PRESERVATION OF LOCAL VILLAGE ROOSTER SEMEN USING RINGER'S DILUENT WITH 3 LEVELS OF GLYCEROL AND ETHYLENE GLYCOL AT CHILLED TEMPERATURE (5 °C)

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THIS THESIS IS SUBMITTED INPARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF AGRICULTURAL SCIENCE- LIVESTOCK PRODUCTION

FACULTY OF SUSTAINABLE AGRICULTURE

UNIVERSITI MALAYSIA SABAH

JANUARY 2017

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL: ~~PRE~~ PRESERVATION OF VILLAGE ROOSTER SEMEN USING RINGER'S DILUENT WITH 3 LEVELS OF GLYCEROL AND ETHYLENE GLYCOL AT CHILLED TEMPERATURE (5°C)

IJAZAH: IJAZAH SARJANA MUDA ~~DENGAN KEPUJIAN~~ SAINS PERTANIAN (PENGELUARAN TERNAKAN) DENGAN KEPUJIAN

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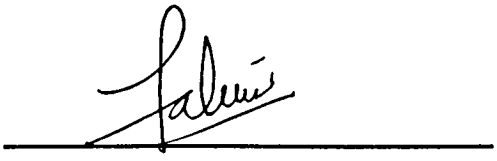

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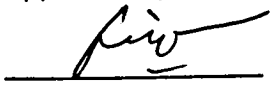

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ACKNOWLEDGEMENT

In the name of Allah, the most gracious and the most merciful

Foremost, I would like to express my sincere gratitude to Head Programme of HG-36 Livestock Production, Dean and entire members of the Faculty of Sustainable Agriculture, for the assistance during my graduate study in Universiti Malaysia Sabah. I also would like to thank my supervisor, Prof. Dr. Abdul Rashid bin Baba and our Final Year Project coordinators for their invaluable advices, patience, motivation, idea contribution and immense knowledge. Their guidance helped me in all the time of research and writing of this thesis along I am conducting this study. My sincere thanks to all my fellow classmates and friends who supported me a lot to finish my study. Finally, I wish to express my deepest gratitude to my parents and family members for their prayers, continuous moral support during the course of my thesis.

Thank you

ABSTRACT

The aim of this study is to evaluate the effect of 3 levels of glycerol and Ethylene glycol on the preservation of rooster sperm's quality and quantity. Ten roosters were selected and sperm from rooster village chicken were selected and sperm ejaculation was collected by abdominal massage method. Sperm ejaculation of three cockerels were pooled and extended using Ringer's Solution and they were categorized into 0%G, 2%G, 4%G, 6%G, 0%EG, 2%EG, 4%EG, 6%EG as treatment groups. Extended semen from three different levels of glycerol were put in chilled (5°C) temperature. Semen evaluation was evaluated hourly over 72 hours. The best post-thaw motility scale for rooster village chicken after 72 hours are 4%EG (16.67%) followed by 2%G (13.33%). The highest percentages of live sperm were obtained with 6%G (27.55%) followed by 2%EG (19.56%) while the best post-thaw abnormal sperm was obtained with 6%G (68.89%) followed by 2%EG (68%). From this, we can conclude that glycerol with 6% level is better than 2% level of ethylene glycol for maintaining sperm viability and abnormality with significant level $p<0.05$.

Key words: Rooster village semen, glycerol, ethylene glycol, sperm quality.

ABSTRAK

Tujuan kajian ini dijalannkan adalah untuk mengkaji kesan kepekatan gliserol dan etelin glikol yang berbeza bagi pemeliharaan kualiti dan kuantiti sperma ayam kampung jantan. 10 ekor ayam jantan kampung telah dipilih dan air mani telah dikumpulkan. Air mani tersebut telah di cairkan mengunakan pelarut Ringer. Mereka dikategorikan kepada 0%G, 2%G, 4%G, 6%G, 0%EG, 2%EG, 4%EG, 6%EG sebagai kumpulan rawatan. Air mani yang telah dicairkan diletakkan dalam suhu sejuk (5°C). Penilaian air mani dilakukanselama 72 jam. Skala pergerakan terbaik untuk ayam kampung jantan selepas 72 jam adalah 4%EG (16.67%) diikuti oleh 2%G (13.33%). Peratusan tertinggi spermatozoa hidup yang diperolehi adalah 6%G (27.55%) dikuti oleh 2%EG (19.56%) manakala sperma abnormal yang terbaik selepas pencairan telah diperoleh dengan 6%G (68.89%) diikuti oleh 2%EG (68%). Melalui keputusan yang diperoleh, maka dapatlah disimpulkan bahawa gliserol dengan kepekatan 6% lebih baik daripada kepekatan etelen glikol 2% untuk mengekalkan survival dan abnormality sperma dalam tahap yang terbaik dengan perbezaan bererti $p<0.05$.

