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Plasmid-Borne Genes

Degradation of Crude Oil

Highlights

Blood Glucose Concentration

Concentration of Albino Rats

Discovering Thoughts, Inventing Future

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Contents of the Volume

i. Copyright Notice
ii. Editorial Board Members
iii. Chief Author and Dean
iv. Table of Contents
v. From the Chief Editor's Desk
vi. Research and Review Papers

1. Identification of Couple of Novel Genes Probably have Relation with Virulence in *Yersinia Enterocolitica* O:8 using Subtractive Hybridisation. *1-9*
2. Effect of N-Hexane Oil Extract of Two Spices on Serum Lipid Profile and Blood Glucose Concentration of Albino Rats. *11-16*
3. Modeling of Non- Newtonian Fluid for Blood Flow in Stenosed Arteries; A Comparative Study. *17-19*
4. Degradation of Crude Oil by Bacteria: A Role for Plasmid-Borne Genes. *21-26*

vii. Auxiliary Memberships
viii. Process of Submission of Research Paper
ix. Preferred Author Guidelines
x. Index

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Identification of Couple of Novel Genes Probably have Relation with Virulence in *Yersinia Enterocolitica* O:8 using Subtractive Hybridisation

By Mahmoud. M. M. Zaky

Port-Said University, Egypt

Abstract - *Yersinia enterocolitica* is one of the important enteric pathogens which are gram-negative rods that are motile when isolated from environment but become nonmotile in mammalian host which cause human disease due to consumption of contaminated water and food and it has an invasivness ability to cross the gastrointestinal mucosa to infect the underlying tissue The pathogenic *Yersinia enterocolitica* always harbouring the important virulence factors, Such as the virulence 70Kbp plasmid which encodes the Yop virulon and the HPI which encodes the Yersiniabactin iron responsible genes, but still other virulence genes in *Yersinia enterocolitica* exists need to be identified and chracterized. Subtractive hybridisation is one of the most powerfull tools for the identification of virulence genes in wide range of bacterial pathogens, and in this study the hybrydization of high pathogenic *Yersinia enterocolitica* O:8 and low pathogenic *Yersinia enterocolitica* O:5 was successful to identify the two novel genes which are probably have relation to virulence. The prepilin peptidase which was proved by PCR in most pathogenic *Yersinia* species, and it is responsible for the faimbrial and pilli formation which has an adhesive and congugative functions which are important for the genetic materials transfere and the other gene is an invasine Inv homolog sequence which has an ORF of invasine Inv of pathogenic *Yersinia enterocolitica* which could be named as *Inv2*.

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Mahmoud. M. M. Zaky

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I. Introduction

Yersinia spp are gram-negative rods that are motile when isolated from environment but become nonmotile in mammalian host. Three species of *Yersinia* cause disease in humans. *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis* These species differ considerably in invasivness, *Y. enterocolitica and Y. Pseudotuberculosis* can ceoss the gastroentistenal mucosa to infect underlying tissue, but infections usually remain localized in the submucosal area. *Y. pestis* is injected into the body by an insect bite and thus does not have to penetrate a body surface on its own, but once inside the body, it spreads rapidly and causes a systemic infections (Heesemann *et al* 1984; Mingrone and Fantasia, 1988; Cornelis, 2002).

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Y. enterocolitica infections have been particularly common among children, and outbreaks have occurred in day-care centers and schools. Symptoms of *Y. enterocolitica* infections vary from a mild form of the disease, characterized by diarrea and abdominal pain, to a more severe from *Yersinia enterocolitica* strains which cause disease are serogrouped using an O1H type of E.coli (Fukushima *et al*, 2001; Denecker *et al*, 2002; Hayashidani *et al*, 2002; Juris *et al*, 2002).

Pathogenic *Yersinia spp* share a common tropism for lymphoid tissue and are markable ability to resist the non-specific immune response of the host their main strategy seems to consist of avoiding lysis by complement, avoiding phagocytosis by polymer-phonuclear leucocytes and macrophages and forming extracellular microcolonies in the infected tissues and they have common basic virulence functions (Tauxe *et al*, 1987, Simmonei *et al*, 1990).

The chromosome of *Yersinia enterocolitica* encodes many virulence factors, such as enterotoxin Yst, Myf fibrille and Inv (Cornelis, 1992, Iriarte *et al*, 1993; Gruizkan *et al*, 1990).

Which have a great influence on the pathogenicity of such micro-organisms and the concept of pathogenicity island explained that particular genomic regions of pathogens, such as *Y. enterocolitica*, carry virulence-associated genes together with loci whose presence strongly indicates horizontal gene transfer of these regions between different species or even genera (Dobrindt *et al*, 1998; Elliot *et al*, 1998; Al-Hasani *et al*, 2001).

The yersiniabactin gene cluster responsible for the manifestation of lethality for mice was named the high-pathogenicity islands (HPI). The functional core of the islands consists of 12 genes, at least six genes encoding iron-responsible proteins (irp1 to 5 and irp9) in *Y. enterocolitica* (YbtE and YbtT are Y. Pestis synonyms for irp4 and irp5, respectively) are involved in biosynthesis of the Yersiniabactin (Heesemann, 1987; Heeseman et al, 1993; Corniel et al, 1996; Pelludat et al, 1998; Rakin et al, 1999; Xu et al, 2000).

Y. pestis, Y. pseudotuberculosis and all pathogenic *Y. enterocolitica* strains harbor a 70-Kb

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plasmid that is devoted to virulence. In *Y. enterocolitica*, this plasmid is called pYV (for plasmid involved in Yrsinia virulence) followed by the identification of strain in *Y. pestis* and *Y. Pseudotuberculosis*, the archetypes are called PCD1 and PIB1 respectively. Most of this plasmid encodes the Yop virulon, a sophisticated virulence apparatus which is conserved in the three species and is considered an archetype of the so-called type III virulence systems encountered in several plant and animal pathogens. (Heesemann and Gruter, 1987; Neubauer et al, 2000; wecks et al, 2002).

