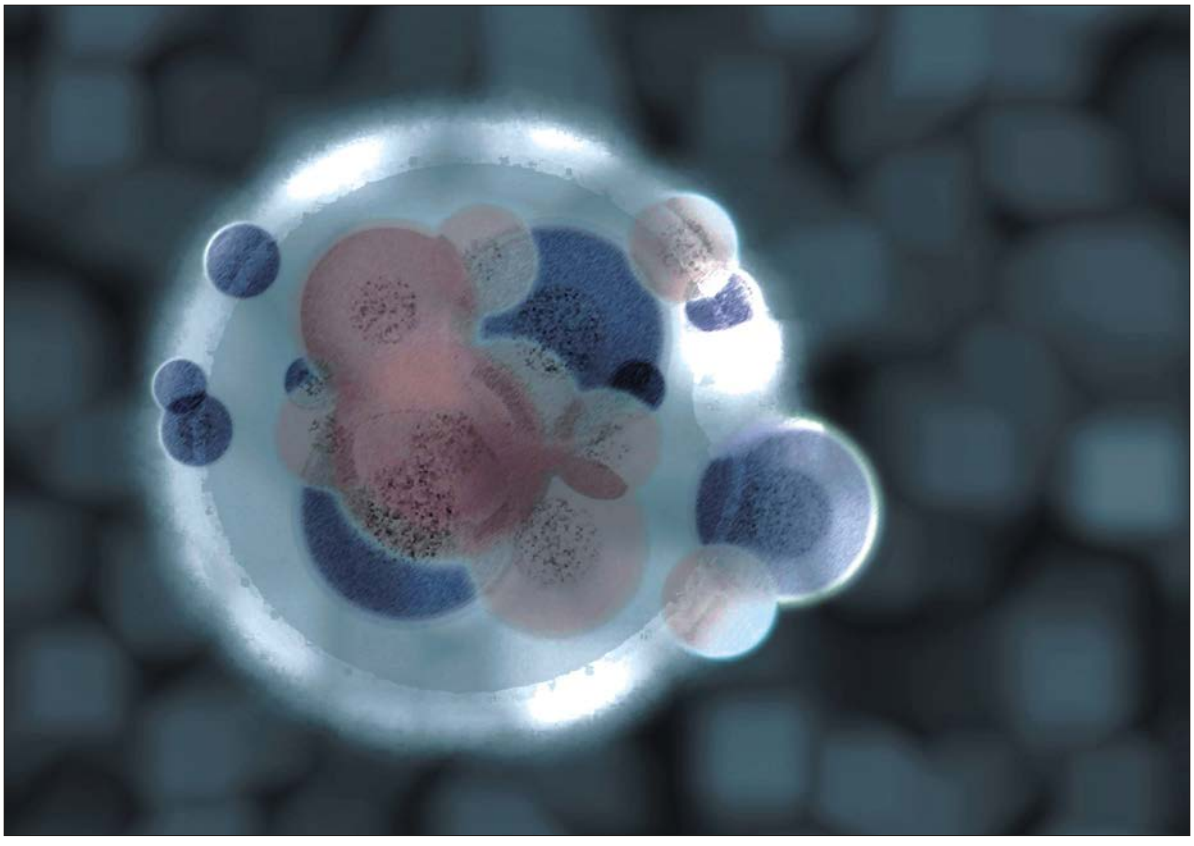

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Isolation, Identification, Antibiogram and Characterization of Bacterial Pathogens of the Silkworm, *Bombyx mori* in South-West Nigeria

