Multidrug Resistant *Staphylococcus* Isolates from the Phyllosphere and Rhizosphere of *Ficus sycomorus* Linn

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Abstract: I report here a microbiological study of the Rhizosphere and the phyllosphere of *Ficus sycomorus* plant, with particular focus on *Staphylococcus species*, to provide the scientific explanation for an age-long observation and mythology of the Yoruba ethnic group of Nigeria, which says " ja ewe opoto kio ri ija eewo" which translates to "pluck the fig leaves and incur the brunt of boils". Various species of microorganisms have been discovered to inhabit different parts of plants. The Phyllosphere and the Rhizosphere of 40 samples of *Ficus sycomorus* plants were investigated to specifically isolate using the dilution plate technique, determine the pathogenicity, and antibiotic resistance of the associated *Staphylococcus* microbiota. In this study, the prevalent bacteria were *Staphylococcus aureus* (60%), *Staphylococcus intermedius* (20%), *Micrococcus sp* (10%) and *Staph. xylosus* (10%) from the Phyllosphere, while *Staphylococcus saprophyticus*, *S. aureus, S. xylosus* and *Micrococcus sp* were recovered from the Rhizosphere at 19.5%, 54.2%, 15.5% and 10.8% respectively. Of all the isolates, *Staph. aureus* from all the samples were resistant to Penicillin, Tetracycline, Ampicillin and Streptomycin. While *Micrococcus sp* was only susceptible to Ampicillin, *Staph. intermedius*, *S. saprophyticus* and *S. xylosus* were resistant to Penicillin. Ciprofloxacin and Augmentin were the most active against the bacterial isolates. Based on the use of extracts of the leaves by some local cheese ("Wara") producers for cud coagulation, as well as in ethnomedicine, the presence of this antibiotic resistant *Staphylococcus aureus* strains have the potentials to elicit preformed toxins and enteric infections if consumed, or elicit boils and abscesses on contact with abraided skin. Such patient may experience treatment failure using the first line antibiotics. This study indicate the potentials of *Ficus sycomorus* plant as a reservoir for dissemination of pathogenic multidrug resistant strains of *Staphylococcus* species in the environment. [Babalola Michael Oluyemi. **Multidrug Resistant *Staphylococcus* Isolates from the Phyllosphere and Rhizosphere of *Ficus Sycomorus* Linn.** *World Rural Observations* 2013;5(3):83-89]. ISSN: 1944-6543 (Print); ISSN: 1944-6551 (Online). http://www.sciencepub.net/rural. 13

Key Words: *Ficus sycomorus*, Phyllosphere, reservoir, *Staphylococcus*, boils, antibiotic resistant.

1. Introduction.

In the last two decades, the impact of opportunistic infections on human health has increased considerably. However little is known about the ecology and pathogenesis of these emerging pathogens. While some opportunists live as human commensals or in aquatic habitats, others originate from terrestrial ecosystem such as the Rhizosphere and Phyllosphere of plants. The aerial habitat of plants colonized by microorganisms is termed the phyllosphere, and the inhabitants are called epiphytes. Most work on phyllosphere microbiology has focused on leaves, a more dominant aerial plant structure (Lindow and Brandl, 2003).

A diverse number of microorganisms that exist as microbial communities on leaves include different genera of bacteria, fungi, viruses, yeasts, algae, protozoa and nematodes. Filamentous fungi exists predominantly as spores while bacteria are by far the most abundant inhabitants of the phyllosphere. Aerial surfaces of leaves are stressful environments for colonizing bacteria due to rapidly changing temperature, relative humidity, repeated alternation between presence and absence of moisture due to rain and dew, as well as limited nutrients (Lindow and Brandl, 2003). Therefore, significant numbers of leaf associated bacteria are true epiphytes rather than transient microbiota. To establish their colony, the majority of Gram positive epiphytes tended to localize within biofilms. Although single cells can be deposited at any given position, multicellular aggregates preferentially form around stomata and trichomes or along veins, responding to moisture, and nutrient concentration (Monier and Lindow, 2004).

The microbial ecology of the phyllosphere has been viewed mainly through the biology of Gram negative bacteria such as *Pseudomonas syringae* and *Erwinia spp*, two of the most ubiquitous bacterial participants of phyllosphere communities. The extreme flunctuations in the physicochemical environments of the phyllosphere over short time scales may select for bacteria species that have unusual and versatile traits that make them fit to colonize plant surfaces. Culture methods to enumerate bacterial populations have revealed high numbers of bacteria strains inhabiting leaf surfaces. For instance 37 genera and 78 species of bacteria were isolated from wheat and 20 genera from mango leaves (Leggard et al. 1995; de Jagger et al. 2001).

