dewormed with fenbendazole at a dose rate of 5-10 mg/kg Dung sample examination revealed high percentage for strongyle ova than *Strongyloides*. This finding is similar to that of Abhijith*et al.* (2018) who reported 58.1 per cent prevalence of strongyles in elephants from South Wayanad Forest Division of Kerala and suggested that the increased prevalence of strongyles was an indicative of potential transmission of parasites via faecal route. Vidya and Sukumar (2002) had reported mixed infection in a study conducted on intestinal parasites in Asian elephants (*Elephasmaximus*) of Southern India. Several genera of strongyles like *Murshidia, Equinurbia, Choniangium, Quilonia, Bunostomum,Grammocephalus*etc. are recorded in Asian elephants (Shahi and Gairhe,2019).However, molecular tools are needed for species identification from faecal samples. Out of the 31 dung samples, none were found to be positive for trematode or cestode

Table1. Haematological profile for elephants with parasitic load

Parameter	Mean±SE		t- value
	With GI parasites n = 7	Without GI parasites n = 24	
Total leucocytes count (x10^3/$mm^{3)}$	14.9±1.06	14.09±1.47	0.288^{ns}
Lymphocytes (per cent)	28.79±2.72	28.41±2.58	0.075^{ns}
Monocytes (per cent)	16.23±2.37	15.19±1.51	0.338^{ns}
Granulocytes (per cent)	57.41±3.52	56.13±3.22	0.114*
Total erythrocyte count (x10^6/mm^3)	2.18±0.13	3.25±0.19	0.773*
Haemoglobin (g/dl)	10.76±0.6	11.76±0.74	0.711^{ns}
Haematocrit (per cent)	31.84±1.93	33.92±2.36	0.458^{ns}
MCV (fl)	107.3±6.43	104.24±2.98	0.470^{ns}
MCH (pg)	36.11±1	36.28±0.83	0.103^{ns}
MCHC (per cent)	34.81±3.12	35.35±1.3	0.184^{ns}
Platelet count (x10^3/mm^3)	360.43±16.97	514.42±59.51	2.489**

p<0.01; highly significant (**) p<0.05; significant (*) p>0.05; non-significant (ns)

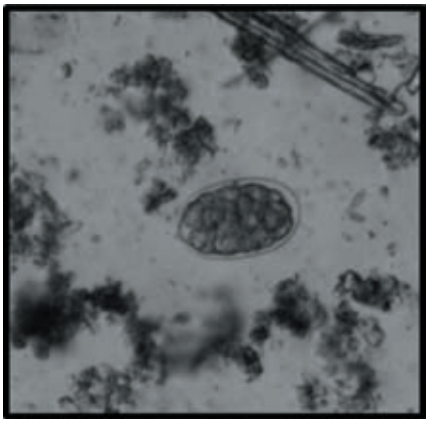

Fig.1. Strongyle ova

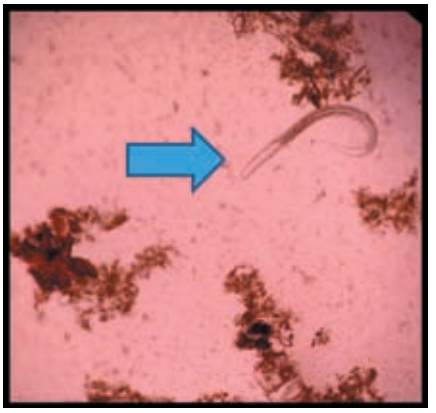

Fig.3. *Strongyloides* larva

ova. Amulya, (2016)identified *Anoplocephala* spp. (cestode) and *Bivitellobilharzianairi* (trematode) in Kerala.In Gujarat,Jani (2008) had found *Fasciola*spp. to be more prevalent in elephants that had access to water bodies and a habit of soil licking. The 31 captive and semi-captive elephants included in the study had no access to water bodies but had a habit of soil licking.

