ENTEROTOXIN AND THERMONUCLEASE PRODUCING POTENTIAL OF BACTERIA ASSOCIATED WITH AFRICAN PALM MAGGOT, *RHYNCOPHORUS PHOENICIA*.

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ABSTRACT: The enterotoxin and thermonuclease producing ability of bacteria commonly associated with *Rhyncophorus phoenicia* larvae, a human protein source referred to as African palm maggot was determined to express their pathogenicity. Of the isolates tested for enterotoxigenicity using micro-slide gel diffusion and optimal sensitivity plate methods, only *Bacillus cereus* APM-1, *Clostridium* sp APM-8, *Enterococcus faecalis* APM-5, *Escherichia coli* APM-3, *Salmonella schothmulleri* APM-9 and *Staphylococcus aureus* APM-6 gave positive results. In thermonuclease test, all the spore - formers (*B. subtilis* APM-102, *B. kaustophilus* APM-4, *B. cereus* APM-1, *Clostridium* sp APM-8), and *Staph. aureus* APM-6 gave positive reactions. Their reactions to both tests however showed marked variation in the number of enterotoxin and thermonuclease production strains of each isolate. Their potential to induce gastroenteritis and ability to resist heat treatment is of serious health risk, particularly to consumers that prefer raw or partially cooked larvae as food.

INTRODUCTION

African palm maggot is the larval stage of the *Rhyncophorus phoenicia*, a member of the large weevil family Curcuhonidae. The insect is commonly found in many West and East African countries (Umoh and Bassir, 1977). Its economic importance in Nigeria, is solely nutritional. In the southern states of Nigeria and especially in the wetlands of the Niger Delta, the inhabitants do supplement their carbohydrate rich foods with palm maggots (Essien *et al*., 2004).

Rhyncophorus phoenicia larva, a voracious feeder lives in dead raffia palm (*Raphia hookeri*) trunks. It is a big sluggish, brown or milky colored maggot characterized by its thick strong head, similar sized thorax segment and a repeating abdominal segment. The maggot is usually eaten raw, cooked or partially cooked by the natives who have little knowledge of the health risks associated with uncooked maggot. There are cases of illnesses particularly dysentery, cholera, typhoid and giardiasis among the wetland inhabitants of the Niger Delta attributed to the consumptions of foods heavily loaded with coliforms and other pathogenic strains of bacteria (Essien *et al*., 2004, Opara and Egwali, 2004). To identify the source and etiological agents of illnesses, a survey of the presence of pathogenic strains of bacteria in the maggot was necessary. This study therefore was aimed at ascertaining the disease causing potential of bacteria commonly associated with palm maggot. The detection of enterotoxin and thermonuclease produced by the isolates, were used as indices of pathogenicity (Dabbah *et al*., 1969).

MATERIALS AND METHODS

Samples Collection

A total of 100 samples of *R. phoenicia* larvae were excavated from decaying stems of raffia palm (*Raphia hookeri*) trunk in a swamp in **U**penekang, an oil producing community along the

Atlantic coast in Ibeno Local Government Area, Nigeria. Live maggots collected and placed in sterile glass jar were immediately transferred to the laboratory for analysis.

Bacteriological Analysis of African Palm Maggot

Prior to bacteriological analysis the maggot samples were repeatedly washed with sterile distilled water and sterilized by immersion in 0.5% sodium hypochlorite. The larval viscera were obtained aseptically by dissection using sterile surgical blade. Ten grams of the maggot viscera was dissolved in 10ml of sterile distilled water for use as the inocula.

Viable aerobic bacterial count was adopted and the bacteria present in the maggot viscera were isolated on nutrient agar (OXOID) plates into which sterile fungizone (50µg/ml) had been incorporated to inhibit fungal growth. Samonella-Shigella agar and cooked meet agar under anaerobiosis were employed for the isolation of salmonellae and clostridia respectively. The bacteria colonies which developed on the plates after incubation at $37^{O}C$ and $60^{O}C$ for 48 hours were randomly picked and purified by sub-culturing on fresh nutrient agar using the streak - plate technique (FDA, 1984). Colonies which appeared on the plates were isolated and then transferred onto nutrient agar slants and stored as stock cultures for further tests.

Representative colonies of pure bacterial isolates were identified by their cultural, morphological and biochemical properties as described by Cowan (1985). The isolates were coded as APM (African Palm Maggot)

Determination of Enterotoxin and Thermonuclease Producing Potential of Bacterial Isolates

The ability of the bacterial isolates to induce gastroenteritis in man was tested using their enterotoxin and thermonuclease producing potential. Enterotoxins from the bacterial cells were tested with the help of cellophane over- agar method of Jarvis and Lawrence (1970) and detected by the micro-slide gel diffusion (MS) method (Baish *et al*, 1989 and Casman *et al*, 1969) and optimal sensitivity plate (OSP) method (Ouchterlony, 1967). Production of thermonuclease was determined by the technique of Lachina *et al.*(1971).