The multiple outbreaks of food – borne illnesses associated with fresh fruits and vegetables has raised concern about the possible preharvest contamination of plants' phyllosphere with human pathogens, and many ornamental plants have been identified as carriers of *Pseudomonas aeruginosa* (Cho et al. 1975). Recently, a substantial number of deaths have been attributed to the consumption of Enterotoxigenic *E. coli* contaminated vegetables in Germany and the outbreak was spreading across Europe in the year 2011. Interactions in the phyllosphere determine the extent to which human pathogens are able to colonize and survive on plant tissues employed as food, such as fresh salad, fruits, and vegetable produce (Whipps et al. 2008; Berger et al. 2010). The relative fitness of some human enteric pathogens in the phyllosphere as well as the wide distribution of *Enterococcus spp* and of common opportunistic pathogens such as *Pseudomonas aeruginosa* and *Burkholderia cepacia* require scientists to broaden our paradigms on plant microbial communities and their significance to the field of health microbiology.

The leaf bacteria flora differs substantially from that of roots. The Rhizosphere forms a unique habitat in terrestrial ecosystem which may select for opportunistic pathogens as these organisms are fiercely competitive for nutrients and produce many antimicrobial metabolites, thereby invariably conferring antibiotic resistances in this microenvironment (Berg et al. 2005). Bacterial living in the rhizosphere can have a neutral, pathogenic or beneficial interaction with their host plant. The majority of Rhizobacterial species which have emerged as pathogens include *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Pantoea agglomerans*, *Serratia liquifasciens, Proteus vulgaris and Staphylococcus epidermidis* from the rhizosphere of oilseed rape and potato (Sessistch et al. 2004). Others are *Staphylococcus aureus*, *Streptococcus pyogenes, Burkholderia cepacia* and *Stenotrophomonas maltophila,* from the rhizosphere of wheat and maize (Germida and Siciliano, 2001; Chelius and Triplett, 2000). Many mechanism involved in the interaction between antagonistic plant associated bacteria and their host plants are similar to those responsible for pathogenicity of bacteria (Rahme et al. 1995) and these mechanisms may also be involved in colonizing the human body (Cao et al. 2001) in addition to their ability to grow at 37^0C. In *Salmonella enterica*, the same virulence genes for attachment and virulence factor in the human host has been described for attachment of the pathogen to a plant surface (Barak et al. 2005).

Ficus sycomorus is a deciduous tree belonging to the family Moraceae. It grows to 50 ft tall, but more typically to a height of 10 – 30ft. The branches are muscular and twisting, spreading wider than they are tall. Fig wood is weak and decays rapidly. The trunk often bears large nodal tumors, where branches have been shed or removed. The bark is green - yellow to orange and exfoliates in papery strips. The sap contains copious milky latex that is irritating to human skin. Fig leaves are bright green, single, alternate and large, about 14cm long by 10cm wide and arranged spirally around the twig. They are more or less deeply lobed with 1 to 5 sinuses, rough hairy on the upper surface and softy hairy on the underside (CRFG, 1996). The tiny flowers are clustered inside the green fruits, that is, it is technically a syconium. Pollinating insects, usually the fig wasp, *Ceratosolen arabicus* gain access to the flowers through an opening at the apex of the syconium. The matured fruit has a tough peel (pure green, green suffused with brown, brown or purple), often cracking upon ripeness, and exposing the pulp beneath (Morton, 1987; Schurrie, 1990). The genus *Ficus* of the *moraceae* family has more than 800 species of which 65 have been recorded in India. The plant is native to Africa, it grows naturally in Nigeria, Lebanon, Cyprus, Madagascar, and has been naturalized in Israel and Egypt (Daniel and Maria, 2000).

