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Isolation and Characterization of Midgut Symbiotic Bacteria from Tsetse Flies (*G.Pallidipes*) and Their Role in Biological Control Methods

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Abstract: *In this study isolation and Characterizations of Mid gut symbiotic bacteria from tsetse flies (Glossina pallidipes) and vector competence were undertaken. 50 flies were collected from Nech SAR National Park and brought to Arba Minch University, Laboratory of genetics and molecular biology. The tsetse flies were sterilized and dissected to isolate the symbiotic bacteria from the midgut. Various morphological and biochemical studies were conducted for characterization of the dominant isolate. Among the culture 13 isolates were found Gram negative, rod shaped, non motile and Catalase negative. The probable isolate found to be Sodalis sp. under the family Enterobacteriacae. The antibiotic sensitivity against these bacteria was performed and chloroamphenicol found to be potent to limit the growth of the isolate. The effect of chloroamphenicol on Tsetse flies were further checked and observed that it has no effect on the fertility of the tsetse but has indirect effect on the reproduction capability of the vector. Here we can conclude that chloroamphenicol could be taken as indirect alternative biological control strategy in tsetse flies.*

Keywords: *Antibiotics, Chloroamphenicol, Glossina, sodalis, Symbiont, Tsetse*

1. INTRODUCTION

Livestock is an integral part of agricultural System in Ethiopia. According to CSA (2013), 82.4% of total population engaged in agriculture, of which, the country stands 1st in Africa and 10th in the world in livestock population. The Domestic animal population of the country is estimated to be 53.99 million cattle, 25.5 million sheep, 24.06 million goat, 0.92 million camel, 50.38 million poultry, 1.91 million horses, 0.35 million mules and 6.75 million donkeys (CSA, 2013). The full utilization of the product from such a huge number of livestock population particularly cattle's have been hampered due to tsetse transmitted trypanosomiasis (Southern Tsetse Eradication Manual, 2010).

The total area infested by tsetse flies is estimated to be 240,000km^2 in Southern, South western, Western, and North western part of the Ethiopia. Five species of tsetse flies, such as *Glossina pallidipes, Glossina fuscipes, Glossina longipennis, Glossina tachnoides and Glossina morsistans* exists commonly in the aforementioned areas. Both sexes of tsetse flies are haematogenous where transmission of trypanosomiasis occurs commonly (Southern Tsetse Eradication Manual, 2010). Therefore, Tsetse transmitted animal trypanosomiasis is a potential treat to livestock agricultural development and productivity in the country.

The trypanosome parasites could be injected when an infective tsetse pierces the skin to take a blood meal from the host. In such a case, trypanosomiasis have become mainly a serious constraint to livestock production in areas of the South west of Ethiopia at altitudes lesser than 1700 m above sea level (Langridge, 1976).Tsetse flies (Diptera: Glossinidae), which feed exclusively on vertebrate blood, harbour three distinct species of endosymbiotic bacteria that presumably play different roles in the flies. These bacteria include *Wigglesworthia glossinidia*, genus *Sodalis*, and genus *Wolbachia.* These symbioses are successful in large part because the above-mentioned bacteria have adapted to the tsetse flies unique viviparous reproductive physiology. Studies have been carried out on tsetse gut microbiota and the contribution of microbial symbionts to the host's nutritional homeostasis (Dillon and Dillon, 2004). Little attention has been given to the economic impact of the tsetse and the symbiont in vector competence in Gamo Gofa, Southern Nations, Nationalities and Peoples Regional State (SNNPRS). For example Arba Minch has about 68,323 cattle population (ATAO, 2013).