Prior to the tests, the bacterial cultures were revived in Brain Heart Infusion broth (BHI, DIFCO). They were then grown for 18h in the same medium, harvested and inoculated into 0.2mol/1 sodium phosphate buffered saline (PBS) at pH 7.0. One ml of this standardized cell suspension when added to 99ml of the menstrum (PBS) gave a final population of approximately $1x10^{8}$ viable cells per ml.

RESULTS

The different bacterial species isolated from the African palm maggot were characterized as shown in Table 1, and were identified as *Bacillus cereus* APM-1, *B. subtilis* APM – 2, *B. kaustophilus* APM – 4, *Clostridium* sp APM – 8, *Enterococcus faecalis* APM – 5, *Escherichia coli* APM-3, *Pseudomonas aeruginosa* APM-10, *Salmonella schothmulleri* APM – 9, *S. paratyphi* APM – 7 and *Staphylococcus aureus* APM-6. Their prevalence in the palm maggot is given in Table 2, while Table 3 shows that some strains of *Staph aureus, B. cereus, En. faecalis, S. schothmulleri* and *Clostridium* species isolated from the maggot have enterotoxigenic potential. The enterotoxigenicity of *E. coli* (60%) was also remarkable. Very few strains of *B. cereus, En. faecalis* and *S. schothmulleri* associated with the maggot gave positive result, while only one strain of *Clostridium* sp was positive. The remaining number of test organisms which were negative by these methods was either lacking in enterotoxin producing ability or inhibited by the test conditions.

Table 1: Cultural, morphological and biochemical characteristics of bacteria isolated from *Rhyncophorus phoenicia* larvae

	Isolate codes									
	APM-6	APM-3	APM-10	APM-1	APM-2	APM-4	APM-5	APM-8	APM-9	APM-7
Isolation Temp.	37^{0}C	37^{0}C	37^{0}C	37^{0}C	37^{0}C & 60^{0}C	60^{0}C	37^{0}C	37^{0}C	37^{0}C	37^{0}C
Colour in agar slant	white to orange	smooth gray	green to brown	white to yellow	white spreading	white to bluish	gray beaded	Anaerobic	bluish gray	grayish glistening
Cell shape	spheres	short rods	short rods	long rods	long rods	long rods	chains	long rods	medium rods	long rods
Cell size (µM)	0.9	1.5	1.6	2.5	2.6	2.6	0.8	3.6	2.0	3.2
Spores	-	-	-	+	+	+	-	+	-	-
Motility	-	-	+	-	+	+	-	-	+	+
Gram rxn	+	-	-	+	+	+	+	+	-	-
Coagulase	+	-	-	-	-	-	+	-	-	-
Catalase	+	+	+	+	+	+	+	-	-	+
Oxidase	-	+	+	-	-	-	+		-	-
Indole production	-	+	±	+	+	+	-	±	-	-
Geletin Liqufaction	+	-	+	+	+	+	-	-	-	-
V.P Test	-	-	-	+	+	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	-	-	+	+
M-R Test	-	+	-	-	-	-	-	-	-	-
Sugar										
Glucose	AG	AG	-	A	A	A	A	AG	AG	AG
Maltose	A	AG	-	A	A	-	A	AG	AG	AG
Mannitol	AG	AG	-	-	A	-	AG	-	AG	AG
Lactose	AG	AG	-	-	-	-	A	-	-	-
Sucrose	AG	A	-	A	A	-	A	-	-	-
Galactose	AG	A	-	-	A	-	A	A	-	-
Probable Organism	*6*	*3*	*10*	*1*	*2*	*4*	*5*	*8*	*9*	*7*

Footnote: + = positive reaction; - = negative reaction; ± = variable reaction
1 = Bacillus cereus, 2 = Bacillus subtilis, 3 = Escherichia coli, 4 = Bacillus kaustophilus, 5 = Enterococcus faecalis, 6 = Staphylococcus aureus, 7 = Salmonella paratyphi, 8 = Clostridium sp, 9 = Salmonella schothmulleri, 10 = Pseudomonas aeruginosa

Table 2: Frequency of incidence of bacterial isolated from *Rhyncophorus phoenicia* larvae

Isolate/Code	Frequency of Occurrence	%
Bacillus cereus APM – 1	40	80
B. subtilis APM – 2	30	60
B. kaustophilus APM – 4	5	10
Clostridium sp APM – 8	5	10
Enterococcus faecalis APM – 5	43	85
Escherichia coli APM – 3	50	100
Pseudomonas aeruginosa APM – 10	23	45
Salmonella schothmulleri APM – 9	5	10
S. paratyphi APM – 7	5	10
Staphylococcus aureus APM – 6	20	40

The frequency of incidence was based on 50 samples of R. phoenicia larvae analysed.