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BW	Body Weight
DVS	Department of Veterinary Service
EG	Ethylene glycol
G	Glycerol
MVC	Malaysia Village Chicken

CHAPTER 1

INTRODUCTION

1.1 Background of study

Chicken meat is one of the main sources of protein for Malaysians. The poultry production industry plays a big role in providing this source of protein to all Malaysians as the demand and consumption of chicken meat is increasing day by day. In term of world population, poultry production industry has helped to solve the issue of food security. According to Livestock Statistics of Department of Veterinary Services (DVS) (2014), production of poultry meat has increased slowly year by year, which was 1.39 million tons in 2013. In addition, the consumption of poultry products in 2014 was 1.46 million tons. From this evidence, we can see Malaysia has reached self-sufficient in poultry meat production as production is higher than consumption or demand.

Due to the education level and healthy lifestyle concern, village chicken has slowly penetrated into the poultry market and became one of the favourite meats by the consumers instead of commercial broiler meat. In Malaysia, village chicken is the local breed that is well adapted to local conditions but low in productivity. It is commonly being reared by local people in rural areas. In general, village producers keep small flocks of between 5 to 20 birds per household (Gueye, 1997). In the current market, the demand for village chicken meat is increasing as people are looking forward to a healthy lifestyle and start to eat organic food. Besides, there is a unique taste of village chicken meat that makes it differ from the commercial broiler meat and also known as high nutritive value However, the increasing demand has caused the lack of good breeding stock of village chicken which is also the main constraint for its low productivity.

In order to increase the productivity, quality and quantity of village chicken, a systematic genetic improvement programme needs to be emphasized such as application of Artificial Insemination (AI) technique. Effort will be mainly focused on optimization on management of cockerel semen storage (Al-Daraji, 2012). Good quality semen can be preserved under a controlled condition so that it can be stored for a long period of time.

1.2 Justification

The increase of demand for the village chicken meat by the consumers has caused the shortage of breeding stock for village chicken. The village chicken sector is having a big challenge nowadays in order to supply the good quality and optimum quantity of village chicken meat. Some of the major problems are low productivity, and poor husbandry system. Therefore, emphasize on it need to be done to improve the genetic basis of village chicken. The commonly used diluent for semen storage is the Ringer's diluent. This research is important to evaluate the effect of different levels of glycerol that associate the survivability of the sperm cells.

1.3 Objective

To evaluate the effect of 3 levels of glycerol and Ethylene glycol on the preservation of rooster's sperm quality.

1.3 Hypothesis

H_o : There is no significant different of different levels of glycerol or ethylene glycol on the the preservation of rooster's sperm quality.

H_a : There is significant different of different levels of glycerol or ethylene glycol on the the preservation of rosster's sperm quality.

CHAPTER 2

LITERATURE REVIEW

2.1 Poultry and village chicken production in Malaysia

Poultry is the term used to designate those species of birds which render men an economic service and reproduce freely under their care (Winter *et al.*, 1960). Examples of poultry species are chicken, turkeys, ducks, quail, geese, swans, pigeon, peafowl, guinea fowl, pheasants and ostrich. Among those species, the most popular in Malaysia are chicken, ducks and quails. Poultry is a big part of Malaysian diet and it is consumed in many forms such as meat, eggs and broth.

At the beginning, the chicken rearing is only spreading in America and Europe. However, along with the increase of world population and development in transportation, the chicken was being commercialized and their genetic potentiality has spread all over the world. The ancestor of the current commercial chicken is believed from the Red Jungle Fowl or also known as *Gallus gallus*. Environmental constraints and sporadic mating involving the wild red jungle fowl eventually lead to the existence of the smaller size village chicken. After experiencing evolutionary and artificial selection forces, the chicken with desirable characteristic is selected to be commercialized and left out to be the village chicken nowadays.

