Maintaining the life cycle of ornithodoros moubata using in vitro feeding

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Abstract: An inexpensive and efficient method for artificially feeding all the life-cycle stages of the tick Ornithodoros moubata on sheep blood in vitro was demonstrated. Two types of membranes were tested: a silicone membrane and a Parafilm membrane. There was a significant difference between the two membranes. Parafilm membrane was the better of the two. An artificial feeding device incorporating a Parafilm membrane was constructed and optimised for tick attachment and feeding. It was found that the ticks fed and engorged successfully through the Parafilm membrane without the need for the addition of a phagostimulant. Repletion and feeding survival rates of O. moubata using two different intervals between feeding were also compared. The tick survival rates for three monthly feeding intervals were significantly higher than for monthly feeding. All life cycle stages attached and began sucking blood within 5-10 minutes, generally becoming fully engorged within 10-90 minutes. Unwanted bacterial contamination in blood of what was avoided by changing the blood every 2 hours during tick feeding. The best feeding and survival rates were achieved with a constant mean blood temperature of 37 °C. The importance of the finding in the maintenance of the ticks in the laboratory was discussed.

Keywords: Ornithodoros moubata, artificially feeding, Parafilm membrane

Introduction

Ticks are obligate blood feeders, mating, moulting and oviposition frequently occurs only after feeding (1).

Accumulating evidence indicates that ticks infect humans and several domestic animals with wild types of pathogens (2, 3). Therefore, an artificial blood feeding technique would be highly desirable to replace the use of experimental animals. Ornithodoros moubata is a tick that parasitizes both humans and domestic pigs. It is naturally endemic in the tropics and poses a threat to the swine industry (4). It transmits severe diseases to the pigs and man, such as Borrelia duttonii, which causes the African human relapsing fever.

It is easily maintained under defined conditions (5) and has a relatively long lifespan that requires at least a year to complete one generation (3). O. moubata tick inhabits burrows made by swine and human dwellings to avoid the high temperatures of the tropics, usually between 40 - 50 ºC (6). The underground habitat provides an optimal temperature of approximately 26 - 29 ºC and a relative humidity of ~80 - 90%; both are required for the survival under natural conditions (7). Humidity and temperature are very important factors influencing the successful maintenance of this species in the laboratory.

It has been reported that the optimal laboratory conditions for the maintenance and the breeding of O. moubata are a temperature between 27 ºC and 30ºC and a relative humidity of 80-90% (8, 9). O. moubata ticks prefer to inhabit dark places and keeping them in glass tubes containing a small amount of sand (10) or in Petri dishes covered by sifted sand (11), or coated with some layers of filter papers (12) is a requirement for effective breeding.

To maintain a successful ticks' colony, the ticks need to be fed with blood. This is done by two methods; either by feeding the ticks on live laboratory experimental animal or by providing the blood, or artificial blood substitutes, through natural and artificial membranes, thus, eliminating or reducing unnecessary animal use (4, 8). Although artificial feeding on blood has several advantages over using live animals, some difficulties still occur.
Firstly, what type of membrane is to be used in the feeding process?
Two membranes proved to be successful. These were silicone membranes (4) and Para-film membrane (13). Secondly, the optimum incubation period between each feeding time has to be determined. Thirdly, the optimum conditions for the maintenance of blood are another problem. These include: temperature and contamination with bacteria. It has been recommended that blood supplemented with ATP, glucose and antibiotic as a feeding stimulus results in an increased rate of attachment and feeding (12, 14). Several researchers pointed that the dissection of salivary gland is an effective method to collect saliva (5, 15, 16). The diagnosis study by Baranda et al. (15) suggested that the component of salivary gland extract of O. moubata can be substance to pilocarpine-induced saliva in some certain stances and that using salivary gland extract instead of saliva did not affect the outcome of experiments. The main aim of this paper was to improve the breeding of O. moubata ticks in captivity, using artificial feeding, and to assess factors affecting the feeding such as the optimal mem-brane, blood temperature, intervals feeding period of in vitro feeding and the repletion time of O. Moubata which affect the feeding process and therefore the breeding of the ticks.