Table 3: Enterotoxin production by bacteria isolated from *Rhyncophorus phoenicia* larvae

Isolate/Code	MS	OSP	% Positive reactions	% negative reactions
Bacillus cereus APM – 1	+	+	20	80
B. subtilis APM – 2	-	-	-	100
B. kaustophilus APM – 4	-	-	-	100
Clostridium sp APM – 8	-	+	5	95
Enterococcus faecalis IAPM – 5	+	+	15	85
Escherichia coli APM – 3	-	+	60	40
Pseudomonas aeruginosa APM – 10	-	-	-	100
Salmonella schothmulleri APM – 9	+	+	10	90
S. paratyphi APM – 7	-	-	-	100
Staphylococcus aureus APM – 6	+	+	80	20

MS – Microslide gel diffusion method
OSP – Optimal sensitivity plate method
% of positive reaction was estimated based on 20 (10 for MS test and 10 for OSP test) different samples per isolate tested.

However, in both cases OSP method was found to be superior to MS method in terms of clarity of precipitation bands obtained with the bacterial isolates. The only enterotoxigenic strain of *Clostridium* species encountered in this study was detected by the OSP method.

Table 4 contains the result of the thermonuclease test. Most strains of the spore-formers namely *B. cereus B. subtilis, B. kaustophilus* and *Clostridium* species and the non-sporeforming bacterium *Staph. aureus* gave positive reactions while most of *En. faecalis, E. coli*, *P. aeruginosa, S. schothmulleri and S. paratyphi* isolates did not produce thermostable deoxyribonuclease enzyme (DNase).

Table 4: Thermonuclease production by bacteria isolated from *Rhynophorus Phoenicia* larvae

Isolate/Code	No. of samples tested	No. of thermononuclease producing samples	% Positive reactions	% negative reactions
Bacillus cereus APM – 1	20	18	90	10
B. subtilis APM – 2	20	16	80	20
B. kaustophilus APM – 4	20	17	85	05
Clostridium sp APM – 8	10	6	60	40
Enterococcus faecalis APM – 5	20	1	5	95
Escherichia coli APM – 3	20	2	10	90
Pseudomonas aeruginosa APM – 10	-	-	-	100
Salmonella schothmulleri APM – 9	-	-	-	100
S. paratyphi APM – 7	-	-	-	100
Staphylococcus aureus APM – 6	20	19	95	5

DISCUSSION

It is evident from these observations that some strains of bacteria associated with *Rhyncophorus phoenicia* larvae are enterotoxigenic. In the present study the optimal sensitivity plate method (OSP) revealed more bacterial strains with exterotoxin producing potential compared to the micro-slide gel diffusion method (MS). Although the bacterial isolates were incubated under actively growing condition (37°C) it is expected that endospores are formed mainly by bacteria under stress. Incubation at 60°C was done mainly to induce the formation endospores by spore forming bacteria. Tijani *et al.* (1976) showed that most enterotoxigenic strains of bacteria produce thermonuclease.

The thermonuclease producing capability of non-enterotoxigenic producing bacteria such as *B. subtilis, B. kaustophilus* and some strains of *Clostridium* species may be linked to their physiological ability to form heat endospores (Manachini *et al*, 1989 and Takami *et al*, 1989). Lachica *et al.* (1971) demonstrated that Dnase could be detected in substrates which permitted growth of the organism but not production of entrotoxin. This might be one of the reasons for the fewer number of enterotoxin positive bacteria (Table 3) than DNase positive bacteria (Table 4). Another reason put forward by Zayaitz and Ledford (1982) is that some bacteria produce proteases, which could only degrade their own DNases. It was concluded however that the degree of inactivation can be stimulated under other sets of conditions including improper processing of foods (Batish *et al* 1989). If low levels of nucleases are present initially in foods; any degradation may be significant, particularly if nuclease is used as an indicator of enterotoxigenic strains of bacteria. The degradation of DNase by the organism's own proteases, by proteases of other organisms, or by proteases in food system itself, should be of concern to the microbiologists. The results of the present study suggest that some spore forming, non-enterotoxigenic bacteria such as *B. subtilis,* and *B. kaustophilus* may also elaborate thermonuclease. Hence due consideration should be given to this aspect while interpreting results of the Dnase test as an attribute of bacterial entrotoxigenicity, because the use of thermonuclease as indicator of enterotoxigenicity of bacteria may be misleading in some cases.