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Abstract: In spite of its socio-economic and cultural importance to silk production, not much work has been done on *Bombyx mori* and silk farming in Nigeria. Of particular interest to this study is bacterial flacherie which is probably the most serious disease of *Bombyx* silkworm. Using a completely randomized block design, moribund (manifesting symptoms of flacherie disease) and dead larvae of three genetically distinct strains of silkworm and their runts were microbiologically analyzed for the presence of pathogenic bacteria on their body surfaces, gut and whole body. Standard Plate Count (SPC) was done by spread plate method. Bacteria isolation was done using standard microbiological procedures while identification of isolates was done using cultural, morphological and biochemical characteristics. Results show that Akure-China strain consistently showed the highest bacteria count regardless of the location of the insect body from which the sample was taken while the hybrid strain showed the lowest SPC values. The bacterial species isolated from the insect samples in this study were identified as follows: *Bacillus badius*, *Citrobacter freundii*, *Citrobacter amalomaticus*, *Enterobacter cloacae*, *Staphylococcus epidermidis* and *Serratia marcescens*. Results from the present study confirms the superiority of individuals of the hybrid strain to the Akure-China and the Akure-Japan strain when evaluated for their ability to prevent multiplication of pathogenic bacteria, an essential requirement for resistance to infection. Although, flacherie-causing organisms are present in the insect population, an outbreak will be avoided if proper hygiene is maintained by the farmers, ensuring that insects are reared under optimum conditions for productivity.

Key words: Flacherie, *Bombyx mori*, sericulture, Nigeria

INTRODUCTION

Although, the origin of textiles production and usage in Nigeria remains unknown, there are radio carbon date evidences of long use of textile as apparel since 10-12th century AD (Olajide *et al*., 2009). The most important fiber used in Nigerian textile production is cotton. On the other hand, silk garments have been made for centuries in West Africa primarily by Yoruba people in Nigeria where the men do the weaving (Clarke, 1998).

'Sericulture' is a term describing three different processes in silk production, namely; cultivation of mulberry plants (moriculture), rearing of silkworms (actual sericulture) and reeling of cocoons for the manufacture of fabrics. While the first two parts of the process are agricultural in nature, reeling of cocoons to spin yarn is distinctly industrial and is carried out either in cottage-type establishments or in large-scale factories called 'filatures'. The main areas of employment generation include mulberry cultivation, silkworm rearing, production and sale of silkworm eggs, silk reeling, threading and weaving, fabrication of machines for both the small scale filature and/or the big time miller. For an oil-rich but developing country such as Nigeria, sericulture provides an alternative to petroleum as an income generating source and serves as a mean of rural revitalization. Sericulture has the potential to help alleviate the high unemployment problem in Nigeria.

Several species of silkmoths belonging to the genera *Anaphe* and *Epanaphe* are the indigenous silkmoth species used in traditional silk production in Nigeria (Asakitikpi, 2007). The fiber obtained from the silkmoths are processed, hand spun into silk threads, washed and soaked in corn starch known locally as Sanyan

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(the king of cloths). The color of the silk is natural beige and is particularly associated with chiefs and kings. The alaari is sanyan dyed in red camwood solution. The cultivation of African wild silk is much simpler than that of the exotic *Bombyx mori* because the worms do not need to be killed before leaving the cocoons. On the other hand, the African wild silk cannot be wound and can be used only in the chappe silk industry and it does not matter that the cocoons have been bored through the moth. This means that every generation of the worm is available for further cultivation. Inspite of occupying such an important position in the economic and cultural history of Nigeria, literature on these species remains scanty.

Globally, *Bombyx* silk remains the most highly demanded type of silk with upto 95% demand rate while other silk types such as Tussah and Eri both developed in India constitute only about 5% of silk sold to the global market. With the foregoing, in order to be competitive in the global marketplace, priority is given to developing *Bombyx* silk while research continues on developing local strains of silkworm types for introduction to the global market. However, a major constraint to *Bombyx* silk production is the scourge of pathogenic diseases.

Silkworm diseases occur due to various biological, chemical, physical, nutritional and environmental causes. The *Bombyx* silkworm does not exist in the wild, being totally domesticated. Excessive inbreeding has lowered drastically the immune factors in this group of insects, making the insects particularly susceptible to diseases and changes in the environment.