1.1. Statements of the Problem

Trypanosome transmission resulted from the multifactors interaction between several organisms, including the pathogen, the vector, wild reservoirs, and the human host. While this interdependence can be complicated, it provides numerous opportunities for interfering with the transmission of the disease (Akman *etal*, 2001). However, current solutions involve treating infected hosts with synthetic chemicals and/or inhibiting further transmission by attempting to reduce the insect vector population with insecticides remain in effective (Akman *etal*, 2001, Docampo and Moreno. 2003; Donelson, 2003).The vector because of the midgut micro-flora ensure vector competence, unless alternative method is designed to control the development of symbionts inside, the vector might cause a serious problem in livestock productivity particularly in Ethiopia. The main problems associated with them are mortality, abortion, decreased in milk and meat production, weakening of draft and ploughing animal (STEP Manual, 2010).

1.2. Significance of the Study

Now a day in Ethiopia the widely used control method of tsetse fly is the use of Sterile Insect Techniques (SIT) and insecticide without the clear understanding of the role of endosymbiotic bacteria. Among the three endosymbiotic bacteria only *Glosinidus sodalis* is culturable in the laboratory (Dale and Maudlin, 1999). This bacterium has the role in facilitating penetration of the midgut membrane and hence entry of the parasite in to the mid gut for maturation and growth. Controlling of this process will have significant impact in vector competence and hence control of vector and the damage due to tsetse transmitted trypanosomiasis. The study of such symbiotic bacteria for tsetse fly vector competence is little or incomplete.

The various species of the tsetse flies and their midgut micro-symbiotic bacteria have not been assessed as such. Hence, the present study will give a focus on characterization of midgut symbiotic bacteria from tsetse flies, its role in vector competence, and to use it as baseline information for biological control methods.

2. OBJECTIVES

2.1. General Objective

- To isolate symbiotic bacteria from the mid gut of *Glossina pallidipes* in the laboratory and evaluate its role in biological control methods.

2.2. Specific Objective

- To isolate symbiotic bacteria from mid guts of tsetse flies.
- To characterize bacterial flora based on biochemical and morphological test.
- To evaluate the role of the bacteria in vector competence
- To identify appropriate antibiotic against symbiotic bacteria to be used for biological control method.
- To cultivate the isolated bacteria
- To determine their sensitivity to antibiotics administered through the blood meal of the fly

3. MATERIALS AND METHODS

3.1. Description of Study Area and Sample Collection

Fifty flies of *Glossina pallidipes* were randomly collected from Nech SAR National Park, Arba Minch. Arba Minch is one of the fifteen woredas and two administrative town of Gamo Gofa zones. It is located at 505 km South of Addis Ababa in Gamo Gofa Zone, Southern Nations, Nationalities and Peoples Regional State (SNNPRS). It is situated in Great African Rift Valley at an elevation of about 1285 meters above sea level according to the Arba Minch town administration office report (ATAO, 2013).