In the rural area, the village chicken is reared traditionally under free range system and some of them are reared at the backyard. The input resource such as cost is at the minimum. The flock is usually being fed freely or scavenge and sometimes supplied with grains and leftover meal. Due to these factors, Malaysia village chicken (MVC) gets the low nutritional feed and affect its growth rate and production. However, village chicken have their own specialty where they have the ability to survive and well adapted to any

harsh environment with highly resistance to disease or illnesses due to the environmental and evolutionary forces. Consumers will select the chicken with a heavier body weight (BW) which is also one of their desirable phenotypic characteristics. Thus, consumer normally purchased village chicken with higher percentage of carcass yield of edible portion to BW as it symbolizing a better economic proposition than those with lowered dressed yields (Azlina and Azahan, 2011).

2.2 Anatomy and reproductive physiology of rooster

The male reproductive system consists of two testicles, epididymis, vas deferens, small penis, cloaca and papillae. The two testicles are located in the dorsal area of the body cavity, just in front of the kidneys (North and Bell, 1990). The testes of birds are intra-abdominal and loosely suspended from the dorsal body wall by short peritoneal folds, the mesorchia, medial, and partly cranial to the kidneys (Jones and Lin, 1993; Lake, 1971). They are both elliptical in shape and light yellow in colour and play important roles in producing sperms and testosterone. Sperm will be only produced when sexual maturity is attained in cockerel, which is about five months of age. The sperm duct carries the sperms from the testes to vas deferens, which then leads the sperms to the papillae. The sperms will finally reach to the copulatory organ through papillae. This process takes about 4 days.

The size of both testicles differs from each other as the left testis usually heavier than the right testis, which is about 0.5-3.0 g. The size of testis can affect the amount of semen produced. The larger the size of the testis, the greater the sperm production (Senger, 2003). Usually, the semen is stored in the vas deferens and being diluted with lymph fluid before it is ejaculated during copulation (North and Bell, 1990). The penis of the cockerel is smaller compared with the waterfowl that has a well-developed, long, and twisted organ. During copulation, the semen is ejaculated into the opening of cloaca of the female. After the semen is deposited, it will find its own way up to the oviduct.

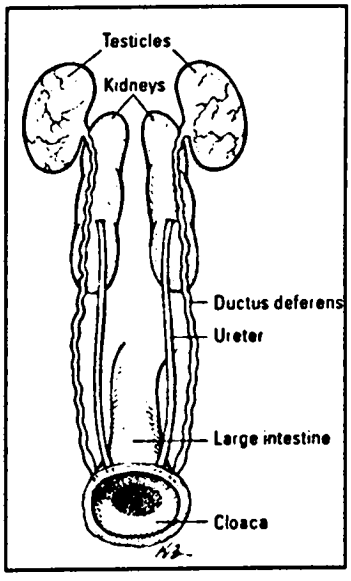

Figure 1: Reproductive organ of rooster

Source: Retrieved from http://www.dummies.com/home-garden/hobby-farming/raising-chickens/starting-with-the-chicken-and-then-the-egg-growth-and-development/

2.3 Spermatogenesis in Avian

Spermatogenesis is the process of producing sperms from the primordial germ cells by the way of mitosis and meiosis. Once the vertebrate primordial germ cells arrive at the genital ridge of a male embryo, they become incorporated into the sex cords. The process of spermatogenesis can be divided into four basic types of germ cells which are spermatogonia, primary spermatocyte, secondary spermatocyte, spermatids (Jones and Lin, 1993). Same as mammals, the sperms production in birds also occur in seminiferous tubules of testes, the starting point of the process.

2.3.1 Spermatocytogenesis

The first stage of spermatogenesis is spermatocytogenesis. Spermatocytogenesis is the male form of gametocytogenesis and results in the formation of spermatocytes possessing half the normal complement of genetic material. A diploid spermatogonium which is formed in spermatocytogenesis, undergoes mitotic division and produces two diploid intermediate cells, called primary spermatocytes. The primary spermatocyte then moves into adluminal compartment of the semi tubules and undergoes meiosis 1, duplicates its DNA.