Four categories of silkworm diseases have been reported in tropical locations like India which shares similar climate with Nigeria. In India, grasserie (viral), flacherie (bacterial), muscardine (fungal) and pebrine (protozoan) are the major mulberry silkworm diseases. Of these diseases, bacterial flacherie is probably the most serious disease of silkworm causing cocoon crop loss to the tune of 40% (Chakrabarty *et al*., 2012).

Bacterial diseases such as bacterial septicaemia, bacterial toxicosis and bacterial gastro-enteric diseases have also been reported in the silkworm in India. Bacteria that induce flacherie include *Bacillus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Streptococcus faccalis*, *Streptococcus liquifacions*, *Staphylococcus acire*, *Staphylococcus epidermidis*, *Bacillus thuringiensis*, *Serratia marcescens, Micrococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Aerobacter cloacae*, *Achromobacter delmarvae,* etc. (Karthikairaj *et al*., 2013). Symptoms of flacherie include loss of appetite, sluggishness of worms with slow growth, shrinkage, swelling of thorax, appearance of brown specks on skin, straightened appearance of body, oral and anal discharge, liquefaction of inner organs, rupturing of skin and oozing out of foul smelling brown liquid (Zhang *et al*., 2013). In addition to the itemized symptoms, there is also a documented relationship between the occurrence of runts in silkworm batches and infection of silkworm by bacterial pathogens. In most cases, the runts develop when infected insects' anal lips become sealed with a sticky soil-colored semi solid thereby preventing the insect from feeding and such individuals shrink lengthwise (Singh *et al*., 2011; Sahay *et al*., 2000).

The silkworm, *Bombyx mori* is an insect with a high socio-economic and cultural value. The present study aims to identify, characterize and describe for the first time the bacterial pathogens of the *Bombyx* silkworm in South-West Nigeria. This is expected to create a base line data for future breeding programs against silkworm diseases in Nigeria.

MATERIALS AND METHODS

Experimental design: The 4th instar larvae of three genetically distinct strains of silkworm maintained at the Ondo State Sericulture Project, Akure, Ondo State, Western Nigeria were sampled in a completely randomized block design for moribund (manifesting symptoms of flacherie disease) and dead larvae. The Ondo State Sericulture Project site is the hub for the collection of silkworm eggs for rearing purposes by silk farmers in South-West Nigeria. The moribund diseased insects showed symptoms such as cessation of feeding, flaccidity, loss of body luster, sluggishness and dysentery. These insects were kept frozen immediately after sampling and maintained frozen until analyzed. The three strains of silkworm are namely, Akure-China (Strain AC), Akure-Japan (Strain AJ) and hybrid (Strain HY). The samples were wrapped in tissue paper to control moisture and kept in Styrofoam containers which were kept in chilled coolers at a temperature of 4°C for transportation to the laboratory.

In addition to full sized 4th instar individuals of the AC, AJ and HY strains that were randomly sampled for isolation of pathogens, runt individuals of these were also randomly sampled in order to investigate the relationship between infection and emergence of runts. The distinct blocks and their assigned codes in parenthesis are shown as follows, Strain Akure-China (AC); Strain Akure-Japan (AJ), Hybrid (HY), Strain Akure-China Runts (ACR), Strain Akure-Japan Runts (AJR) and Hybrid Runts (HYR).

Isolation of bacteria: Bacteria were isolated from three sources on the body of the insects as follows.

Outer body surface of the insect: To obtain samples from this batch of insects, the insect was surface sterilized in 70% ethanol and washed 3 times with sterile distilled water. The insects were then triturated in sterile water blanks using glass rod. The suspension was serially diluted upto 10-6 then 0.1 mL of the suspension was inoculated unto nutrient agar using the spread plate method and incubated at 37°C for 24 h. This set up was observed for colony growths and the total number of colonies observed was recorded to determine the standard plate count from 3 replicates. Distinct colonies were subsequently isolated for further analysis.