Regular deworming is considered to be the main reason for the reduced presence of gastrointestinal parasite in the captive and semi-captive elephants. The captive and semi-captive elephants in Kerala were dewormed at an interval of 4 months after checking the faecal sample. Fenbendazole is a broad spectrum anthelmintic used against gastrointestinal parasites like *Giardia*, round worms, strongyles, etc. for captive elephants (Tiwari and Rao, 1996). In captive and semi-captive elephants, parasitic infection may aggravate because of space limitation as they were confined

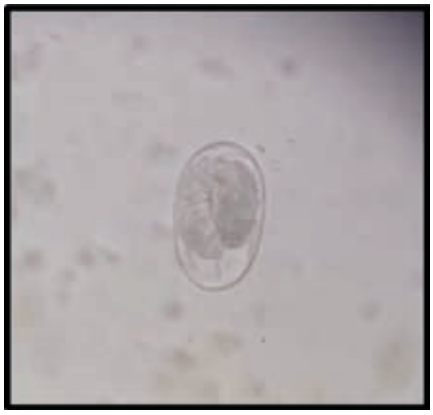

Fig. 2. *Strongyloides* ova

in close proximity (Abeysekara*et al*., 2018). Saseendran*et al*.(2003)reported that 10 per cent captive elephants in Kerala, that were dewormed annually had strongyle infection.

The haematological parameters of elephants infected by helminth parasites were compared to that of apparently healthy elephants (Table1). Significant low levels of total erythrocyte count (2.18±0.13 x10^6/ mm^3) and haemoglobin (10.76±0.6 g/dl) as well as significant high levels of granulocytes (57.41±3.52 per cent) were noticed in elephants harbouring parasites.Haematological findings suggestive of anaemia is in agreement with Sarode*et al*. (1991) who also reported low significant reduction in both erythrocyte count and haemoglobin in elephants due to helminth infection. Significant eosinophilia (57.41±3.52 per cent)could be observed in the affected animals and it can be due to hypersensitivity reaction towards parasites as observed by Jani(2008).

Summary

The effectiveness of deworming depends on the environmental conditions affecting viability and behaviour of the parasite ova, feeding, movement and defecation patterns of the host. Therefore, management practices play a significant role in reducing parasitic infection. Further studies on gastrointestinal parasites in Asian elephants of Kerala is essential to find out the presence of other nematodes, cestodes and flukes as reported from other state of India.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Surgical management of dystocia due to dicephalic sternophagus tetrabrachius tetrapus dicaudatus monster in a Murrah buffalo

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Abstract

Successful delivery of a conjoint monster, dicephalic sternophagus tetrabrachius tetrapus dicaudatus monster by caesarean section and partial fetotomy in a Murrah buffalo is reported.

Keywords: *Dystocia, conjoint monster, sternophagus, bicephalic, tetrabrachius, tetrapus, dicaudatus, caesarean section.*

In bovines, the incidence of dystocia is quite high than other domestic animal species (Dutt *et al.,* 2021). Monsters are mostly observed in cattle with an overall incidence of one in 100,000 bovine births. They are rare in other species (Roberts, 1971) but reports in buffaloes are meagre. The incidence of monstrosities encountered for cow is 0.5 per cent (Craig, 1930) whereas an incidence of 7.9 per cent to 12.8 per cent has been reported for riverine buffalo (Singla and Sharma 1992). The conjoined twins are two fetuses joined together and arise typically from a single ovum and are monozygotic that occur due to incomplete division of a fertilized ovum (Roberts, 2004). Such conjoint fetuses at the time of parturition warrants the need of obstetrical intervention and feto-maternal disproportion is consistently the most frequent overall indication for caesarean section. The present report describes a case of dystocia due to dicephalic sternophagus tetrabrachius tetrapus dicaudatus monster in a Murrah buffalo and its delivery through caesarean section.

A seven-year-old pluriparous buffalo in sternal recumbency with a history of ruptured water bag 10 hours ago along with hanging snare from vulvar lips was brought to veterinary clinical complex of the Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. According to owner, excessive traction was applied by the field paraveterinarian staff to the presented forelimbs of the fetus with snare for about 5-6 h but attempts made by local paraveterinarian staff went futile.