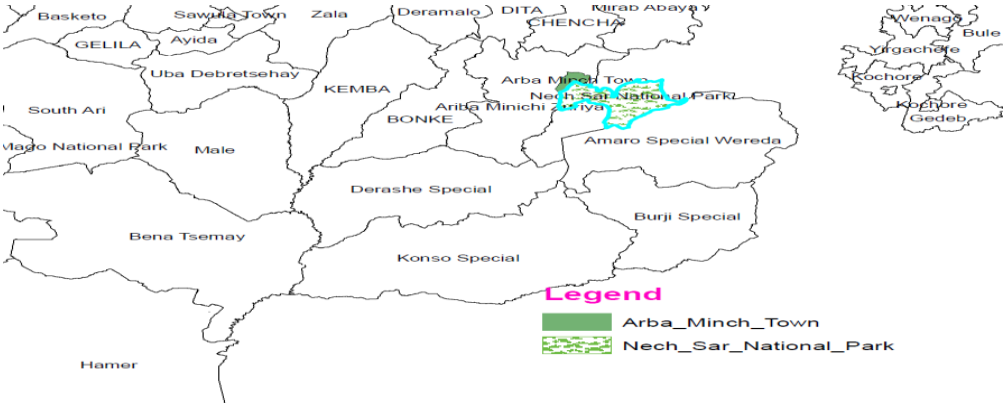

Figure3. *Map of the study area*

3.2. Study Methodology

3.2.1. Materials

The lists of equipment and chemicals used includes trap, phenol, acetone, cow urine, adult tsetse fly, pin, rubber and grease, fly dissection kit, test tubes, auto clave, vortex shaker, magnetic stirrer, ph meter, incubator, weighing balance, flasks, laminar air flow cabinet, eppendroffs tubes, Ethanol, phosphate-buffered saline (pbs), inoculating loop, petridish, microscopic slide, oil immersion, refrigerator, stereo microscope, hot plate, sodium hypochlorite, distilled water, Bunsen burner, glass rod, feeding cage and membrane for feeding.

3.2.2. Methods

- Tsetse Capture and Identification

Tsetse flies were identified by using a modified entomological key (Buxton, 1955). The tsetse fly species *Glossina pallidipes* was randomly collected from Nech Sar National Park, Arba Minch. Then the adult tsetse flies were taken to the laboratory, Arba Minch University for dissection and further experiments.

The method for collection of the flies is shown in figure 4.

Figure4. *Collection of flies from the Nech SAR National park by using NGU trap*

- Dissection of Tsetse

A newly collected adult flies were selected and dissected for midgut micro-biota isolation and identifications. Dissections were made under sterile condition (FAO, 1982). For this individual insect was then surface sterilized with a 70% ethanol solution and rinsed three times in sterile phosphate-buffered saline (PBS). Midgut of each insect was then carefully removed using clean forceps and homogenised in 100µl of sterile PBS using eppendroff tubes and was kept in -4^{0}C freeze. The whole procedure was performed using stereo microscope (10× magnification).

- Culture Media Preparation and Isolation of Midgut Symbiotic Bacteria

The Mitsuhashi and Maramorosch insect (MMI) media was prepared by using the chemicals mentioned below in Table 1.

Table1. *Chemical composition of MMI insect culture media*

Ingredients	Grams/Liter
Calcium chloride dihydarate	0.19
Magnesium chloride anhydrouse	0.046
Potassium chloride	0.2
Sodium chloride	7
Sodium phosphate monobasic	0.173
D(+)glucose	4
Casein hydrolysate	6.5
Yeast extract	5
agar	15

The midgut homogenate was cultured for isolation of symbionts. The Mitsuhashi and Maramorosch insect (MMI) culture medium was used in this study using the streak plate method and a pure isolated bacterial colony was obtained (Dale and Maudlin, 1999). All cultures were maintained at 37°C under aerobic conditions for 24 hours. Distinct colonies were taken for further purification of isolates. Individual bacterial colonies were re-streaked then a single pure colony was isolated. Colonies with distinct morphologies, colours and margins were picked and sub-cultured to obtain pure bacterial isolates. Pure colonies harvested were stored at −4°C.

- Screening of Symbiont Isolate

Screening and characterization of bacterial isolates were evaluated based on morphological and biochemical property of the isolates. Here various biochemical (citrate utilization, methyl red, voges-Proskauer test, indole test, starch hydrolysis, casein hydrolysis test and catalase) and morphological (Gram staining, Motility, colony Shape, Size and color tests) were implemented. Then, the identification was made according to Berg's manual of determinative bacteriology (appendixI). All procedures for the biochemical test were shown on appendix V and VI

- Antibiotic Susceptibility Test

Using aseptic technique a sterile swab from a broth culture was taken by gently pressing and/or rotating the swab against the inside of the tube, using the swab Muller-Hinton agar was streaked and then the plate was allowed to dry for five minutes. Using a flame sterilized forceps a disc was pressed gently to allow disc containing a specific antibiotic to the plate and finally the plate was incubated over night at a temperature of 37^0c (Mohantly, 2010).

The antibiotics used for the sensitivity tests in different plates were streptomycin (10µg), tetracycline (30 µg), and penicillin g (10 µg), ampicillin (10 µg), chloroamphenicol (10 µg), gentamycin (10 µg), erythromycin (15 µg) and cloxacilin (30µg) (manufacture's recommendation). With this method, zone of inhibitions were measured for each of the antibiotics using a ruler, and then high zone of inhibition was taken for further study (Mohantly, 2010).