Although the concentration of the bacterial inoculum responsible for thermonuclease production and enterotoxigenicity was not ascertained in this study, researchers have shown that in many foods, the first detectable amounts of thermonuclease in *Staph aureus* occurred in the region of 10^5 to 10^6 cells per gram, while enterotoxins were first detected after a population of 10^6 to 10^7 cells per gam (Tijani *et al*., 1976). The same researchers also reported that the presence of detectable amount of thermonuclease in *Staph aureus* is an indication that growth has reached a population of 10^6 cells/g. However, preliminary studies on the microbiological

properties showed that these values are even higher than the total aerobic bacterial count of 2.5 x 10^4 viable cells per gram and coliform count of 1.3 x 10^4 viable cells per gram observed in freshly collected African Palm maggot (Essien *et al*, 2004).

CONCLUSION

It is obvious that gastroenteritis which sometimes follow consumption of raw palm maggot or meals in which palm maggot are served might involve a consortium of microbial toxins. For instance enterotoxigenic strains of *B. cereus, En. faecalis, E. coli, Staph aureus* and *S. schothmulleri* found in the maggots have been implicated in cases of food poisoning (Jay, 1978 and Nester *et al*, 2001). The ability of the isolates to produce thermonuclease is a pointer to the resistibility of the bacteria to heat treatment and the danger associated with the consumption of raw or partially cooked palm maggots.

REFERENCES

Batish, V. K., Nataraji, B. and Grover, J. E. (1989). Variation in the behaviour of enterotoxigenic *Staphylococcus aureus* after heat stress in milk. *Journal of Applied Bacteriology,* 66: 27 – 35.

Casman, E. P., Bennet, R. W., Dorsey, A. E. and Stone, J. E. (1969). The microslide gel double test for detection and assay of staphylococcal enterotoxins. *Health Laboratory Science,* 6: 185 – 198.

Cowan, S. T. (1985). *Cowan and Steel's manual for identification of medical bacteria.* (2nd edn.) Cambridge University Press, England.

Dabbah, R., Moats, W. A. and Mattick, J. F. (1969). Factors affecting resistance to heat and recovery of injured bacteria. *Journal of Dairy Sciences,* 52: 608 – 614.

Essien, J. P., Etang, B. B. and Udoukpo, F. C. (2004). Nutritional potential and bacteriological health risk of human consumption of African palm maggot (*Rhyncophorus phoenieia*, (Insecta; coleoptera) from the Niger Delta wetlands, Nigeria. *Living Systems and Sustainable Development,* 1: 25 – 30.

FDA (1984). Bacteriological analytical manual (6th Edn) AOAC Publication, Arlington, VA, USA.

Jarvis, A. W. and Lawrence, R. C. (1970). Production of high titres of enterotoxins for the routine testing of Staphylococci. *Applied Microbiology,* 19: 698 – 699.

Jay, M. (1978). *Modern food microbiology* 2nd Edition. Dvan Nostrand Company. New York. pp. 135 – 142.

Manachini, P. L., Fortina, M. G. and Parini, C. (1989). Thermostable alkaline protease produced *Bacillus thermoruber* – a species of *Bacillus*. *Applied Microbiology*, 28: 409 – 413.

Nester, E. W., Roberts, C. E., Pearsall, N. N. and Nester, M. T. (2001). *Microbiology: a human perspective* McGraw Hill New York pp 72 – 74

Opara, K. N. and Egwali, E. C. (2004). Human health implications of wetlands conservation in Nigeria, *Living Systems and Sustainable Development*, 7: 7-8

Ouchterlony, O. (1967). Immuno-diffusion and immunoelectrophoresis. *In*: Handbook of Experimental Immunology ed. Weir D. M. Oxford and Edinburgh. Blackwells. pp. 665 – 702.

Lachica, R. V. F., Genigeorgis, C. and Hoeprich, P. D. (1971). Metachromatic agar diffusion methods for detecting Staphylococcal nuclease activity. *Applied Microbiology,* 21: 588 – 587.

Takami, H., Akiba, T. and Horikoshi, K. (1989). Production of extremely thermostable alkaline protease from *Bacillus* species no. AH-101, *Applied Microbiology and Bacteriology*, 30: 120 – 124

Tijani, B. R., Cords, J. and Gramoli, J. (1976). Screening for Staphylococcal enterotoxins in foods. *Food Technology,* 30: 64 – 74.

Umoh, I. B. and Bassir, O. (1977). Lesser known sources of protein in some Nigerian peasant diets. *Food Chemistry*. 2: 315 – 321.

Zayaitz, A. E. K. and Ledford, R. A. (1982). Proteolytic inactivation of thermonuclease activity of *Staphylococcus aureus* during recovery from thermal injury. *Journal of Food Protection,* 45: 625 – 626.