Materials and methods

O. moubata ticks used were obtained from specimens at the University of Glasgow, UK and were free from infection. They were maintained in Petri dishes with 2 filter papers or sand, which prevented them from exposure to direct light. The Petri dishes were covered with perforated lids and kept under the following standard conditions: temperature 28 ± 1 ºC, relative humidity of 60 - 90%, with a 16 hour light /8 hour darkness cycle. They were fed defibrinated sterile fresh blood from sheep. The blood was refrigerated at 4 ºC and was used within a week. No supplements were added to the blood. It was pre-heated to 37 ºC before using it in the feeding. The in vitro feeding of O. moubata followed the procedure described by (12) with modifications. Petri dishes, containing O. Moubata, were initially placed on a hot plate for 30 minutes at 30 ºC to activate them. A feeding apparatus was developed, tested and adopted. It consisted of a glass Petri dish (diameter 9.0 mm and wall height 1.5 mm). The Petri dish was filled with 30 ml of sheep blood. The glass Petri dish was placed on a hot plate for 15 minutes for warming. Approximately 40 adult ticks, or 100 nymphs, were placed in a 60 ml container with a white polyethylene

screw cap polystyrene 60 ml volume container (diameter 5 mm and height 7 mm). The container was sealed at the open top end with a stretched membrane. It was then inverted onto the glass Petri dish so that the membrane was in direct contact with the blood. Care was taken to avoid introduction of air bubbles between the membrane and the blood. The ticks were able to pierce it easily with their hypostome to take up the blood meal. To maintain a constant mean temperature of 37 ºC and to evenly distribute the blood, the feeding unit was placed on a heating magnetic agitator with a magnetic stirrer in the Petri dish. The blood was changed every 2 hours to avoid bacterial contamination (Figure 1).

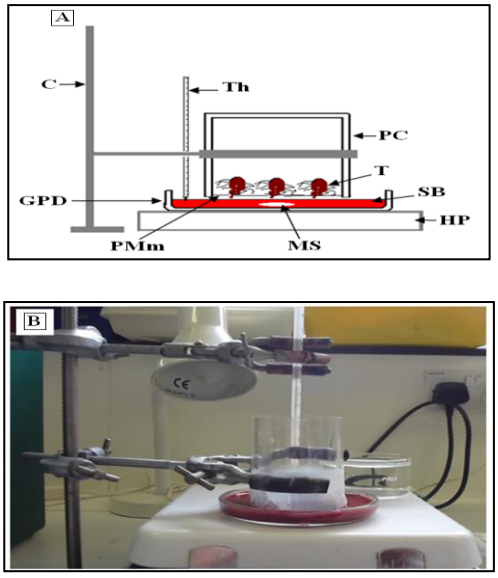

Figure 1: Schematic illustration of the membrane artificial ticks feeding device.

C, clamp; GPD, glass Petri dish; PMm, Parafilm M membrane; MS, magnetic stirrer; HP, hot plate; SB, sheep blood; T, ticks; PC, polyethylene container; Th, thermometer. B. Photograph of the apparatus for artificial feeding taken during the experiment, the lower receptacle (glass Petri dish filled with approximately 30 ml of sheep blood stirred using with magnetic stirrer) and the upper receptacle (container where the ticks are situated). The apparatus was placed on a hot plate.