- Insect Feeding and Effect of Antibiotic

According to the standard guide line manual of FAO/IAEA(FAO, 1982), a Gamma cell sterilized blood which was collected from abattoir was given to the 140 tsetse flies treated with antibiotic for 70 and untreated for the other 70 flies on the feeding membrane as shown in figure 6.

Figure5. *Placing of field collected flies into a feeding cage*

Figure6. *Feeding of flies (A/Membrane feeding of flies B/ Fed flies)*

For this study the blood was mixed with antibiotic (Chloramphenicol 30 µg/ml, cloxacillin 30 µg/ml and Gentamycin10 µg/ml) and the control was prepared without the antibiotics. The effect of antibiotic on the growth of symbiotic bacteria which has role in transmission of trypanosomiasis and hence helps in vector competence was monitored for 21 days. Then after, the flies were also dissected to check the presence of the symbiotic bacteria, fecundity and longevity. Accordingly, the death of isolate, fecundity and longevity of flies in both experimental and control groups were counted and compared in order to see the effect of antibiotic.

- Study Design and Data Analysis

Cross-sectional type of study was used in this study. The entire data source was recorded in Excel spread sheets and then it was displayed using tables and graphs. All the experiments were conducted in duplicates.

4. RESULTS

4.1. Isolation and Characterization of Symbionts

The 50 adult tsetse flies from various corners of Nech SAR national park were collected for this study. Randomly selected tsetse flies were sterized and dissected for isolation and characterization of symbiotic bacteria harboring in the midgut of the insect. Around 100 colonies were isolated in stepwise isolation procedures; of which 16 isolate were motile and the 84 are non-motile. Then 100 colonies were further characterized based on their morphological and biochemical properties. The result is shown in table 2 and figure 8.

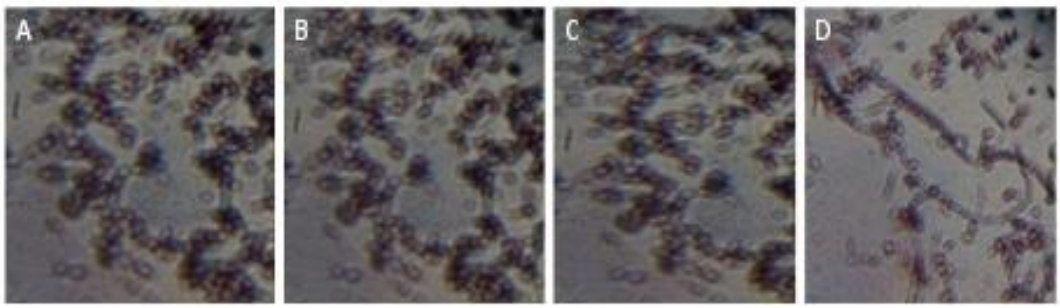

Figure7. *Gram stained morphology of bacteria A) Gram negative cocci rod B) gram negative cocci and rod C) Gram negative cocci D) Gram negative yellow*

The morphological features of the isolates such as Gram-negative, non-motile, rod-shaped nature were similar to enterobacteriacae family based on Bergey's manual of determinative bacteriology (appendix I). The result confirmed that the probable isolate dominantly dwell in tsetse flies midgut was *Sodalis* sp. (table 2).Then the aforementioned isolates further examined for their biochemical properties.

Table2. *Morphological characteristics of the bacteria*

No of Isolates	e Morphological characterization				
	Microscopic characteristics		Colony characteristics		
	Gram staining	Motility	Shape	Size	color
16	G -ve	motile	Rod	small	Yellowish
18	G +ve	Non motile	Rod	small	Yellowish

30	G +ve	Non motile	Circular	small	Yellowish
28	G -ve	Non motile	Rod	small	Yellowish
8	G +ve	Non motile	circular	small	Yellowish

The result of the biochemical tests is shown below in table 3 and figure 8. It was further confirmed that common isolate harboring in the midgut of the tsetse flies is *Sodalis sp*.