It resulted in producing two haploid secondary spermatocytes. The secondary spermatocytes will later divide into haploid spermatids. Each cell division from a spermatogonium to a spermatid is incomplete; the cells remain connected to one another by bridges of cytoplasm to allow synchronous development. It should also be noted that not all spermatogonia divide to produce spermatocytes; otherwise, the supply of spermatogonia would run out. Instead, certain types of spermatogonia divide mitotically to produce copies of itself, ensuring a constant supply of spermatogonia to fuel spermatogenesis (Fishelson *et al.,* 2007).

2.3.2 Spermatidogenesis

Spermatidogenesis is the creation of spermatids from secondary spermatocytes. Secondary spermatocytes produced earlier rapidly enter meiosis II and divide to produce haploid spermatids. The brevity of this stage means that secondary spermatocytes are rarely seen in histological studies.

2.3.3 Spermiogenesis

Within this process, the spermatids begin to form a tail by growing microtubules on one of the centrioles, which turns into basal body. These microtubules form an axoneme. The anterior part of the tail (called midpiece) thickens because mitochondria are arranged around the axoneme to ensure energy supply. Spermatid DNA also undergoes packaging, becoming highly condensed. The DNA is packaged firstly with specific nuclear basic proteins, which are subsequently replaced with protamines during spermatid elongation. The resultant tightly packed chromatin is transcriptionally inactive. The Golgi apparatus that surrounds the condensed nucleus becomes acrosomes.

After that, the sperm maturation process occurs due to the influence of testosterone, known as spermiogenesis or spermateloisis (Jones and Lin, 1993). The remaining unnecessary cytoplasm and organelles will be removed. The excess cytoplasm, known as residual bodies, is phagocytosed by surrounding Sertoli cells in the testes. The spermatozoa formed now is mature but lack motility, rendering them sterile. A process called spermiation will take place then, where the mature spermatozoa are released from the protective Sertoli cells into the lumen of the seminiferous tubule. The non-motile

spermatozoa are transported to the epididymis in testicular fluid secreted by the Sertoli cells with the aid of peristaltic contraction. While in the epididymis the spermatozoa gain motility and become capable of fertilization. However, transport of the mature spermatozoa through the remainder of the male reproductive system is achieved via muscle contraction rather than the spermatozoon's recently acquired motility.

2.4 Semen characteristics

One advantage of avian reproductive system is that the sperms can still survive or viable at body temperature compared with mammal where the sperms are not viable at body temperature. Due to this physiology condition, the mammal's male reproductive tract is at the outside of the body while the cockerel's reproductive tract is located entirely inside the body. Spermatogenesis will occur at 41°C in cockerel which is slightly different from mammal that produced sperms when scrotal temperature in range of 24-26°C (Tuncer *et al.*, 2006). Spermatozoa from the cockerel show a long pointed headpiece, followed by a long tail and its pH is between 7.0-7.4 (North and Bell,1990).

2.5 Semen collection technique

There are some techniques that can be practiced to collect the semen from rooster in order to improve the genetic basis of poultry through AI. Before carrying out the real process of semen collection, the rooster needs to be trained first. The purpose of this training is to make the rooster become familiar with the methods carry out by the AI technician and at the same time letting AI technician familiar with semen collection so that the next process can be done smoothly. The whole semen collection process needs to be done carefully so that the quantity and quality of collected semen can be maximized as contamination from the collecting equipment, foreign material such as blood and cloacae products might ruin the result (Lukaszewicz, 2008).

According to Burrows and Quinns (1937), the abdominal massage technique is described as a non-invasive method. It is a traditional and effective way to collect semen from rooster and yet being used until today. The rooster must be taken and handled gently from the cage with minimal stress. The abdomen region is massaged and gently stroked the back of the bird from behind the wings towards the tail with firm rapid strokes. Through this method, it leads to copulatory organ erection and ejaculation of

semen occurs. Then, the cloaca is squeezed and semen is expressed through external papillae so that the semen can be collected into the container or tube. Once the initial excitement is missed, the reflex massage is difficult to elicit, together with ejaculation. When it is stimulated for the first time, the cockerel tends to defecate and urinate but this behaviour would not last longer until they are adapted.