Series of experiments were carried out to optimize the membrane feeding process. Two different membranes, the Silicone and the Parafilm membranes, were tested in the new in vitro feeding apparatus. To evaluate the difference of the attachment of the tick's rate to the membranes, these were tested with and without the addition of phagos-timulant such as an animal hair. The phagostimulant added was a fragment of cow hair. The silicone membrane was prepared as described by (17) and modified to aid attachment of O. moubata by rendering the silicone softer and thinner. Aquarium silicone sealant clear 310 ml was mixed with vegetable oil at a ratio of 4 : 1 using a glass rod. The mixture was placed on a layer of kitchen plastic film (70 x 120 mm) and care was taken to disturb the mixture to a very thin layer approximately (2.0 mm thick) using a plastic ruler. A layer of plastic mosquito net was applied on the mixture, the sealant with mosquito net was allowed to polymerise at room temperature for 16 hours to ensure complete drying. Subsequently, the silicone membrane was cut into squares (5.0 mm x 5.0 mm) and washed with 70% ethanol for 15 minutes followed by 3 changes of hot distilled water at 90 °C for 40 minutes. The squares of silicone membrane were left to dry at room temperature for 10 hours prior to use. The Parafilm was prepared by stretching tight Parafilm "M" laboratory film to produce membranes of the desired thickness. In each of the experiments to evaluate the membranes, two groups of ticks were used. Each group consisted of 20 ticks: 1 female, 1 male and 3 tick from each nymphal stage N1- N6. The ticks were then fed using the new apparatus, but with a different membrane each time. Each experiment was repeated 5 times. Once two or three ticks were attached to the

membrane and began to feed, others joined them and attached to the same site. Engorged ticks were identified by an increased body size of between 4 - 6 fold upon placing on a life stage scale. They were then removed with forceps and washed in distilled warm water to remove any remaining blood before they were returned to clean Petri dishes. They were placed on a hot plate at 30 ºC for approximately 30 minutes. The Petri dishes were removed and kept at constant temperature and humidity as described above.

The survival of the ticks was observed during the first 7 days after feeding. The survival rate of the ticks after feeding was calculated using the following equation:

$$\text{Survival rate (\%)} = \frac{\text{Number of the survived ticks}}{\text{Number of total fed ticks}} \times 100\%$$

The interval between feeding time is an important factor in the maintenance of ticks. Six groups of ticks were prepared. Each group consisted of 20 ticks as follows: 1 female, 2 males and 3 ticks from each nymphal stage N1-N6. The six groups were fed using the developed in vitro feeding apparatus. Three groups were fed once a month over a period of 6 months. The other three groups were fed every three months for three years.

To determine the optimum blood temperature for artificial tick feeding, 5 groups of starved ticks were formed as follows: 1 female, 2 males and 3 ticks from each nymphal stage N1- N6. They were then uninterruptedly fed with sheep blood at temperatures of 27, 32, 37, 42 and 47 °C for an hour. Subsequently the percentage feeding of each group was calculated. The experiment was repeated 5 times and an average of the 5 trials was calculated. Because the repletion time of ticks is a very important factor for their survival, engorging nymphs were collected after every 10 minutes for one hour: i.e. 10, 20, 30, 40, 50, 60 minutes. The engorged adults were collected every 30 minutes at: 30, 60 and 90 minutes of engorging. The tick survival rate was calculated weekly.

Statistical Analysis

SPSS programme (version 19) was used. The independent samples t-test was used to compare two groups of data. Analysis of one-way variance (ANOVA) was used to compare more than two groups of data. In order to determine where the significant difference exactly came from, the Multiple Post-hoc test (Turkey) was used.

Results

Mating of ticks was observed by the naked eye after females were replete during the feeding time. Female parasites began to lay eggs after 12 days of feeding. A single female produced batches of eggs of about 100-150 at a time. The eggs hatched after 2 - 3 weeks into immotile larvae. Ecdysis did not occur until 2 - 3 weeks after feeding. All the nymph stages moulted 10-14 days after feeding and developed to the next stage. After tick engorgement, all the life cycle stages of ticks were inactive and rapidly evaded the light by burying themselves between the sheets of filter paper or in the sand.

Figure 2 shows that the mean percentage of ticks successfully fed on Parafilm membranes (91.0%) was highly significant than those fed on silicone membranes (9.0%), $p = 0.0001$. The nymphal stages never

attached to any membrane. No difference in tick attachment rate was observed with or without using the cow hair as stimuli with membranes.