II. Materials and Methods

a) Bacterial Strains and Growth Media

The bacterial strains used are *Yersinia enterocolitica* O:8 and O:5 in the subtractive hybridisation and *E.coli* for carrying plasmid with subtracted DNA fragments and *E.coli* DH5α in the transformation and the growth media was L.B media considering the incubation of *Yersinia* strains at 27° and *E.coli* at 37°.

b) Bacterial Plasmids

In subtractive Hybridization pMOSBlue with 2.8 Kbp to carry the subtracted DNA fragments in *E.coli* and pSB315 which carry the Kanamycin cassette which is 1Kbp.

c) Subtractive Hybridisation

Subtractive hybridisation is a powerful technique that has been applied to research in many different fields for studying the eukaryotic systems. The application of subtraction techniques using clontech PCR-select bacterial genome ubtraction Kit user Manual PT3170-1, typically focused on differential gene expression differences between two cDNA populations-rather than differences between genomes. This is because eukaryotic genomes are too complex for existing subtraction technologies. In contrast, bacterial genomes are considerably smaller, and are even less complex than many eukaryotic cDNA populations. Thus subtraction methods can be used to identify sequences that are present in one bacterial genome but are absent in another.

Although there are several different methods, the basic theory behind subtraction is simple. The genomic DNA sample that contains the sequences of interest is called “tester” and the reference sample is called “driver”. Tester and driver DNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized DNAs represent tester-specific sequences.

Traditional subtractive hybridization methods involve several rounds of hybridization and require large amounts of DNA. In contrast the clontech PCR-select bacterial genome subtraction Kit overcomes these and other technical limitations of traditional subtraction procedures. Like PCR-select cDNA subtraction, this kit is based on the suppression subtractive hybridization (SSH) method, the PCR- select procedure requires only 1.5-2 μg of genomic DNA, takes only 2-3 days. Suppression PCR prevents undesirable amplification while enrichment of target molecules proceeds.

d) Sequencing of the DNA Fragments

- PCR for the fragments (Subtracted fragments of *Y. enterocolitica* O:8 carried on the plasmid pMos blue) using the primers T7 and U19

240 μl	buffer (10x)
240 μl	dNTPs
24 μl	primer1
24 μl	primer 2
9.6 μl	tag polymerase
1822.4 μl	distilled water

90 μl for each tube (24) + 10 μl DNA and the running in the PCR set with 30-35 cycles.

- Purify the PCR product (DNA) using the PCR purification Kit.
- Run on the Gel.

e) Preparation of the DNA for Sequencing

- 3 μl PCR product (DNA)
- 1 μl primer (5pmol / μl)
- 4 μl Big dyes
- 12 μl distilled water
- Then run in the PCR set.
- Sequencing was done using ABI prism Big Dye terminator cycle sequencing ready reaction kits, in the diagnostic lab, Max von pettenkofer Institute University of Munich Germany.
- The data were analysed with computer using the NCBI bast x and TIGR blast program.

f) Isolation of Plasmids

The plasmids were isolated with nucleobond application Kit using the ready buffer solutions S1, S2, S3, N2, N3 and N5.

The bacterial culture is harvested by centrifugation at 300 to 5000 rpm for 5-10 min at 4°C.

g) Electroporation

With ice cold environment 40 μl from competent cells plus 3.5 μl DNA (plasmid was diluted 1:10). Then electroporation was done in electroporation cuvetts with gene pulser. L.B. medium was added (1 ml) and incubated for 1hr in 37°C, then 100 μl was platted in L.B. medium with antibiotics and incubated overnight. Some colonies were selected and platted on L.B medium with antibiotics overnight and then screened for the plasmids.

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h) Isolation of the plasmid pMos blue from E.coli

The plasmid pMos blue of the subtracted clone No 75, which carries the invasine homology was isolated from *E. coli*, and it was 2.8 Kbp and then cut with the restriction enzyme EcoRI

i) Isolation of the kanamycin kassette from the plasmid pBs 315

The pBs 315 plasmid was isolated from *E. coli*, then cut with the restriction enzyme Hinc II to give the kanamycine kassette band which is 1000 bp, and was cut from the gel and purified

j) Mutagenesis using linear transformation ET recombination

The principle of this method is the design of the required sequence (Invasin) with about 50 nucleotides which forms homology arms to the selection marker (kanamycin).

- Then PCR amplification and purification of the PCR product (linear DNA).
- Digestion with restriction enzyme DpnI.
- Direct transformation of the linear DNA into competent cells of the Nalidixin mutant of *Y. enterocolitica* to produce point mutation (homologous recombination) into the chromosomal DNA.
- Plat the mutants on the nalidixin, kanamycin L.B. medium plates
- Prove the insertion of the desired sequence with PCR (Wagner and Koszinowski, 2002).

III. Results

a) Subtractive hybridization and Sequencing of the Y. enterocolitica subtracted DNA fragments

96 subtracted clones of *Y.enterocolitica* O:8 which carried on the plasmid pMos blue were detected with PCR, and revealed different sizes (Fig. 1). Then purified and sequenced to show different important homology to translated proteins, which include some virulence factors (Table. 1), such as prepilin peptidase (Clone NO.3) and invasin (Clone NO.75).

b) Prepilin peptidase detection in different Yersinia strains

Prepilin peptidase was detected in different *Yersinia* strains such as *Yersinia enterocolitica, Y. pseudotuberculosis* and *Y. pestis* by PCR, using primers designed from the subtracted fragment DNA of the clone No 3 which have homology to prepilin peptidase and PCR revealed specificity of this protein to all strains of *Y. enterocolitica* (Fig. 2).

c) Detection of invasine homolog in Yersinia enterocolitica

PCR was applied on the subtracted DNA fragment No.75 which has homology to invasine using the proper primers T7 and U19, which is carried on the plasmid pMos blue, the DNA fragment was about 900 bp (Fig.3,4). and from the Data base of the genomic sequencing of the *Yersinia enterocolitica*, using blast matching it was found that the sequence has an ORF of *inv* gene (Fig.4)

d) Transformation of the pMos blue plasmid which carries the kanamycine kassette into E.coli DH5α