Gut of the silkworm: The intestine of previously surface sterilized insects in 70% ethanol and washed 3 times with sterile distilled water was carried out by placing the surface sterilized specimen on a dissecting tray that has been previously disinfected with 95% ethanol. The specimen was fixed at both ends with two pins and the specimen cut with scissors along the back and pulling with tweezers the digestive tract. The cutting equipments were disinfected by dipping in 70% ethanol. The extracted digestive tract was shredded and deposited in an eppendorf tube containing 200 μL of sterile saline solution. For each sample, serial dilutions of upto 10-6 was inoculated unto nutrient agar and the total number of colonies recorded and average calculated from triplicates. Distinct colonies were subsequently isolated for further processing.

Whole body of the cadaver: Cadaver that has been previously surface-sterile was placed in mortal and pestle that was sterilized by placing in a dry heat oven for 4 h at 160°C. The cadaver was ground with mortal and pestle in 10 mL sterile saline water and an aliquot of the suspension was taken for serial dilution upto 10-6, 0.1 mL of the suspension was inoculated unto nutrient agar and the total number of colonies recorded and average calculated from triplicates. Distinct colonies were subsequently isolated for further processing.

The isolates obtained from the above procedure were coded for the sites from which such isolates were obtained from the insect body e.g., if an isolate was from the body surface of an insect sampled from the Akure-China strain, it is coded ACBS; from the insect's gut, ACG; from the insect's whole body, it is coded ACW.

Microbiological analysis: The distinct colonies on the plates were counted using a colony counter and the mean bacteria count per mL was obtained by dividing the average value of colony forming units on enumerated plates by the volume of sample dispensed unto nutrient agar and multiplied by the dilution factor. To identify the isolated organisms, distinct isolates were then screened based on the size, colony morphology, color, gram's staining reactions and biochemical characteristics of the isolates determined by subjecting the isolates to tests such as methyl red, vogues-praskauer, citrate, urease, indole, motility, catalase, oxidase and sugar fermentation tests. Differential media such as Mannitol salt and MacConkey agars were used to confirm identification of suspected organisms. Identified organisms were further characterized by *in vitro* sensitivity testing (antibiogram) against 5 mcg novobiocin antibiotic disks. This was carried out using the Kirby-Bauer method and results compared with Clinical Laboratory Standards Institute (CLSI) values (CLSI, 2007).

RESULTS

The highest number of bacterial isolates was recovered from individuals of the Akure-China strain with a total number of 32 isolates recovered while the lowest number was recovered from the hybrid strain. Generally, the highest numbers of isolates were recovered from the insect body surface while the lowest numbers of isolates were recovered from the gut of all categories of insects that were examined. More isolates were recovered from the runts than the hybrid and Akure-Japan strains (Table 1).

The hybrid strain exhibited the lowest colony count regardless of whether the sample was obtained from the body surface, gut or whole body. On the other hand, Akure-China strain consistently showed the highest

Table 1: No. of isolates from various strains of silkworm

	Sampling location			
Strains	Body surface	Gut	Whole body	Total
Akure-China	16	8	8	32
Akure-Japan	4	7	3	14
Hybrid	9	1	1	11
Akure-China runts	8	6	10	23
Akure-Japan runts	7	6	12	25
Hybrid runts	5	5	8	18
Total	49	33	42	123

Different strains of silkworm reared at 24-26°C, were frozen immediately after sampling, thawed out and bacterial isolates were obtained from the body surface, gut and whole bodies of the insects

Table 2: Bacteria colony count from various strains of silkworm

	Sampling location on insect body		
Strains	Body surface	Gut	Whole body
Akure-China	$2.96\times10^{6}\pm0.47$	$10.03\times10^{8}\pm0.64$	$0.79\times10^{8}\pm0.32$
Akure-Japan	$2.44\times10^{6}\pm0.29$	$2.96\times10^{6}\pm0.12$	$1.46\times10^{7}\pm0.22$
Hybrid	$1.00\times10^{4}\pm0.20$	$1.00\times10^{4}\pm0.10$	$1.00\times10^{4}\pm0.31$
Akure-China runts	$2.00\times10^{7}\pm0.12$	$0.42\times10^{7}\pm0.01$	$0.66\times10^{7}\pm0.71$
Akure-Japan runts	$1.00\times10^{7}\pm0.01$	$0.52\times10^{7}\pm0.01$	$2.00\times10^{7}\pm0.10$
Hybrid runts	$1.72\times10^{7}\pm0.10$	$0.91\times10^{7}\pm0.10$	$0.44\times10^{7}\pm0.10$