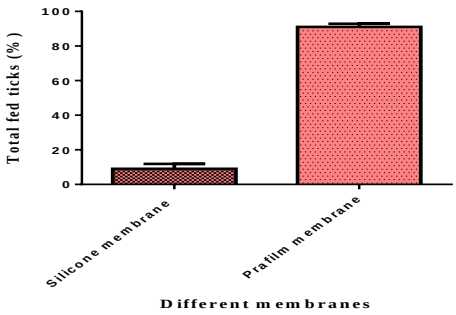

Figure 2: The comparison of artificial membranes.

Two non-living mem-branes; silicone and Parafilm used to artificially feed O. moubata. The bars represent mean ± SD for five replicates per variable according to percentage of tick fed. Data analysis showed a significant difference between using silicone and Parafilm membranes for tick attachm-ent and feeding.

The results of the feeding experiments at monthly and six months intervals are presented in **Error! Reference source not found.**. The mean survival rate after monthly feeding was poor (33.0%) when compared with survival rates (97.0%) of ticks fed every 3 months. The difference was highly significant (p = 0.0005). The statistical analysis showed a significant variation within N1 and N2 (*t*-test = $F_{1,207}$ = 22.323, p = 0.0001) and N3 (t-test = $F_{6,17}$ = 13.623, p = 0.001), the mean number of ticks surviving for three months and monthly feeding regimes, were (176.38, 155.36 & 85.29) and (16.33, 12.5 & 9.0) respectively. The optimal temperature suitable for artificial feeding was found to be 37 ºC (Figure 4). This was determined by the mean number of fed and unfed ticks (p = 0.0001).

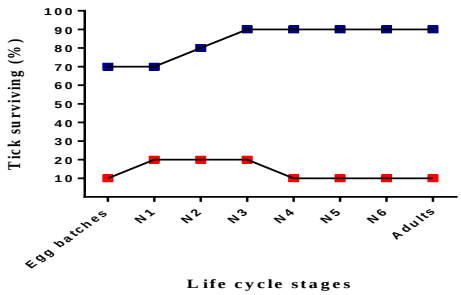

Figure 3: Comparison of breeding rates between monthly (Red, squares) and three months feeding intervals (blue, squares) for tick maintenance.

The histogram representing the perce-ntage of total ticks survival of each life stages separately, including egg batc-hes produced (egg batches), as well as nymphal stages; nymph1 (N1), nymph 2 (N2), nymph 3 (N3), nymph 4 (N4), nymph 5 (N5), nymph 6 (N6) and adults. There was a remarkable difference in the mean value of the egg batches, N1, N2, N3, N4, N5, N6 and adult.

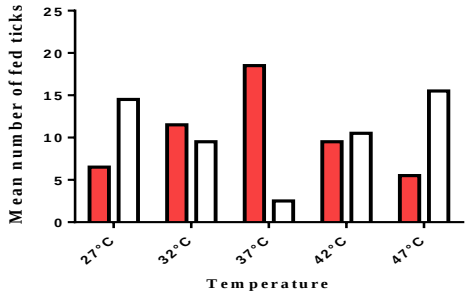

Figure 4: Effect of blood temperature on the fed ticks using *in vitro* feeding system.

Five groups of ticks were tested during artificial feeding experiments using sheep blood. The bars represent the mean value of fed (Orange bars) and unfed ticks (White bars) within the five different blood temperature conditions ranging from 27 ºC to 47 ºC. Two-way ANOVA analysis showed that the optimal temperature of the blood in artificial feeding, based on the mean value of fed ticks, is 37 ºC.

The rate of tick repletion life stages is represented in **Error! Reference source not found.**. Ticks attached to the Parafilm membrane within 10-90 minutes. The time of engorgement was associated with the size of life stages. Male ticks became engorged within 60 minutes, whereas females required approximately 90 minutes to become fully engorged. Most of the nymphal stages attached rapidly to the Parafilm membrane and became engorged within 15 - 50 minutes (**Error! Reference source not found.**).