In Africa, some traditional Cheese ("Wara") producers use the leaf extract and the latex of this plant for cud coagulation during processing (Oh Mahony, 1988; O'Connor, 1993). In addition, others use the leaves and the roots in ethnomedicine for therapeutic purposes, as the extracts from the leaves, the fruits, the bark and the roots were found to contain flavonoids, terpenoids, saponins, steroids and anthraquinones (Ahmadu et al. 2007). Previous studies have focused on enteric infections emanating from plant associated bacteria with less emphasis or non at all on skin infections, soft tissue and wound infections from plant associated microflora. Studies on *Ficus* species were more on the Phytochemical and antibacterial analysis of the leaf and stem bark extracts rather than the microflora. The objective of this study was to isolate and determine the antibiotic susceptibility of *Gram positive cocci* bacteria associated with the phyllosphere and Rhizosphere of *Ficus sycomorus* plant. The motivation for assaying for Gram positive cocci on this plant is a Yoruba (Nigeria) mythology which says that contact with the leaves of the fig tree usually leads to outbreak of rash of boils.

2. Materials and methods

2.1 Sampling

Forty (40) stands of the *Ficus sycomorus* were identified in various locations in South West Nigeria and marked for sampling. Sampling was done in the morning by aseptically plucking the leaf samples

randomly from each plant, transferred into sterile plastic bags and transported to the laboratory in an icebox for analysis within 24 hours.

2.2 Processing of samples

Epiphytic *Staphylococcus* was isolated from the leaves according to the method of Marchi, et al. (2005) with minor modifications. Ten (10) grammes of the leaves were placed in 100ml sterile Erlenmeyer flasks containing 40ml of 0.05M sterile Phosphate buffer (pH 7) and shaken for 2hrs at room temperature. This stock solution was serially diluted in 0.05M sterile phosphate buffer. Inoculum (1ml) was taken from 10^{-2} dilution to seed plates of Mannitol Salt Agar (MSA) and Baird- Parker Agar (Merck), all in replicates and incubated at 37^0C for 48 hrs. The resultant isolates on MSA were purified and characterized by Gram reaction, Coagulase, Catalase, Motility, Haemolysis, Gelatin and sugar fermentation tests. The pure isolates were characterized and identified according to Bergey's Manual of Determinative bacteriology (Holt et al., 2000) and further speciated by the API Staph-Ident system (API Laboratory Products Ltd, St Laurent, Quebec, Canada). Staphylococcal colonies showing lecithinase activity on Baird- Parkers medium were streaked on Nutrient Agar and rapidly tested for agglutination by the Staphyslide test.

Antibiotic susceptibility testing of the isolates

The following antibiotics (Difco) and their sensidisk concentrations were tested against the isolates by disk diffusion on Mueller-Hinton Agar (Oxoid), using the standard method of CLSI (2005). Tetracycline (10µg), Ampicillin (10µg), Penicillin (16mg), Streptomycin (10µg), Augmentin (3µg), Ciprofloxacin (5µg), Gentamicin (10µg), Erythromycin (25µg) and Amoxicillin (25µg). Inoculum of each pure isolate was prepared in a direct broth suspension of Nutrient broth, incubated at 35^0C for 18 hrs and standardized by adjusting its density to match the 0.5 McFarland turbidity standard using Barium sulphate ($BaSO_4$).

3. Results

Plate 1 shows a sample of the *Ficus sycomorus* Linn plant. All the forty (40) samples of *Ficus* plant yielded four *Staphylococcus* species from both the Phyllosphere and the Rhizosphere, as identified by the Bergey's Manual of determinative Bacteriology (Holt et al., 2000). The plate dilution method was efficient in characterization of the bacteria population on the phyllosphere (Yadav et al., 2010) and the method recovered abundant *Staphylococcus* from the leaf surface. From all the tested samples *Staphylococcus aureus*, *S. intermedius, Micrococcus spp and Staph. xylosus* were recovered from the Phyllosphere at 60%, 20%, 10% and 10% respectively, and the rate of recovery remain thesame during on and off fruiting season. *S. aureus*, *S. saprophyticus*, *S. xylosus* and *Micrococcus spp* were recovered from the Rhizosphere at 19.5%, 54.2%, 15.5% and 10.8% respectively (Table 1).

Plate 1: Sample of *Ficus sycomorus* plant

Table 1: Occurrence of *Staphylococcus* Isolates on *Ficus sycomorus* Linn (nf: not found)

Isolates	Phyllosphere	Rhizosphere
	No. of +ve samples &% Isolate	No. of +ve samples & % Isolate
Staph. aureus	40 (60%)	40 (54.2%)
Staph. intermedius	40 (20%)	Nf
Micrococcus spp	10 (10%)	30 (10.8%)
Staph. saprophyticus	Nf	35 (19.5%)
Staph. xylosus	10(10%)	35 (15.5%)

Of all the isolates, *Staphylococcus aureus* was the most prevalent from the Phyllosphere as well as the Rhizosphere, followed by *S. intermedius* which was only recovered from the Phyllosphere. *Micrococcus* sp was isolated from the phyllosphere and the Rhizosphere while S. *saprophyticus* was only recovered from the Rhizosphere (Table 2). Coagulase negative *Staphylococcus epidermidis* was not recovered from both the Phyllosphere and the Rhizosphere.