2.6 Preparation of diluents

2.6.1 Diluents in poultry

Poultry semen is characterized by viscose and highly concentrated. Semen extender or diluents is used to increase the volume of semen especially those animal produce less number volume of semen per ejaculation in order to inseminate more female animal such as hen per semen ejaculation in rooster. It is one of the important procedures for approaching successful AI depending on extent to which semen can be preserved and stored to maintain the fertility (Sukumarannair *et al.*, 2004). Semen diluents are based on the biochemical composition of chicken and turkey semen (Chauduri et al., 1988). Glutamic acid, the most prominent anionic constituent of avian seminal plasma, became a standard component of diluents (Lake and McIndoe, 1959).

It can be stored in terms of short and long term condition. Short term storage of semen (less than three days) can be stored as liquid while long term storage of semen (more than three days) can be stored in frozen condition depending on favour of poultry breeder (Loo, 2015). Semen stored and preserved under extenders shows good quality sperm. Therefore, an appropriate osmolarity levels, energy source and pH which is same as seminal plasma is provided to spermatozoa where the extender is prepared prior to this condition. Availability of special buffered salt solutions can be used as rooster semen extender whereby variable of components can be included depending on the types of semen extender (Loo, 2015).

Adrohep, Beltsville thawing solution (BTS), Cornell University extender (CUE); Honey, egg yolk, Palm wine plus "Nche" (*Saccoglotis gabonensis*) (Pitso, 2009; Umesiobi, 2004) and coconut water are the example of existence of semen extenders includes conventional and renowned local extenders respectively (Pitso, 2009). The spermatozoa

can be protected from cold shock injury especially during cooling using extender by adding egg yolk or skimmed milk. Furthermore, Buffers are needed in order to against the low pH and also help to prevent any changes, maintain and stabilize the pH in range of 7.0-7.4. Examples of buffers include sodium citrate, sodium phosphate and TRIS. Egg albumin, skimmed milk, coconut water, gentamicin sulphate or antibiotics such as streptomycin can be added into extender as additives (Siudzin´ska and Łukaszewicz, 2008).

2.6.2 Semen preservation in poultry

The range of cryoprotectants used for chicken spermatozoa includes glycerol, dimethylsulphoxide, dimethylacetamide, ethylene glycol, dimethylformamide, and propyleneglycol (Hammerstedt and Graham, 1992). In comparing the efficacy of different compounds for their cryoprotective activity, or for their toxicity towards chicken spermatozoa , several factors should equilibration be taken into consideration: cryoprotectant concentration, equilibration temperature, equilibration time, freezing rate, freezing method, and post-thaw treatment. The most toxic and least effective cryoprotectant is uniquely, contraceptive for intravaginally, inseminated chicken spertomazoa (Hammerstedt and Graham, 1992).

2.6.3 Technique for semen preservation

2.6.3.1 Room temperature (RT)

There is low possibility to maintain the fertility rate for diluted semen under *in vitro* for more than 30 minutes, where the quality of undiluted of semen roosters degraded in 30 minutes depending on its initial quality (Lake, 1971). Inactivation of sperm motility in domestic chicken (*Gallus gallus*) happened under increment of temperature from 30-40°C. Moreover, changes in the external medium are highly correlated to several factors such as temperature, pH and ion composition (Bonato *et al.*, 2012). Under this circumstance, the sperm motility will be restored at 40°C under alkalization of external pH with addition of calcium (Ca) (Bonato *et al.*, 2012). Thus, the effect of storage medium pH, storage temperature and their interaction need to be taken into consideration to maintain sperm viability and activity *in vitro*.

2.6.3.2 Chilling temperature

In order to achieve higher rate of fertility, freshly diluted semen of poultry species should be used within 20 minutes (Figueiuredo *et al.*, 1999). As dilution of rooster semen shows decreased in percentage of dead sperm in stored semen (Abu *et al.*, 2013). Motility of rooster semen shows the lowest percentage when stored at 41°C compared to those semen stores at 25°C, 18°C or 5°C under undiluted or diluted condition (Peters *et al.*, 2008). However, precaution must take into consideration in avoiding semen undergone cold shock injury.