Table3. *Biochemical characterization of bacteria*

Isolates	Biochemical test characterization						Casein hydrolysis
	Catalase	Citrate utilization	Methyl red	VP test	Indole Test	Starch hydrolysis	
22	-ve	+ve	-ve	+ve	-ve	-ve	-ve
14	-ve	+ve	-ve	-ve	+ve	-ve	-ve
36	-ve	+ve	+ve	-ve	+ve	-ve	-ve
13	-ve	+ve	+ve	-ve	+ve	-ve	-ve
15	-ve	+ve	+ve	-ve	+ve	-ve	-ve

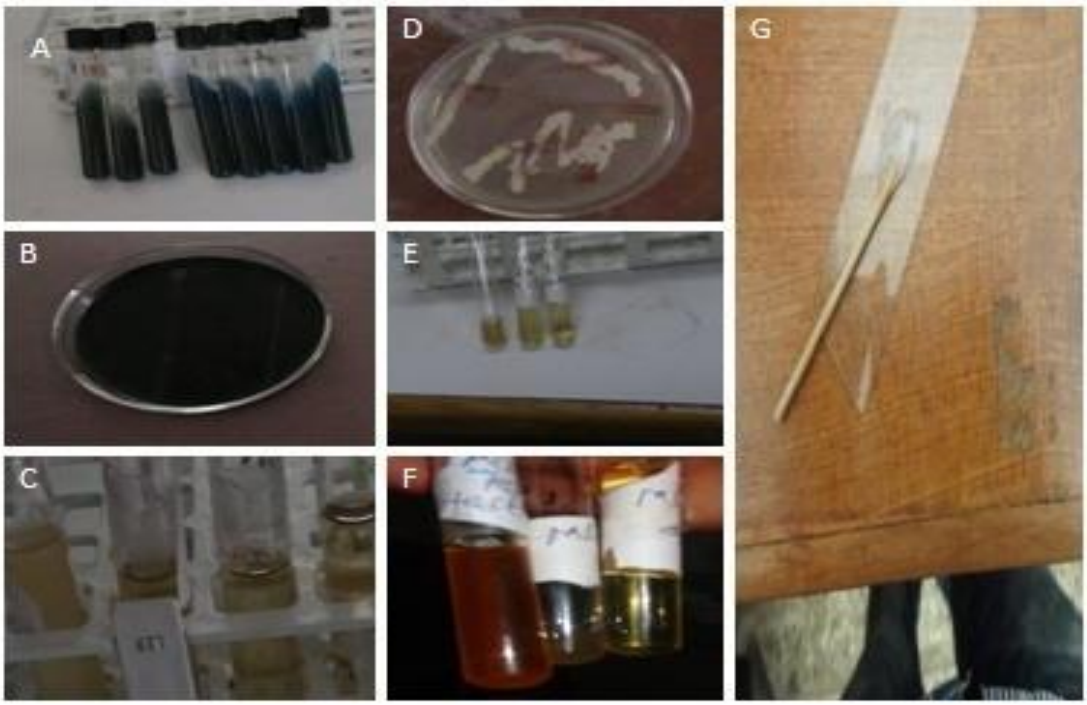

Figure8. *biochemical tests: A) Citrate utilization test B) Starch hydrolysis test C) Indole test D) casein hydrolysis test E) Vogus proskauer test F)Methyl red test G) Catalase test*

4.2. Effects of Antibiotics on the Isolate Sodalis Sp

Antibiotic susceptibility tests were conducted using various antibiotics streptomycin (10µg), tetracycline (30 µg), penicillin g (10 µg), ampicillin (10 µg), chloroamphenicol (10 µg), gentamycin (10 µg), erythromycin (15 µg) and cloxacilin 30µg in µg /ml against *Sodalis* sp. The zone of clearance was recorded. The result showed that chloramphenicol is the best antibiotic so as to control growth of *Sodalis* sp. However, penicillin g and streptomycin were found to be ineffective (Table 4).