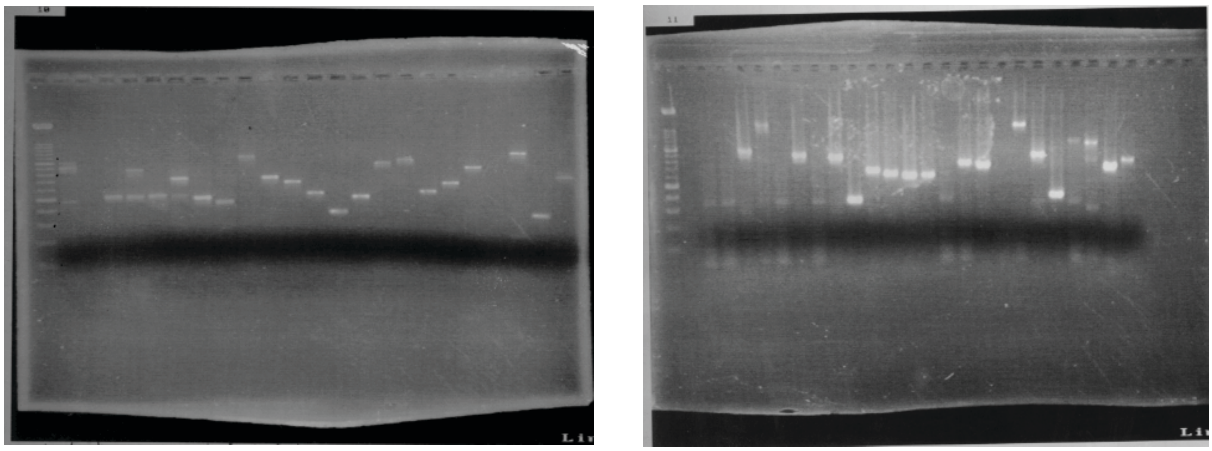
The kanamycine kassette was ligated with the plasmid pMos blue 75 which carries the invasine homology, and then transformed into *E. coli* DH5α, and the transformants were grown well on the L.B. medium plates containing the antibiotic Kanamycine, then the ligated plasmid was isolated again from *E. coli* (Fig.5)

e) Mutagenesis of Y. enterocolitica using E.T recombination

The proper primers were designed from the kanamycine kassette sequence and the sequence of invasine homology which is carried on the plasmid pMos blue 75 to make 50 nucleotides homology arms to the kanamycine kassette. With the application of PCR, the DNA fragment 1000 bp was obtained from the DNA obtained from the cooked cells of *E.coli* DH5α and the resulted DNA was electroporated into the competent cells of the Nalidixin mutant *Y. enterocolitica* WA314, and the transformation was positive where the mutants were grown on L.B. medium containing the antibiotics Nalidixin and kanamycine.

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(A) (B)



Mr ABCD EF GH I J K LM NO PQ RS TUVW Mr ABCDEFGHIJKLMNOPQRSTUVWX

Fig. 1 : Detection of the subtracted fragments of DNA of *Y. enterocolitica* O:8 using PCR. Mr = 100 bP DNA marker. (A) A to W = the subtracted fragments from 1 to 23. (B)A to X = the subtracted fragments DNA from 73 to 96

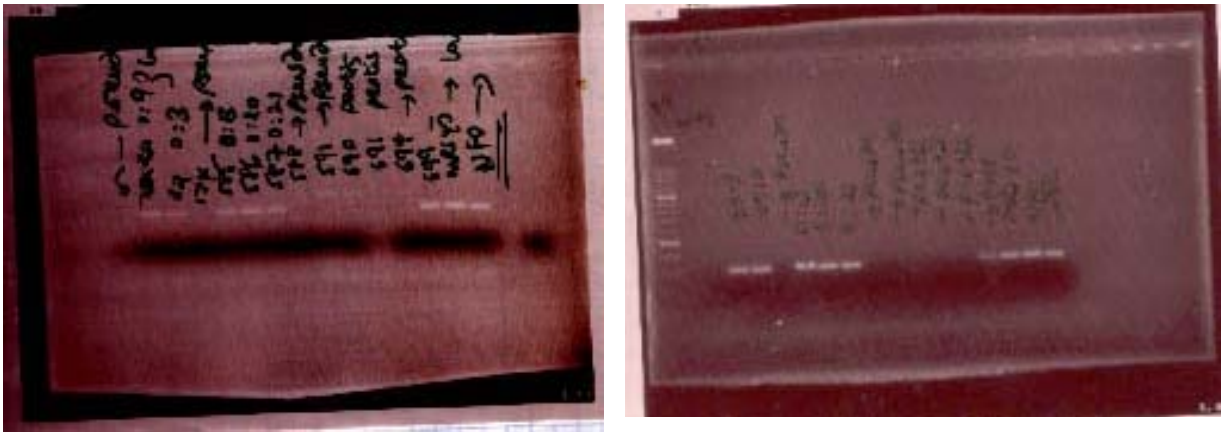
Table 1 : Results of sequencing of the subtracted fragments DNA of the pathogenic *Y. enterocolitica* O:8

Clone No.	Size bP	Significant similarity homologous protein
3	483	Prepilin peptidase
5	610	Putative transposase for Ins
10	623	Putative transposase / yersinia
30	589	Phospho-N-acetymuranoyl-pen
31	725	Cell wall surface anchor
32	590	Orf hyoth
34	590	DNA-Damag-inducible protein
37	637	Hemin transport protein HMV
38	783	Cytochrome C oxidases
39	789	Hypothetical protein
40	999	(M74027) mucin (Homo sapiens)
41	999	(M74027)mucin (Homo sapiens)
42	644	Protein Y HHw > gi 125 180891
43	600	Hypothetical protein PA5101
44	624	(AP002552)deoxyribopyrimidin
45	516	(AE005566-1glycogen ph
49	757	(AE006974) PE- PGRS family protein
51	653	(AF068066) cytochrome c
54	646	(AB005245) ATP- dependent RNA
55	649	(AP002839) hypothetical protein
59	692	Extension homolog F28A2180
62	672	(K03503) threonine dehydratase
64	451	(Y14835) beta- galactosidase
65	660	(Aj231116)257r (*Vibrio cholerae*)
66	745	(Ac006585)hypothetical
70	626	Beta-galactosidase-complent-protein
73	645	Orf, hypothetical protein
75	632	Putative invasin
76	654	Ribonuclease G
77	688	Orf. Hypothetical protein

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78	676	Conserved hypothetical protein
79	784	(M7 40279 mucin (homo sapiens)
81	643	NADP-alcohol dehydrogenase
83	624	(AF 285784) Clp protease
84	629	(Aj 414143) UDP-N-actylmuramoyl
86	644	(L37382) beta- galactosidase-comple..
88	801	(M7 4027) mucin (Homo sapiens)
90	628	Transcriptional regulator (lys..
92	401	(AJ 414153) putative long-chain..
93	641	(AJ 414146) putative surface ant..
94	629	KIAA0579 protein (Homo sapiens)
95	573	(Y08949) tipA (synthetic construct)
96	628	(AJ 414156) conserved hypothetic..