100 μL of inocula at dilutions 10^{-2}, 10^{-5}, 10^{-9} from each of the different strains was inoculated in duplicates on to Nutrient Agar (NA) by spread plate method, NA plates were incubated at 37°C for 18-24 h before reading the plates

bacteria count regardless of the location of the insect body from which the sample was taken. The bacteria colony count from runts of the 3 distinct strains ranged between 0.42×10^{7} -2.00×10^{7}, this is considerably lower than the number recorded for the Akure-China strain which exhibited colony count as high as 1.03×10^{8} and 0.79×10^{8} when samples were taken from the insect gut and the whole body, respectively. Individuals of the Akure-Japan strain however displayed lower colony count ranging between 2.44×10^{6} -1.46×10^{7} (Table 2).

As shown in Table 3 and 6, organisms were identified from a total of 123 isolates obtained in this study using a combination of tests based on cultural characteristics such as the size, colony aspect ratio, color, gram's staining reactions and results from biochemical tests. The organisms are *Bacillus badius*, *Citrobacter freundii*, *Citrobacter amalomaticus*, *Enterobacter cloacae*, *Staphylococcus epidermidis* and *Serratia marcescens*. The result of *in vitro* sensitivity testing (antibiogram) of the isolated organisms against 5mcg novobiocin is shown in Table 4. The test organisms displayed their characteristic antibiotic susceptibility patterns when compared with the CLSI values.

Table 6 shows *S. epidermidis* as the organism with the highest frequency of occurrence of the identified organisms, followed by *C. amalomaticus*, *E. cloacae*, *S. marcescens*, *C. freundii* and *B. badius* in descending order. Moreover, *S. epidermidis* is the only organism found to be present in all the strains examined in this study while *C. freundii*, *E.cloacae* and *S. marcescens* each occurred in 4 of the strains of insect. *B. badius* and *C. amalomaticus* were found to occur in only 2 of the insect strains namely, Akure-China and Hybrid runts (Table 6).

DISCUSSION

Results from the present study confirms the superiority of individuals of the hybrid strain to the

Table 3: Identification of isolates using cultural, cell morphology, gram staining and biochemical tests

Gram stain	Cell morphology	Methyl red	Indole	Vogues-proskauer	Catalase	Oxidase	Motility	Glucose	Lactose	Fructse	Galactose	Mannitol	Simmon citrate	Phenyl alanine acid	H_2S	GAS	Urease	Suspected organism
+	R	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-	+	*Bacillus badius*
-	C	+	-	-	+	-	+	+	+	+	+	+	-	-	-	-	+	*Citrobacter freundii*
-	C	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-	*Staphylococcus epidermidis*
-	C	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	*Enterobacter cloacae*
-	C	+	-	+	+	+	+	+	-	+	-	+	+	-	-	-	+	*Citrobacter amalomaticus*
-	R	+	-	-	+	+	+	-	-	+	-	+	+	-	-	-	-	*Serratia marcescens*

+: Positive, -: Negative

Akure-China and the Akure-Japan strain when evaluated for their ability to prevent rapid multiplication of pathogenic bacteria, an essential requirement for resistance to infection (Table 1). However, these distinctions are not so clear when the total number of bacterial isolates from the runts was compared to those isolated from the Akure-China batch with individuals presenting no apparent diminutive size such as observed in the runts (Table 1 and 2). Inspite of displaying higher loads of recovered bacterial from their body surfaces, guts and whole body, these individuals were still able to grow to normal size, although with observable symptoms of flacherie. It appears that the life stage of the insect at the point of infection is more critical to the emergence of runts, rather than the available amount of bacteria inoculates. High infection during the early larval instars of the insect would most likely lead to the emergence of runts than when such infections occurred later in the life cycle of the insect. The most devastating effect of infection at this stage occurs when the infected insects' anal lips become sealed with a sticky soil-colored semi solid thereby preventing the insect from feeding and such individuals shrink lengthwise (Singh *et al*., 2011).