Table 2: The prevalence of *Staphylococcus* on *Ficus sycomorus*

Isolates	Phyllosphere	Rhizosphere
Staph. aureus	+++ (100%)	+++ (100%)
Staph. intermedius	++ (50%)	Nf
Micrococcus spp	+(25 %)	+ (25%)
Staph. saprophyticus	Nf	++ (50%)
Staph. xylosus	+ (25%)	++ (50%)

Table 3: Percentage incidence of antibiotic resistance among *Staphylococcus* and *Micrococcus* isolates from *Ficus sycomorus* Linn

Phyllosphere Isolates	Percentage of resistant bacterial isolates to the antibiotics:								
	Tet	Amp	Pen	Str	Aug	Cip	Gen	Ery	Amo
Staph. aureus	70	73	75	40	20.5	1.2	60.5	20	55
Staph. intermedius	10	5	75	5	10	0	67.5	55	45
Micrococcus spp	50	5.5	50	40	2.2	0	5.3	5.1	5.5
Staph. xylosus	55	50	60	20	10	1.2	45	55	30.2
Rhizosphere Isolates									
Staph. aureus	60	80	80	60	25	2.0	65	25.5	40.5
Micrococcus spp	55	5.5	50.8	45	2.5	0	5.5	5.4	5.8
Staph. saprophyticus	30	30	60.2	25	20	0	55	50	50.5
Staph. xylosus	60	52	61	25	12	0	50	52	32.5

Table 3 shows the susceptibility of the Staphylococci isolates to the various antibiotics. All the isolates were susceptible to Ciprofloxacin, followed by Augmentin, while resistance to Tetracycline, Ampicillin, Penicillin and Streptomycin ranged between 5% (in *Staph. intermedius*) and 80% (in *Staph. aureus*). Resistance to Gentamicin, Erythromycin and Amoxicillin ranged between 67.5% and 5.1%.

4. Discussions

The reservoir of any organism, in the epidemiology of any bacteria disease is very important (Daniel et al. 2002). The pathogens live and or multiply in the reservoir, which may be animate or inanimate objects, on which their survival depends. The aerial leaf surface is a critical reservoir, subject to rapid and large fluctuations in temperature, humidity and osmotic pressure that restrict bacterial proliferation on leaf surfaces. In addition, competition for limited water and nutrients makes it difficult for transient microorganism to survive on leaf surface (Mercier and Lindow, 2000). With this study, the detection of *Staphylococcus aureus* in all the forty samples of *Ficus sycomorus* indicate a permanent resident microflora rather than a transient relationship. Moreover, during fruiting and off season, the rate of recovery of the isolates remain the same.

Simple sugars such as glucose, fructose and sucrose are the dominant carbon sources on plants that have been examined and are thought to simply leach from the interior of plants (Derridj 1996; Mercier and Lindow, 2000).Similar condition in addition to exudation of these nutrients from both the rotten and fresh fruits logically provide the carbon sources and sustenance of the microbiota in this plant species. Insects may be an important epidemiological factor in the transmission of the organisms. In particular, flies which rely on faecal material and rotten fruit as a protein source to develop eggs (Lauzon, 2003) may act as vectors of some of the isolated pathogens. For example, both the vinegar fly (*Drosophila melanogaster*) and the Mediterranean fruit fly (*Ceratitis capitata*) shown to transmit *E. coli* to wounded and intact apples (Sela et al. 2005) were found abundantly around the plant fruits. Most importantly, the sycamore fig tree and a tiny species of wasp the *Ceratosolen arabicus* are utterly dependent on each other as the only insect capable of pollinating the *Ficus sycomorus* fig tree. The wasp lives for only up to two days, flying up to 160 km shuttling between fig trees (Ahmed et al. 2009) and may in the process transmit the *Staphylococci* species. Both the pollinating and non pollinating insects associated with the fig fruits and trees provide unparalleled opportunities for the evolution of bacteria virulence.