2.6.3.3 Freezing temperature

Cryopreservation is one of the assisted reproductive technologies besides AI and semen holding which invented to disseminate and preserve germplasm of livestock species (Bonato *et al.*, 2012) especially chicken, which the assisted technologies was firstly established on it (Blesbois and Brillard, 2007; Donoghue and Wishart, 2000; Lake, 1986). It was also serving as sperm bank and was one only efficient method for preserving the genetic resource using species specific cryoprotectants under ex situ management for endangered species and breeds of livestock as well as in livestock farm animal, such as avian species (Ehling *et al.*, 2012). North America, The Netherlands and France are the three countries which launching and operating the program of national avian gemplasm cryobank (Blesbois *et al.*, 2008; Blesbois, 2006).

CHAPTER 3

METHODOLOGY

3.1 Location of study

This study was carried out at the semi-intensive poultry house and laboratory of Faculty Sustainable Agriculture (FSA).

3.2 Duration of study

This study was conducted for two months, which is from early of August to end of September 2016.

3.3 Rooster and its management

Ten local roosters were selected from the area within Sandakan, Sabah. All ten roosters were kept in the cages for easier management and handling. They were fed with commercial poultry feed and water *ad libitum* throughout the experimental period.

3.4 Semen collection

Before start the semen collection technique, the roosters were allowed to adapt to the experimental site for about 2 weeks as training for semen collection was carried out. Abdominal massage technique was being used to collect the semen according to Burrows and Quinn (1937). The semen was collected once a week. Through this technique, two persons are required to help each other. The first person, handler held and collected the

semen, while another person, the operator massaged and stimulated the rooster. The right hand's palm of holder was used to rest the keel of rooster so that the head of cockerel is between the side and elbow of holder. While holding the rooster, the leg of the rooster was ensured to be free to move under this condition. The other hand of holder (left) was used to collect the semen using 1 mL syringe or collection vessel.

The operator held the leg loosely but firmly using his right hand while his left hand was used to stroke the back of cockerel from neck to tail. This will elicit a reflexive raising of the tail and overt signs of excitement. Male organ swelled and protruded outwards and downwards after a few strokes. White colour of semen was seen in the central furrow of the organ. The semen was milked down by applying a slight pressure along the sides of the cloacal opening (vent) and collected using syringe. The collected semen was placed into a container with temperature 41°C of water to maintain the temperature during the time and distance transport to laboratory (Mphaphathi *et al.*, 2012).

3.5 Preparation of reagents

3.5.1 Ringer's solution

After the semen was collected, it was mixed with formulated Ringer's solution according to Helmenstine (2014) for dilution as shown in Table (1).

Table 1: Measurement of chemicals for preparation of Ringer's solution.

Chemicals	Measurement (g)
Sodium chloride (NaCl)	9.0
Potassium chloride (KCl)	0.4
Calcium chloride ($CaCl_2$)	0.3
Dextrose	1.3
Sodium bicarbonate ($NaHCO_3$)	0.2

Sodium chloride (NaCl), Potassium chloride (KCl), Calcium chloride ($CaCl_2$) and Dextrose was added to 1 L of distilled water. In order to adjust the pH of solution to pH 7.0-7.4, Sodium bicarbonate ($NaHCO_3$) was added into the solution. The solution was then being filtered with 0.22 µm filter and taken for autoclaving process in order to meet ideal results of experiment.

3.5.2 Preparation of semen cryoprotectant (glycerol)

2%, 4% and 6% glycerol in TRIS

(a) 2% glycerol
- 2 ml of glycerol was put in blue cap bottle followed by addition of Ringer's solution up to 100 ml.

(b) 4% glycerol
- 4 ml of glycerol is was in blue cap bottle followed by addition of Ringer's solution up to 100 ml.

(c) 6% glycerol
- 6 ml of glycerol was put in blue cap bottle followed by addition of Ringer's solution up to 100 ml.

3.5.3 Eosin-Nigrosin stain

Eosin-Nigrosin stain was prepared based on formulation proposed by Lukaszewicz *et al.* (2008). The preparation was based on the formulation in Table 2.

Table 2: Measurement of chemicals for preparation of Eosin-Nigrosin stain.

Chemicals	Measurements (g)
Eosin B	1.0
Nigrosin	5.0
Sodium citrate	3.0

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