The bacterial species isolated from the insect samples in this study were identified as follows: *Bacillus badius*, *Citrobacter freundii*, *Citrobacter amalomaticus*, *Enterobacter cloacae*, *Staphylococcus epidermidis* and *Serratia marcescens* (Table 3 and 5). Endo-spore forming, gram positive Bacilli and non-spore forming, gram negative organisms of the family Enterobacteriacae as those identified in the present study are known to be soil dwelling and usually become infectious due to poor hygiene, as these organisms may be fed to the insects through soil-contaminated leaves. Similar organisms have been reported to be isolated elsewhere from these insects in literature (Sakthivel *et al*., 2012; Priyadharshini *et al*., 2008; Nataraju *et al*., 2005).

Apart from infection from feeding of contaminated leaves, the rearing condition is another important factor responsible for the development of flacherie. Nataraju *et al*. (2005) reported that rise in temperature and high humidity leads to malfunction of the insect's alimentary canal, thereby encouraging flacherie. Moreover, Manimegalai and Chandramohan (2005) reported that apart from serving as a direct source of infection when soiled with bacteria, indirectly, poor quality leaves with poor nutritive value are unable to provide the essential nutrients required for the insect to produce the necessary antibacterial proteins required to prevent a high rate of multiplication of infectious bacteria and the development of bacterial flacherie.

In conclusion, it is comforting to know that a fairly resistant strain of *Bombyx mori* is available to silk farmers in Nigeria in the hybrid strain evaluated in this study and that there is yet no incidence of reported outbreak of flacherie in Nigeria. This condition would however continue only if proper hygiene is maintained by the farmers, ensuring that insects are reared under optimum conditions for productivity. Moreover, study should be intensified on developing more disease-resistant and higher-yielding strains of silkworm that are fully adapted to the Nigerian climatic conditions.

Table 4: Antibiogram with 5 mcg Novobiocin

Organisms	Antibiotic susceptibility pattern
Bacillus badius	S (100)
Citrobacter freundii	S (100)
**Staphylococcus epidermidis*	S (100)
Enterobacter cloacae	R (100)
Citrobacter amalomaticus	R (100)
Serratia marcescens	R (100)

S: Susceptible, R: Resistant. Antibiotic susceptibility pattern was obtained by comparison with Clinical Laboratory Standards Institute (CLSI) values

Table 5: Frequency of occurrence of identified organisms

Organisms	Occurrence (%)
Bacillus badius	7.3
Citrobacter freundii	7.3
Staphylococcus epidermidis	40.6
Enterobacter cloacae	13.8
Citrobacter amalomaticus	20.0
Serratia marcescens	11.0
Total	100.0

Table 6: Comparative frequency of occurrence of identified organisms among the insect strains

Strains	*Bacillus badius*	*Citrobacter freundii*	*Staphylococcus epidermidis*	*Enterobacter cloacae*	*Citrobacter amalomaticus*	*Serratia marcescens*	Total
Akure-China	14.0	7.0	50.0	3.6	7.0	18.4	100
Akure Japan	0.0	0.0	57.0	43.0	0.0	0.0	100
Hybrid	0.0	12.0	32.0	32.0	0.0	24.0	100
Akure-China runts	0.0	0.0	83.3	0.0	0.0	16.7	100
Akure-Japan runts	0.0	12.0	32.0	32.0	0.0	24.0	100
Hybrid runts	16.7	22.2	16.7	0.0	44.4	0.0	100
No. of occurrences	2.0	4.0	6.0	4.0	2.0	4.0	

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