(A) (B)



Mr AB CD EFG HIJ KLMNO Mr AB CDE FGHI

Fig. 2 : Detection of prepilin peptidase gene in different *Yersinia* strains using PCR

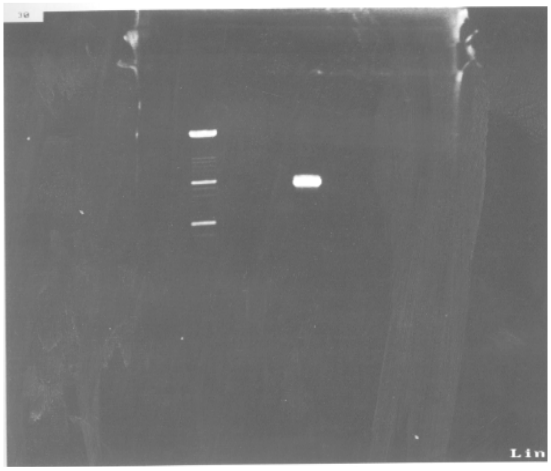
(A)

Mr = 100 bP DNA marker
A = *Yersinia pseudotuberculosis*
B = *Y. enterocolitica O:9*
C = *Y. enterocolitica O:3*
D = *Y. pseudotuberculosis*
E = *Y. enterocolitica O:13*
F = *Y. enterocolitica O:20*
G = *Y. enterocolitica O:21*
H = *Y. pseudotuberculosis*
I = *Y. pseudotuberculosis*
J = *Y. pestis*
K = *Y. pestis*
L = *Y. pestis*
M = *Y. enterocolitica*
N = *Y. enterocolitica*
O = *Y. enterocolitica*

(B)

Mr = 100 bP DNA marker
A = *Y. enterocolitica O:9*
B = *Y. enterocolitica O:3*
C = *Y. enterocolitica O: 13*
D = *Y. enterocolitica O:20*
E= *Y. enterocoliticaO:21*
F = *Y. enterocolitica*
G = *Y. enterocolitica*
H = *Y. enterocolitica*
I = *Y. enterocolitica*

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M A

Fig. 3 : detection of the subtracted fragment DNA (75) which is invasine homolog and carried on pMos blue plasmid using PCR. M = 100 bP DNA marker. A = The subtracted fragment DNA (75)

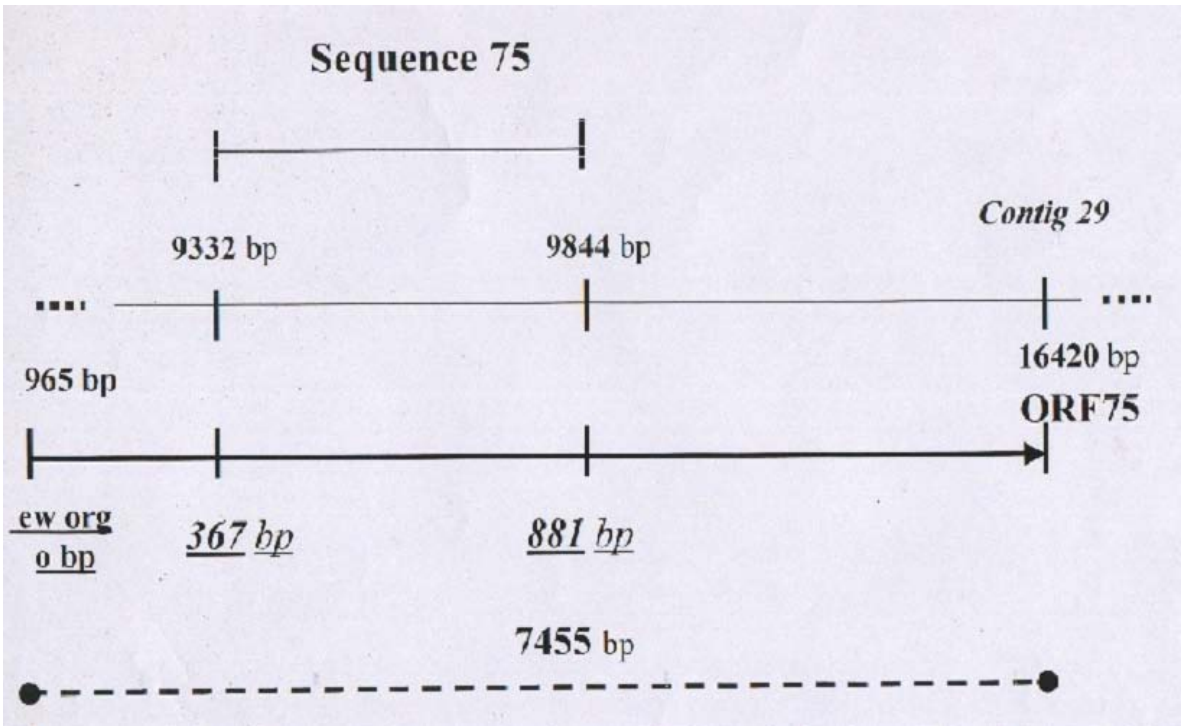
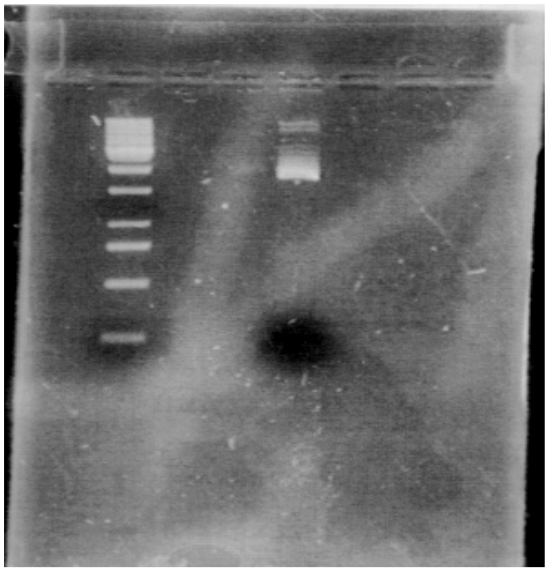


Fig. 4 : Sequence of subtracted fragment DNA-sequence No. 75 which is homologous to a part of ORF of inv-gene from the data-base of *Y. enterocolitica*

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Mr A

Fig. 5 : Detection and isolation of pMos blue plasmid (75) from E.coli DH5α after ligation with Km cassette. M = 100 bP DNA marker. A = pMos blue plasmid (75) after ligation with Km cassette

IV. Discussion

For most bacterial pathogens virulence is a multifactorial process requiring two general classes of determinants. The first encompasses genes that participate in physiological processes necessary for survival in host and non-host environments and these genes are generally found in both pathogenic and non-pathogenic organisms. The second class of virulence genes specifies traits that are unique to pathogens, and not surprisingly, these genes are rarely detected in non-pathogenic organisms, based on the initial characterization of plasmids from *Yersinia* and *Shigella*. Such sequences were originally thought to be confined to extrachromosomal elements but recently, several virulence cassettes have been mapped to the chromosome of pathogenic organisms. These segments of the chromosome, termed pathogenicity islands (Groisman and Ochman., 1996; Thoerner *et al*, 2003).