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On clinical examination, it was found that the temperature of dam was 101ºF, heart rate was 54 beats per minute and respiration rate was 23 per minute.A thorough per-vaginal examination after epidural anaesthesia with 5 ml of 2 per cent lignocaine and proper lubrication with liquid paraffin revealed the presence of two foetuses joined at the sternal region in anterior longitudinal presentation and dorso-iliac position with fourforelimbs and two foetal heads in the birth canal. The case was diagnosed as dystocia due to conjoint foetal monstrosity.As the case was previously handled at field level for 5-6 h, the birth canal was dried up.The animal was in sternal recumbency,the fetus was diagnosed as monster and per-vaginal delivery seemed to be impossible.Considering the physical condition of dam,an emergency caesarean section was conducted.

The animal was restrained in right lateral recumbency and incision was done at paramedian site just parallel to milk vein (four fingers lateral to milk vein) under local infiltration with 2 per cent lignocaine HCl and after preparing the site aseptically. Due to excessively large size and abnormal shape of fetuses, both fetuses were retrieved by careful staged foetotomy (Fig.1) and foetal membranes were removed manually.The uterus was sutured using vicryl no. 3 in two layers by Cushing and lambert suturing patterns, respectively. The first and second muscle layers were sutured by using vicryl no. 3 in lock stitch suture pattern. Skin was sutured with silk using cross mattress suture pattern.

Eight Cleanex®boli (Nitrofurazone, Metronidazole, Urea and Povidone iodine; Boehringer Ingelheim) were placed intrauterine.The animal was treated with Inj. Oxytocin 50 I.U. in 1 liter of normal saline solution IV; Inj. Calcium-magnesium-boro-gluconate 450 ml I/V , Inj. Ceftriaxone 4g I/M, Inj. Chlorpheniramine maleate 227.5 mg I/M, Inj. Flunixinmeglumine1000mg I/M, Inj. Metronidazole 5000 mg/1000 ml and Inj. Vit. B complex 10 ml I/M. Antibiotic, Vitamin-B complex and anti-inflammatory drug were advised for seven days whereas Inj. Metronidazole was recommended for three days. Antiseptic dressing with povidone iodine was advised for 12 days and skin sutures were removed on day 12 post surgery.The animal had uneventful recovery.

Grossly the monster possessed two normal heads, with separate nostrils, eyes and ears, eight limbs and two tails and the fetuses were conjoint over sternum.The sex of two fetuses was same (male). Post-mortem examination of monster revealed complete development of visceral organs and musculoskeletal system in both the conjoint fetuses.

Monstrosity is due to congenital embryonic duplication of germinal layer arising from single ovum (Kumar and Reddy, 2008) that bring about monozygotic fetus with partial or complete duplication of body structures. Conjoint twins develop after the development of embryonic plate (Whitlock *et al.,* 2008).The embryonic disk starts to differentiate on the 13th day of conception and if the split occurs after day 13, the twins share body parts besides sharing their chorion and amnion (Finberg, 1994). Normal per-vaginal delivery of such types of conjoint twins is difficult due to abnormal feto-maternal disproportion resulting in dystocia. Conjoint twins are always identical twins and of the same sex (Arthur *et al.,* 2001). Such twins are usually due to non-inherited defects and often lead to severe dystocia (Roberts, 2004). Dystocia due to conjoint twin monster have been reported as rare cases in buffaloes (Selvaraju *et al.,* 2002; Singh *et al.,* 2013a). Foetotomy remains a good alternative to hysterectomy or caesarean section for relieving a foetal monster resulting in dystocia (Vermunt, 2009). The buffaloes subjected to caesarean section have evidenced a lower survival rate (45.1%) in

Fig. 1 Conjoint dicephalic tetrabrachius sternophagus tetrapus dicaudatus monster

contrast to those with/without partial foetotomy (Singh *et al.,* 2013b). So it's imperative to diagnose the foetal monstrosity at initial stages of parturition and in manipulated and delayed cases of monstrosity, the caesarean section becomes the last resort.

Summary

The present report describes a case of dystocia due to dicephalus sternophagus tetrabrachius tetrapus dicaudatus monster in a Murrah buffalo and its delivery through caesarean section.The paper discusses the causes of foetal monstrosity.The need to diagnose the condition at initial stages of parturition as caesarean section becomes the last resort in manipulated and delayed cases of monstrosity is also emphasisied.

Conflict of interest

The authors declare that they have no conflict of interest.

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