Table4. *Antibiotic susceptibility of the isolates*

Antibiotic	Unit	Zone of inhibition	Sensitivity isolate
Penicillin G	10 µg	19mm	Sensitive 2
Streptomycin	10 µg	16mm	Sensitive 2
Erythromycin	15 µg	31mm	Highly sensitive 2
Gentamycin	10 µg	28mm	Highly sensitive 2
Cloxacilin	30 µg	29mm	Highly sensitive 2
Ampicillin	10 µg	22mm	Sensitive 2
Chloramphenicol	30 µg	32mm	Highly sensitive 2
Tetracycline	30µg	25mm	Sensitive 2

4.3. Effects of Antibiotics on the Vector

For this study 140 tsetse flies were collected and brought to Arba Minch University to evaluate the effect of antibiotics based on the order of manufacturers (µg/ml) (Gentamycin 100ml, Cloxacilin 500mg and Chloramphenicol 500mg) that showed maximum clearance of the *Sodalis* sp. growth. The study was conducted preparing the blood meal mixed with antibiotics and the controls were prepared without the antibiotic. All experiments were maintained in separate identical cages and incubated at 25 °C for 21 days. Then after, flies were removed, sterilized and dissected. The bacterium were isolated and transferred to culture media and maintained at 30°C. Throughout the experiment the number of larvae laid (fecundity), longevity and number of flies dying were recorded.

Seventy flies were feed with blood meal treated with antibiotics Chloroampenicol 500mg of these 10 had died by the day 21 (five were removed for dissection and culturing) but Cloxacillin and Gentamycin treated 70 flies resulted in death. In addition, from the other seventy control flies 6 flies died and 5 flies were removed for dissection at the same day. Flies from both control and treated groups died, because of starvation, none of the control and treated group died with blood in the alimentary canal (Figure.9). Five control and four experimental flies produced larvae on the 10th day (figure.10). However, Gentamycin 100ml, Cloxacilin 500mg treated flies doesn't produced any larvae.

The study showed that chloroampenicol can be a potential antibiotic clear the *Sodalis* sp. and make the tsetse incompetent.

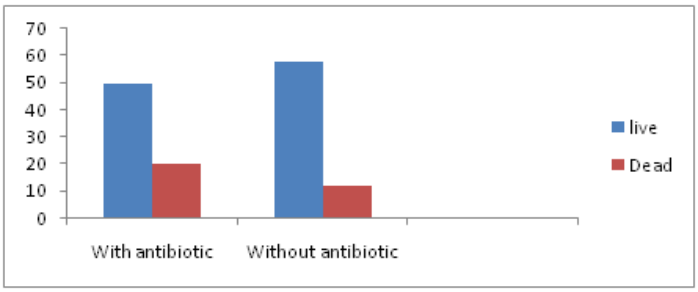

Figure9. *Comparison of feeding of flies with and without antibiotic*

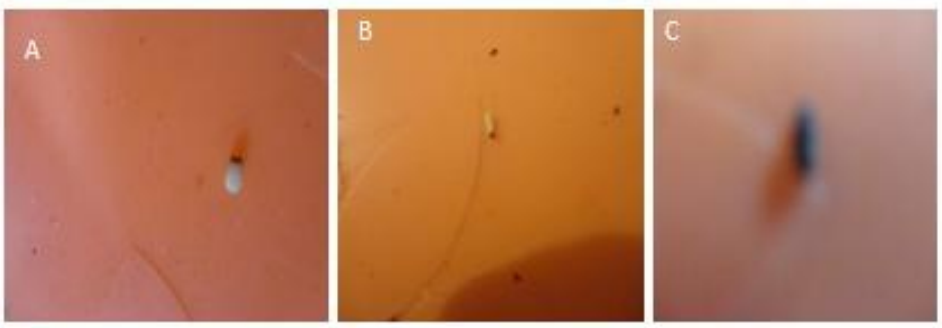

Figure10. *Tsetse larvae development A) experimental B) control C) Pupae*

The study further assessed the effect of the antibiotic on the *Sodalis* sp. isolated from experimental and controls tsetse flies midgut. The result showed that the experimental group of tsetse flies had no *Sodalis* sp. grown, however the control group had *Sodalis* sp as shown in figure.11.