Subtractive hybridization of *Y. enterocolitica* O:8 and O:5 and sequencing of the subtractive fragments after cloning, revealed many homology. The most important fragments which gives indication of the homology to virulence genes is prepilin peptidase for clone No 3. The bifunctional enzyme pripilin peptidase (pilD), is the key determinant in both type-IV pilus biogenesis and extracellular protein secretion. Pripline peptidase cleave, among other substrates, the leader sequence from pripline-like proteins that are required for type II protein secretion in Gram-negative bacteria type 4 pili, that are surface organelles required for diverse activities that contribute to board attributes, such as genetic transfer, virulence and environmental persistence of a wide array of Gram-negative bacterial pathogens (Lory and Strom, 1997; Lapointe and Taylor, 1999; Liles *et al.*, 1999).

Sequencing of the subtractive fragment clone DNA No 75 revealed homology to the Orf of Invasin gene (Inv), where the three pathogenic species of the genus *Yersinia*, invade host tissues and cause disease syndromes, ranging from gastroenteritis *(Y. enterocolitica* and *Y. pseudotuberculosis*) to plague (*Y. pestis*).

Early studies showed that pathogenic strains of *Y. enterocolitica* could invade eukaryotic cells in vitro whereas nonpathogenic strains could not. A molecular analysis of cellular penetration led to the cloning of the chromosomally encoded invasion gene (Inv) of *Y. pseudotuberculosis* Invasin, the Inv gene product, initiates the entry process directly by attaching to mammalian cell receptors, which include multiple members of the integrin superfamily subsequently, the homologous Inv gene from *Y. enterocolitica*. (Pepe and Miller, 1993; Dersch and Isbery, 1999; Grassl *et al*, 2003).

In this study, the homologous of Invasin of *Y. enterocolitica* O:8, which is not found in the other low pathogenic strains could be another Invasine, which could be called (Inv2) and this possible for the first time to be another unknown virulence gene of *Yersinia* spp., and could be the first step to discover another gene cluster which is related to virulence and that needs more work in the light of pathogenicity island concept. Particularly using the Et recombination to block the original Invasine with the kanamycine (Km) cassette and production of mutant lacking one of the most important virulence determinants, reduce the virulence of *Y. enterocolitica*. Such mutants produced by point mutation of *Y. enterocolitica* could be useful in the production of mutants applied as vaccines for pathogenic *Yersinia* infections.

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Effect of N-Hexane Oil Extract of Two Spices on Serum Lipid Profile and Blood Glucose Concentration of Albino Rats

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Abstract - Consumers are concerned with their health and physical fitness and are seeking for alternative plant products with potential for providing nutrients with enhanced health benefits. Hence, this study investigates the effect of mixture of Ehuru *(Monodora myristica)* and Njasang *(Ricinodendron heudelotii)* oil extract on serum lipid profile and blood glucose concentration of albino rats. The spices were processed into fine flour and the oil was extracted with n-hexane as the solvent. A total of twenty five rats weighing 125-160g were separated into five groups of five each to represent control, olive oil and varying concentrations of the spices. After acclimiatization for one week, experimental administration of the extract was carried out daily for 28 days. Blood samples were collected by cardiac puncture into tubes. A portion of the blood was used for fasting blood glucose determination. Serum was separated from the other portion and used for assay of lipid profile using standard kit methods. The results obtained showed percentage fatty acid yield for Ehuru and Njasang as 79.54 and 81.0 (polyunsaturated) and 13.40 and 15.0 (monounsaturated) respectively. Fasting blood glucose assay showed that only rats in group 1 (6.46mmol/L) became significantly ($p<0.05$) hyperglycaemic while groups 2-4(6.03, 5.98 and 5.53mmol/L) showed a hypoglycaemic effect with respect to control (6.13mmol/L).

Keywords : *ehuru, njasang, glucose, serum lipid, rats.*

GJSFR-C Classification : *FOR Code: 270599*



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Ogunka-Nnoka C. U. [α] & Igwe F. U. [σ]

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Keywords : ehuru, njasang, glucose, serum lipid, rats.

I. Introduction

The need to maintain good health is the driving force in the search for alternative oil seeds of spices with high medicinal and nutritional potentials. Spices, depending on the part of the plant being used can be classified into fruits, seed, leaves or floral parts and bulbs used to season food due to their distinctive flavor and aroma (Manay, 1987), as well as for therapeutic purposes (Sirinivasan and Sambaiah, 1991). Herbs and spices are integral part of the daily diet. Dietary spices influence various systems in the body such as gastrointestinal, cardiovascular, reproductive and nervous systems resulting in diverse metabolic and physiological actions (Angerer et. al., 2002). There is an increasing demand for edible oils with essential fatty acids. Achinewhu et al; 1995; investigated chemical composition of thirty wild spices indigenous to Nigeria and observed that they contained high amount of fats as well as essential oils.

Research have been able to show a relationship between edible oils and cardiovascular disease. It has been reported severally that oils with high amounts of cholesterol and other saturated fatty acids tend to cause hyperlipidaemia (accumulation of lipid in the walls of the arteries)causing a narrowing of the blood vessels and an attendant effect on blood pressure (Guthrie and Picciano, 1995). Data from experimental and epidemiological studies show that elevated levels of Low density lipoprotein (LDL) – cholesterol and Triglycerides and low levels of High density lipoprotein (HDL) - cholesterol are major risk factors of coronary heart disease (Besong et. al., 2011). It has also been shown that glucose and fatty acids appear to interact in health and disease as they work together to regulate the expression of several enzymes involved in carbohydrate metabolism thereby affecting blood glucose levels. Ugochukwu et. al., 2003 reported that high levels of triglycerols and total serum cholesterols often accompanying diabetic condition were significantly decreased by the ethanolic extract of G. latifolium. Spices like cinnamon, cloves, bay leave and turmeric have insulin-potentiating effect in vitro (Khan et. al., 1990). So, these spices might have a role in lipid metabolism. Seed oil from spices has been reported as good sources of essential oil. However, no oil from a single source can be suitable for all purposes. Spices also, are rarely consumed singly, they are consumed either as mixture of two or more spices to improve their quality. It is, therefore, important to understand the influence of such combination using n-hexane oil extract of ehuru (*Monodora myrstica)* and Njasang (*Ricinodendron heudeloitii*) oil seeds. Literature on their therapeutic properties in vivo is scanty, hence, this present study is considering the effect of the oil extract mixture of both spices on serum lipid profile and blood glucose concentration of albino rat.