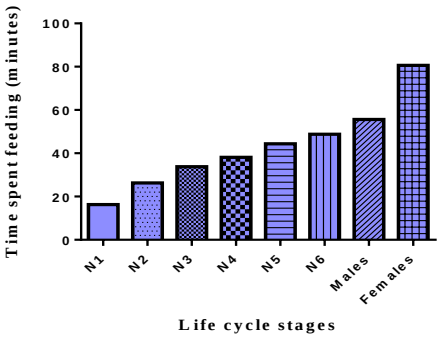

Figure 5: Length of engorgement time of the fed life cycle stages of *O. moubata*.

The time spent feeding in minutes. The histogram representing the means of total ticks engorgement of each life stages separately; nymphal stages; nymph1 (N1), nymph 2 (N2), nymph 3 (N3), nymph 4 (N4), nymph 5 (N5), nymph 6 (N6), males and females.

There was a significant difference between male and female engorgement times (t-test $=F_{1, 203} = 28.023$, $p = 0.0001$).

Discussion

The present work proves the possibility to maintain and breed O. moubata ticks in the laboratory without feeding them on live animals. Several trials of artificial feeding were tested and adopted using Parafilm membrane and blood from pigs, cattle and sheep (9, 18). Mango and Galun (18) concluded that blood from pigs, cattle and sheep were similar when used for in vitro feeding. The present investigation supports the previous findings that the use of defibrinated sheep blood satisfies the nutritional needs of the ticks (8, 12). The Parafilm membranes permit good attachment rate and repletion of soft ticks when compared with the silicone membranes. This finding is consistent with the previous findings of Hokama (8) and Ruheta (9) but it contrasts the results of Osborne and Mellor (4) who stated that artificial feeding of O. moubata using a silicone membrane was a success. This discrepancy could be due to the different methods used in the preparation of the membranes. The membrane used in this study was prepared following the procedure by Krober and Guerin (17) with some modifications. They used the membrane to feed Ixodidae ticks. Another reason for the discrepancy could be the possibility that the use of such a membrane is not appropriate for the feeding of Argasidae ticks regarding the morphological differences between the mouthparts of those families (19, 20). Parafilm membrane offers the advantages of both being readily available and cheap. The findings proves that a non-living membrane can be used without phagostimulant for the in vitro feeding system of O. moubata. This contradics the findings by Hokama (8) who demonstrated that O. coriaceus soft ticks did not feed through Parafilm membrane unless guinea pig hair was used as a phagostimulant.Previous studies demonstrated that the establishment of breeding the ticks in a laboratory colony had only limited success (9). The poor survival rate of the ticks could be due to using a

monthly feeding regime. A feeding period of three months is appropriate for the maintenance and breeding of the ticks in captivity. Jobling (10) reported that the mean time of feeding of females was longer than all the nymphal stages, and twice as lengthy as in males. The length of feeding time plays a vital role in reducing the mortality and increasing tick production. Once a female is fully engorged, it maintains attachment to its host whilst waiting to mate. The repletion of females causes an obvious acceleration in the rate of oviposition (1). Thus, the present results agree with the early studies on ticks.

It has been reported that the temperature of blood is very important and must be maintained within the range of 36 - 39 °C, and that bacterial contamination is a decisive issue in the breeding of ticks in the laboratory (8, 21). The present finding supports the conclusion that constant temperature is critical. The optimal blood temperature for tick attachment and feeding is 37 °C. The frequent changing of blood avoids the bacterial contamination. The modification of the maintenance conditions to favourable ones results in an increase in the feeding of ticks and their survival rates. Thus, the modifications described is effective in maintaining the life cycle of O. moubata in vitro, and will avoid the use of live animal hosts in the feeding of ticks in the laboratory.