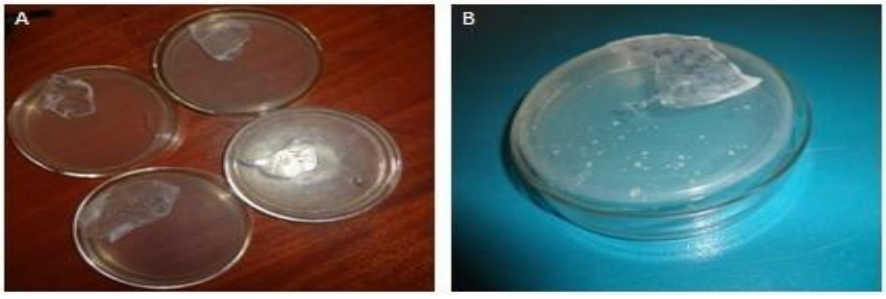

Figure11. *Growth of Sodalis sp isolated A) Experimental B) Control*

5. DISCUSSION

Tsetse flies are the major problem both productivity and quality of livestock in Ethiopia. Various studies showed that the vector competence is the result of the symbiotic bacterial harboring particularly in the midgut. Unlike other insect symbionts such as the intracellular *Wigglesworthia* and *Wolbachia* bacteria, *Sodalis* bacteria commonly found in tsetse flies midgut. The functional role of *Sodalis* symbiosis in tsetse is unknown; however, some studies showed that its elimination by streptozotoicin is associated with decreased longevity in the progeny (Dale and Welburn, 2001). Other studies also confirmed that *Sodalis* is implicated in the host's ability to establish trypanosome infections (Welburn and Maudlin 1991; Welburn and Maudlin, 1999). *Sodalis* has been detected in the milk gland organ and thus may be transmitted to the progeny via the milk gland secretions and Vector competence (Dale and Maudlin, 1999; Dale and Welburn, 2001).

Hence our study focus on the search of symbiotic bacteria from midgut of tsetse flies collected from Nech SAR National park, Ethiopia. The isolation and characterization of the bacteria mentioned was undertaken by morphological and biochemical test using Bergey's manual of determinative bacteriology.

The study considers 50 tsetse flies collected from various corners of the park were subjected to sterilization and dissection in order to isolate the bacteria from the midgut. Around 100 distinct colonies were isolated in a stepwise procedure. Base on the morphological features 16 colonies were motile and the rest were non-motile (Table .2). In terms of colony shape around 62 were found to be rod shaped and the rest were nearly circular. The color of the entire colony was found to be yellow (Table.2). From the result showed that the isolates were showing characteristics similar to Enterobacteriacae family most likely 28 of the 100 colonies showed similarity to the genus *Sodalis.*

On the basis of biochemical features various tests were conducted of which 13 were catalases negative and the rest were positive. This negative catalase test particularly confirmed that the probable isolate is *Sodalis* sp as shown in table 3. Toh *etal.*, 2006, showed the result similar to our findings .