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II. Materials and Methods

a) Plant Materials

The seeds of ehuru (*Monodora myristica*) and njasang (*Ricinodendron heudeloitii*) were purchased from the herb sellers at mile 3 market, Port Harcourt, Rivers State Nigeria. The seeds were cleaned, ground and then sieved to 300mm mesh. The sieved spices were then stored in an air tight container and stored in desiccators for subsequent analysis.

b) Animals

Twenty five (25) albino rats of both sexes weighing 125-160g were obtained from the animal house of the Department of Biochemistry, University of Port Harcourt, Rivers State, Nigeria. They were housed in cages of five groups of five each and acclimatized for one week with food and water ad libitum at room temperature (26 - 28^{0}C) throughout the 28 days of the experiment. The standard laboratory animal care was followed in this study (CIMOS, 1985).

c) Oil Extraction from Ehuru and Njasang

Soxhlet extraction was carried out using n-hexane. Fifty (50) gram of ground seeds were placed in a cellulose paper and extraction lasted for 8 hours. The oil was then recovered by evaporating the solvent using rotary evaporator (Bligh and Dyer 2009). Fatty acid composition of the seed oil of the spices was determined using NUCON series 5700 gas chromatography equipped with the flame ionization detector and stainless steel packed column, having internal diameter 2mm and length 2.0cm. About 0.1ml of oil was converted to the methyl ester by using the borontrifluoride and extracted in 1ml hexane before being injected into the Gas Chromatography (GC). The detector temperature was programmed for 200^{0}C with a flow rate of 25ml/min. The injector temperature was set at 200^{0}C. Column temperature was programmed from 70^{0}C to 200^{0}C with the increasing rate of temperature 6^{0}C/min. Nitrogen was used as the carrier gas. Hydrogen flow 40ml/min and air flow 60ml/min were used. The peak was identified by measuring the retention time of the samples and comparing it with the standard under the same operating conditions.

d) Experimental Procedure

Rats were administered with the mixture (prepared in equal proportion) of the oil extract orally at 0.5% per kg body weight in doses of olive oil: test sample (1:3, 1:2 and 1:1) for groups, 1, 2 and 3 respectively. Whereas group 4 was administered with 2.0ml olive oil and group 5 which served as control received 0.9% oral normal saline solution. All animals were observed daily. After 28 days of treatment blood samples were collected by direct cardiac puncture. The blood was transferred into a sterilized sample container and a portion was used for fasting blood glucose assay; while, the other portion was allowed to stand at ambient temperature and immediately after clotting, it was centrifuged at 2000G for 5 min. the sera were carefully separated into a clean container and kept in the refrigerator at 4^{0}C and analyzed within 24hr.

e) Glucose Assay

Fasting blood glucose concentrations were measured by using the glucose oxidase method with an automated glucose analyzer (Roche Diagnostic GMbH, Mannheim, Germany)

f) Lipid Profile

Serum lipid profile which includes high density lipoprotein (HDL) -cholesterol, total cholesterol (TC) triglygerides (TG) and low density lipoprotein (LDL) -cholesterol were determined by standard enzymatic colorimetric techniques which involves test kits procured from Randox Laboratories.

g) Weight Measurement

Body weights of the rats were measured three times in a week for 28 days. Daily changes in body weights as percentages were recorded. The percentage of daily changes in the body weights was calculated according to the following formula.

Change in body weights (%) =

$$\frac{100 \text{ x } (\text{weight}_{\text{final}} - \text{weight}_{\text{initial}})}{\text{Weight}_{\text{initial}}}$$

h) Statistical Analysis

Results were reported as mean ±SD. All data were analyzed using the analysis of variance. When analysis of variance revealed a significant effect, means were separated using Duncan's new multiple range test (Wahua, 1999).

III. Results

a) Fatty acid Profile

The fatty acid composition of Ehuru and Njasang spices is summarized in Table 1. Ehuru and Njasang showed the following fatty acid percentages. 79.54 and 81.0% for polyunsaturated fatty acids, 13.40 and 15.0 for monounsaturated fatty acids and 7.10 and 5.50 for saturated fatty acids respectively.

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Table 1 : Fatty acid composition of Ehuru and Njasang

Fatty acid	No. of carbon	Oil seed (%)	
		Ehuru	Njasang
Caprylic	8.0	1.0	-
Capric	10.0	0.55	1.90
Lauric	12.0	1.40	1.20
Myristic	14.0	1.60	2.30
Palmitic	16.0	5.42	ND
Stearic	18.0	2.02	ND
Myristoleic	14.1	5.30	14.74
Palmitoleic	16.1	5.42	ND
Oleic	18.1	2.44	ND
Linoleic	18.2	34.48	26.76
Linolenic	18.3	41.45	32.20
α-linolenic	18.3	2.20	ND
Eicosapentaenoic	20.5	ND	20.60
Total fat		38.72	43.20

ND ⇒ Not Detected

b) Effect of oil seed spices on blood glucose

The result on the effect of oil extract of Ehuru and Njasang on blood glucose is shown in Fig. 1. Only rats in group 1 (6.40mmol/L) became significantly ($P<0.05$) hyperglycaemic with respect to the control group. The hyperglycaemic effect was dose-dependent. There was no significant ($P<0.05$) difference with rats in groups 2, 3 and 4 compared to control.

c) Effects of oil seed spices on lipid profile

Table 2 shows the general serum lipid profile after administering the oil extract of the spices to the rats for 28 days. Total cholesterol (TC), LDL-cholesterol, and LDL/HDL - cholesterol ratio values were low for test samples (group 1-3) and olive oil (group 4) compared to control. The decrease was significant ($P<0.05$) for all samples. HDL-cholesterol (1.15-1.55mmol/L) increased with increase in extract administration. The increase observed in HDL was significant ($P<0.05$). Triglyceride (TG), slightly increased for test samples compared to control, but the increase was not significant at $P<0.05$.

Table 2 : Serum Lipid Profile

Group	Lipid Profile (mmol/L)				
	TC	TG	LDL	HDL	LDL/HDL
1 (1:3)	2.14^{b}	0.65^{a}	0.48^{b}	1.55^{a}	0.31^{c}
2 (1:2)	2.12^{ab}	0.62^{b}	0.44^{c}	1.36^{b}	0.32^{c}
3 (1:1)	2.08^{c}	0.60^{b}	0.43^{c}	1.20^{c}	0.36^{b}
4 (olive oil)	1.95^{d}	0.58^{c}	0.40^{d}	1.15^{d}	0.35^{b}
5 (control)	2.20^{a}	0.60^{b}	0.58^{a}	1.02^{e}	0.97^{a}

Values are expressed as mean ± SD Means in the columns not followed by the same superscripts differ significantly ($P\leq0.05$).

d) Body weight changes

The rats gained weight throughout the experimental period as shown in Fig. 2. The weight increase in groups 4, 3 and 2 were significant ($P\leq0.05$) and very steady compared to groups 1 and 5.