The role of staphylococci species in both nosocomial and community acquired infections have been stressed but cannot be overemphasized. *S. aureus* has been known to play a vital role in invasive skin diseases including superficial and deep follicular lesion. However, the role of *Ficus* plant as a reservoir of community acquired staphylococcus have not been hitherto microbiologically described until this present study. *Staphylococcus aureus* has the highest occurrence and the most prevalent isolate from both the phyllosphere and the rhizosphere of all the samples tested. The staphylococci isolates were coagulase

positive strains except S. *saprophyticus* and *S. xylosus*. They were catalase positive and showed high lecithinase activity on Baird – Pakers Agar thereby indicating their pathogenic potentials. Studies on phytochemical constituents of other *Ficus* species claimed antibacterial activities of the stem bark extracts against *Staphylococcus aureus* in ethnomedicine (Makhija et al. 2010). However, this is contrary in *Ficus sycomorus* as a number of test *S. aureus* were resistant to the leaf extract, stem bark extracts and standard Gentamicin (Adeshina et al. 2010). Therefore the existence of the multidrug resistant strains in this study may in part be explained by the inactivity of the phytochemical constituents against the strains, as well as development of natural resistance.

Among the Staphylococcus isolates in this study, only *S. aureus* has been majorly implicated in human infections that range from boils, carbuncles, furuncles, food poisoning, abscesses, wound infections, pneumonia, osteomyelitis, endocarditis and mastitis. In a prospective cohort study of traditional Fulani cheese producers who uses the leaf extracts of the Ficus plant for cud coagulation, observed incidence of rash of boils was higher than the users of *Calotropis procera* extracts for the same purpose (Babalola unpublished data). The higher association further explains and give credence to the current findings. It has been shown that *Staph xylosus* are capable of colonizing and infecting humans (Bannerman, 2003). *Staphylococcus intermedius* has been found to produce enterotoxin and may therefore be involved in food poisoning (Becker and Keller, 2001) while *S. saprophyticus* has been implicated in urinogenital infections especially in sexually active young women (Fihn et al. 1998).

One natural reservoir of opportunistic pathogens is the rhizosphere, the zone around roots that is influenced by the plant. The detection of *Staphylococcus aureus* in the rhizosphere of *Ficus sycomorus* lend credence to earlier reports which grouped *S. aureus, P. aeruginosa, Burkholderia spp, and S. maltophila* as the majority antagonistic rhizobacteria which have emerged as pathogens of human in skin and wound infections (Berg et al. 2005).

All the isolates showed considerable resistance to the tested antibiotics except Ciprofloxacin and Augmentin. Coagulase negative staphylococci (CoNS) are ubiquitous opportunistic pathogens of humans causing both community acquired and hospital acquired infections. CoNS carry a wide variety of multidrug resistance genes on plasmids which can be exchanged and spread among different species of staphylococci including *Staphylococcus aureus* and *Staph. intermedius* (Neihart et al.1988). Antimicrobial resistance genes may also reside on smaller plasmids, such as those found in class 1 staphylococcal plasmids (Novick, 1989). They are composed of small (1-5Kb), multicopy, non- conjugative plasmids, encoding resistance to chloramphenicol, erythromycin, neomycin, streptomycin and tetracycline, and often mobilized by conjugative plasmids or by transduction (Novick et al.1986; Udo et al.1992). In this study, these phenomena in addition to production of beta lactamase, may have taken place between *Staph aureus*, *Staph intermedius*, *S. xylosus* and *S. saprophyticus* and be responsible for the resistance patterns of the isolates both from the phyllosphere and the rhizosphere. The occurrence and production of diverse antibiotic substances by some highly competitive rhizobacteria in the rhizosphere also explains the frequent detection of bacteria with multiple antibiotic resistances in this microenvironment.

The ingestion via use of the plant extracts in ethnomedicine or infection by these multidrug resistant isolates can lead to treatment failures as a result of their resistance to these first line antibiotics that are often prescribed by physicians. This study scientifically explain and uphold the folklore and mythology which says "pluck the fig leaves and incur the wrath of boils" as the staphylococcus laden leaves may elicit boils and abscesses on contact with abraided skin.

Acknowledgements

All glory to GOD, the greatest scientist yesterday, today, and forever, the watershed of all intellects. I thank Prof J.A Akinyanju for his contribution in the preparation of the manuscript.

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9/10/2013