The effects of antibiotics such as penicillin g, streptomycin, erythromycin, gentamycin, cloxacilin, ampicillin, chloramphenicol and tetracycline in recommended doses against *Sodalis sp* were evaluated. The result showed that Gentamycin, Cloxacilin and Chloramphenicol were the potent antibiotics that control the growth of *Sodalis*. Of which Chloramphenicol scored the highest zone of clearance in our study (Table 4). On the other hand, Penicillin G and Streptomycin were shown insignificant effect on the growth of the bacteria. Research also reported that penicillin antibiotics were not be able to affect the intracellular forms of the *Sodalis* symbionts (Beard, 1993). In their report, the penicillin antibiotics do not impair the transmission of *Sodalis* to progeny as well. Molecular biological study confirmed that the resident *Sodalis* population in the milk is apparently cleared; intracellular forms remain within the milk gland cells (Beard, 1993; Dale and Welburn, 2001).

The effect of the antibiotics particularly Chloramphenicol on the vector growth and development were further evaluated. For this study 140 new tsetse flies were collected and the experiments were carried out in two groups experimental and control. The experimental group includes 70 flies fed on blood meal treated with antibiotic. Around 10 flies were found dead within 21 days of incubation. From the experiment there is no significant difference between the control and the experimental groups. The cause for the death of the tsetse flies seems normal due to starvation and the change in environment (Figure 9). In addition some of the tsetse flies both from the experimental and control groups were able to lay larvae within 10 days of the incubation time. Hill *etal.*, 1973 reported the treatment of tsetse flies with antibiotics affects the fertility of the females similar to our study.

These result showed us a direction to check the change in the dynamics of the microbiota populations. The insects both from the control and experimental groups were taken sterilized and dissected to assess the presence of microbes from the midgut.The results were observed to contain more growth of *Sodalis* on control and no growth of bacteria on the experimental groups (Figure 11).

According to the report by, Beard, (1993), the presence of intact *Wigglesworthia and Sodalis* in the bacteriome organ of antibiotic-treated females enables the maintenance of host fertility and results in a fecundity index comparable to that of control flies. The functional role of *Sodalis* symbiosis in tsetse has been reported to be associated with decreased longevity in the progeny (Dale and Welburn, 2001).

Other studies also confirmed that *Sodalis* is implicated in the host's ability to establish trypanosome infections and proliferation (Welburn and Maudlin 1991; Welburn and Maudlin, 1999). All these findings confirmed that *Sodalis* is the vital principle for vector competence and Chloramphenicol can be taken as an alternative method to control the effect of tsetse on livestock population as a semi biological control.

Beard, (1993) reported that few *Sodalis* cells transmitted to intrauterine larvae can establish infections and proliferate to reach normal adult density levels of pupal development period. In addition he further explained that, multiple routes of transmission exist for *Sodalis*, including transovarial and paternal routes. Huebner and Davey (1974) also found bacteroids in the ovaries of *G. austeni* indicating that transmission from one generation to the next was transovarian.

6. Conclusion and Recommendation

The total area infested by tsetse flies is estimated to be 240,000km^2 in Southern, South western, Western, and North western part of the Ethiopia. Five species of tsetse flies, such as *Glossina pallidipes, Glossina fuscipes, Glossina longipennis, Glossina tachnoides and Glossina morsistans* commonly exists in Ethiopia. The tsetse flies competence largely dependent on symbiotic microorganisms.

Here in this study the isolation and characterization of the symbiotic bacteria were considered. Different morphological and biochemical tests confirmed that the probable species was *Sodalis* sp which is the principal in vector competence. The effects of antibiotics on the isolate were carried out and chloramphenicol found to be potent to control the growth of *Sodalis*. In addition, the effect of Chloramphenicol on the vector was checked, and the growth and development remains normal. These confirmed that Chloramphenicol indirectly can be taken as an alternative biological control strategy to tsetse spread. Hence, based on our findings the following recommendation and future directions should be considered

- Wider geographic and ecological niches to study symbiotic bacteria from Tsetse fly
- Molecular and biotechnological studies on symbiotic bacteria.
- The relationship between insecticide/pesticide resistance and the presence of the symbiotic bacteria should priority areas.
- The difference in the harboring symbionts in other species of tsetse.
- The effect of vector competence other than symbionts
- The role of symbionts other than *sodalis* should be assessed.

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