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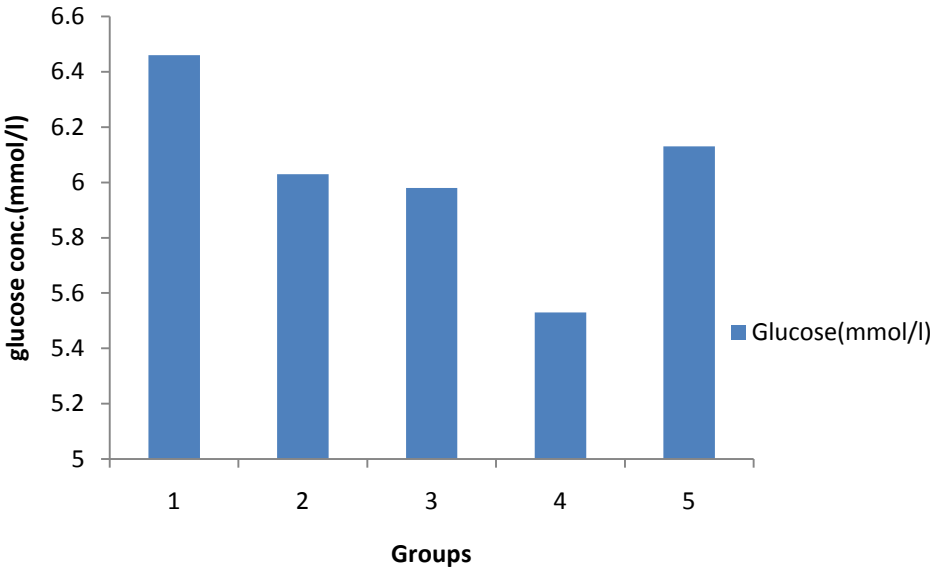


Fig. 1 : Glucose conc.(mmol/l) in the various groups

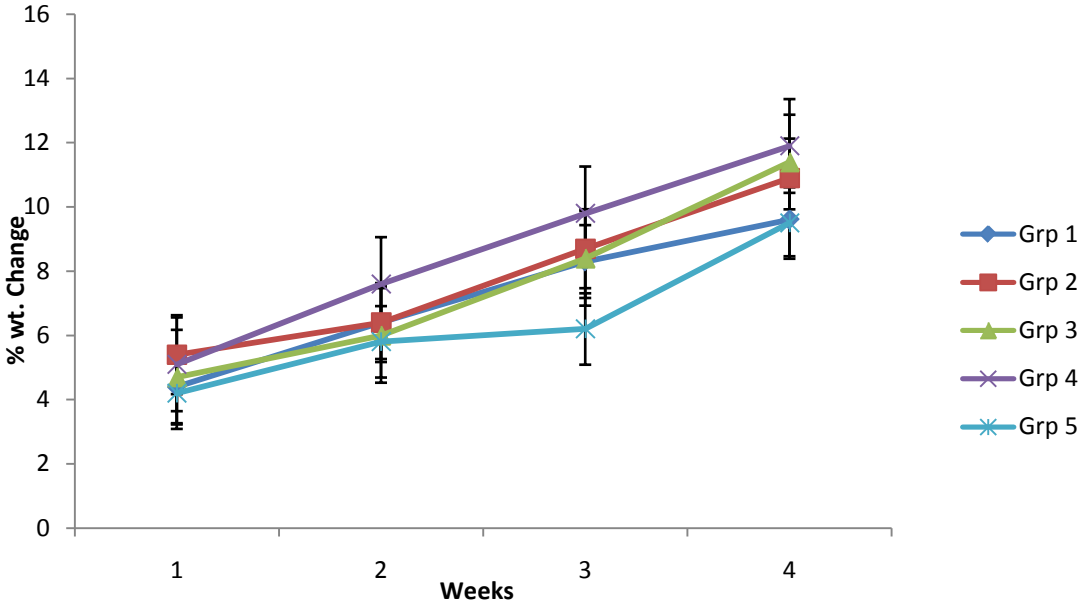


Fig. 2 : Weekly body weight changes in the rats

IV. Discussion

Table 1 reported the fatty acid composition of Ehuru and Njasang. The relative abundance of mono and polyunsaturated fatty acids present in these samples reveals their potentials in nutrition with enhanced health benefit. Linoleic, oleic and linolenic acids present in these extract are fatty acids that have a cholesterol lowering effect which prevents coronary heart disease and artherosclerosis (Colussi et. al., 2007). Linoleic acid as well as its derivatives serves as structural components of the plasma membrane and as precursors to regulate specific cellular metabolic functions and gene expression (Jump, 2002). The fatty acid composition of these samples is also similar to other vegetable oils such as canola oil, olive oil and sun flower oil (Matos et. al., 2009).

Data from this present study show that n-haxane oil extracts of mixed spices exert hypoglycaemic effect as well as hyperglycaemic effect at higher concentration. Chang and Johnson 1980, reported similar results. The mechanism by which spices lower plasma glucose and the interaction of high fat diets on glucose metabolism has not been fully elucidated (Khan et. al., 1990). Although fatty acids are said to be structural components of membrane lipids such as glycolipids. High lipid levels have been associated with increase in serum glucose level which is as a result of insulin inhibition (Raza and Movahed, 2003; Harvey and

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Champe, 1994). However some plants like cinnamon, ginger, bay leaves and garlic have been reported to have beneficial effects in the treatment of both diabetes and cardiovascular diseases (Khan et. al., 2003). Ugwuja et al; 2008 also reported similar result using equal proportions of mixture of curry, garlic and ginger.