In conclusion, modifying of the maintenance conditions results in an increase in the fed ticks being fully engorged and in their survival rates. The maintenance conditions to be adjusted are the incubation period between each feeding time, the blood temperature and contamination, and the length of feeding time of different life cycle stages. This feeding assay can also be utilized to carry out advanced investigations on a variety of relevant topics. Furthermore, the replacement of live animals by using blood feeding units would make it possible to establish high through put methods to examine products against pathogens which the ticks transmit**.**

References

1. Gregson J (1964) problems pertaining to culture procedure for some Ixodid ticks. Bull org mond. 591-594.
2. Jasinskas A, Jaworski DC and Barbour AG (2000) Amblyomma americanum: specific uptake of immunoglobulins into tick hemolymph during feeding. Exp Parasitol. 96: 213-221.
3. Jongejan F and Uilenberg G (1994) Ticks and control methods. Rev Sci Tech 13: 1201-1226.
4. Osborne RW and Mellor PS (1985) Use of a silicone membrane feeding technique in the laboratory maintenance of a colony of Ornithodoros moubata. Tropical animal health and production 17: 31-38.
5. Oleaga A, Escudero-Poblacion A, Camafeita E and Perez-Sanchez R (2007) A proteomic approach to the identification of salivary proteins from the argasid ticks Ornithodoros moubata and Ornithodoros erraticus. Insect Biochem Mol Biol. 37: 1149-1159.
6. Estrada-Pena A and Jongejan F (1999) Ticks feeding on humans: a review of records on human-biting Ixodoidea with special reference to pathogen transmission. Exp Appl Acarol. 23: 685-715.
7. Morel PA and Oriss TB (1998) Crossregulation between Th1 and Th2 cells. Crit Rev Immunol. 18: 275-303.
8. Hokama Y, Lane R and Howarth J (1987) Maintenance of adult and nymphal Ornithodoros coriaceus (Acari: Argasidae) by artificial feeding through a parafilm membrane. J Med Entomol. 319-323.
9. Ruheta MR, Noboru I and Kozo F (2005) Advances in the artificial feeding of Ornithodoros moubata (Acari: Ixodidae) and the follow up of its life cycle after feeding on fetal bovine serum. Protozool. 57-62.
10. Jobling B (1925) A contribution to the biology of Ornithodoros moubata, Murray. welcome bureau of scientific research, London.
11. Schwan EV, Hutton D, Shields KJ and Townson S (1991) Artificial feeding and successful reproduction in Ornithodoros moubata moubata (Murray, 1877) (Acarina: Argasidae). Exp Appl Acarol. 13: 107-115.
12. Lucius R and Textor G (1995) Acanthocheilonema viteae: rational design of the life cycle to increase production of parasite material using less experimental animals. Appl Parasitol. 36: 22-33.
13. Rennie L, Wilkinson PJ and Mellor PS (2000) Effects of infection of the tick Ornithodoros moubata with African swine fever virus. Med Vet Entomol. 14: 355-360.
14. Krober T and Guerin PM (2007) An in vitro feeding assay to test acaricides for control of hard ticks. Pest Manag Sci. 63: 17-22.
15. Baranda JA, Perez-Sanchez R, Oleaga A, Manzano R and Encinas-Grandes A (2000) Purification, N-terminal sequencing and diagnostic value of the major antigens of Ornithodoros erraticus and O. moubata. Vet Parasitol. 87: 193-206.
16. Garcia-Varas S, Manzano-Roman R, Fernandez-Soto P, Encinas-Grandes A, Oleaga A and Perez-Sanchez R (2010) Purification and characterisation of a P-selectin-binding molecule from the salivary glands of Ornithodoros moubata that induces protective anti-tick immune responses in pigs. Int J Parasitol. 40: 313-326.
17. Krober T and Guerin PM (2007) In vitro feeding assays for hard ticks. Trends Parasitol. 23: 445-449.

18. Mango CK and Galun R (1977) Ornithodoros moubata: breeding in vitro. Exp Parasitol. 42: 282-288.
19. Sonenshine D (1991) Biology of ticks. Oxford University Press.
20. Sauer JR, McSwain JL, Bowman AS and Essenberg RC (1995) Tick salivary gland physiology. Annu Rev Entomol. 40: 245-267.
21. Waladde S, Ochieng S and Gichuhi P (1991) Artificial-membrane feeding of the Ixodid tick, Rhipicephalus appendiculatus, to repletion. Exper. Appl, Acarol. 297-306.