Variation in the amount of dietary fat has been reported to affects plasma cholesterol as well as coronary heart diseases. Dietary saturated fatty acid increase the total blood cholesterol level as dietary polyunsaturated fatty acids decrease it. (Guthrie and Picanno, 1995; Sardesai, 1992; Norum, 1992). The oil seed extract of mixture of ehuru and njasang lowers the serum total cholesterol (TC) and low density lipoprotein (LDL) - cholesterol. The observed decrease is probably due to high percentage of polyunsaturated fatty acids and monounsaturated fatty acid present in the samples. Although, saturated fatty acids raise the level of LDL. The mechanisms involved are not completely understood, however the receptor on liver cell membrane that binds LDL cholesterol appears to be suppressed by saturated fatty acids. For example, when LDL receptor activity decreases by the presence of saturated fatty acids, LDL catabolism decreases and blood level of LDL increase and vise versa (Guthrie and Picciano 1995; Fraser, 1994). The insignificant increase in Tryglyceride (TG) observed in all the groups may be attributed to the presence of saturated fatty acids especially in Ehuru sample. It has also been reported that sometimes there may be a defective effect on the enzyme (lipase) that degrades triglyceride (Enig, 1993). Besides, Norum 1992 reported that individual saturated fatty acids differ in their ability to change serum lipid. The serum HDL-cholesterol level significantly increased. This indicates that these mixtures can promote decreased risk of coronary heart disease (Colussi et. al., 2007). Also, the LDL/HDL cholesterol ratio decreased significantly. This ratio is thought to be the atherogenic index of lipoprotein (Ajayi et. al., 2011) compare to an increase in serum total cholesterol level which is said to be associated with increased risk of atherosclerosis. The lower the LDL/HDL ratio the less atherogenic the lipoprotein profile is thought to be (Murray et. al., 2003). The hypolipidaemic and hypoglycaemic effect observed in this study agree with the reports of Khan et al, 2003; Gorinsterin et al; 2006; Ugwuja et. al., 2010. However, at higher concentration slight hyperglycaemic and hyperlipidaemic effect was observed. While group 4 treated with Olive oil showed a lowered glucose and serum lipid levels.

The body weight gain observed in this study may be attributed to the nutrient potentials of these spices, some of which include the stimulatory effect on the digestive system. Platei and Srinivasan, 1996, reported that ginger enhances the digestive activities of enzymes like the intestinal lipase, sucrase and maltase.

V. Conclusion

Observations made from this study prove that mixture of oil extract of Ehuru and Njasang at culinary dose has beneficial health effect on blood glucose and lipids. The combination of spices as is currently practiced is then encouraged.

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Modeling of Non- Newtonian Fluid for Blood Flow in Stenosed Arteries; A Comparative Study

By Mohammed Musad

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Abstract - In this paper the mathematical model have been developed for the computation of pressure gradient, viscosity, yield stress and wall shear stress and the influence of stenosis in the rheology of blood, where the blood flow is assumed to behave like a couple stress fluid, peripheral layer plasma (Newtonian fluid) and core layer of suspension of erythrocytes (Non-Newtonian fluid). The non-Newtonian fluid in the core region of the artery is assumed as a Herschel-Bulkley fluid. The results predicts that wall shear stress has directly proportional relation to the length of stenosis, yield stress, viscosity and pressure gradient respectively, and inversely proportional relation with the value of power model index *n*. The obtained results for wall shear stress in this paper have been compared to the results obtained by Musad and Khan (2010). It is observed that for the range of the height stenosis 8×10^{-5} to 10×10^{-5}, the wall shear stress in case of Herschel-Bulkley fluid is considerably lower than these in case of Casson fluid.

Keywords : *blood flow, wall shear stress, non-newtonian fluid, symmetric stenosis, herschel-bulkley fluid.*

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Abstract - In this paper the mathematical model have been developed for the computation of pressure gradient, viscosity, yield stress and wall shear stress and the influence of stenosis in the rheology of blood, where the blood flow is assumed to behave like a couple stress fluid, peripheral layer plasma (Newtonian fluid) and core layer of suspension of erythrocytes (Non- Newtonian fluid). The non-Newtonian fluid in the core region of the artery is assumed as a Herschel-Bulkley fluid. The results predicts that wall shear stress has directly proportional relation to the length of stenosis, yield stress, viscosity and pressure gradient respectively, and inversely proportional relation with the value of power model index *n*. The obtained results for wall shear stress in this paper have been compared to the results obtained by Musad and Khan (2010). It is observed that for the range of the height stenosis 8×10^{-5} to 10×10^{-5}, the wall shear stress in case of Herschel-Bulkley fluid is considerably lower than these in case of Casson fluid.

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I. Introduction

The blood flow through arteries with stenosis is considered as non-Newtonian Misra, J.C *et al* 1993. Non- Newtonian flow is the flow that does not obey the Newtonian relationship between shear stress and shear rate. In non-Newtonian flow the slope of shear stress versus shear rate curve is not constant and so it is not a straight line but a carve and the viscosity of fluid decreases with increasing of shear rate. Blood flow problems are more complicated than the problems of fluid flow in engineering for some reasons like unusual curvature of blood vessels, unusual large numbers of branches and unusual fluid properties of blood.

Blood flow characteristics in arteries can be altered significantly by arterial disease, such as stenosis. The abnormal and unnatural growth in the arterial wall that develops at various location of the cardiovascular system under diseased conditions is called stenosis. This can cause serious circulatory disorders by reducing of occluding the blood supply. For instance, stenosis in the arteries supplying blood to brain can bring about cerebral strokes. Likewise in coronary arteries it can cause myocardial infarction leading to the heart failure Musad (2012). The effect of severe stenosis calcified atherosclerotic vessel is less elastic than a healthy vessel. In a less elastic atherosclerotic vessel, the ability of the vessels to expand in order to accommodate the volume of ejected blood at the onset of the cardiac cycle is diminished, and consequently blood flow through the diseased vessel may be substantially reduced. In order to maintain proper levels and rates of blood flow, the wall shear stress will increase. Thus there is complementary relation between the growth of stenosis and the flow of blood. Very high wall shear stress can activate the platelets which can cause thrombosis and may totally block the vessels. The wall shear stress distribution is an important diagnostic factor for examining the blood flow characteristics through the arteries. Accurate predictions of the distribution of the wall shear stress are particularly useful for the understanding of the effect of blood flow on endothelial cells.

Srivastava (2003) considered the effects of stenosis assuming blood to behave like couple stress fluid, peripheral layer plasma Newtonian fluid, which is acceptable for high shear rate flow (shear rate $>$ 10 sec^{-1}) in layer arteries and with viscosity coefficients $\mu_{p,}$ and a core region of suspension of erythrocytes an non-Newtonian fluid, which is acceptable for low shear rate flow (shear rate $<$10 sec^{-1}) through small diameter arteries and with viscosity coefficients $\mu_{c.}$. Sanker and Lee (2007) have assumed the non-Newtonian fluid as Herschel-Bulkley fluid.

Kapur (1985) reported that Herschel-Bulkley fluid model is the fluid model with non-zero yield stress and it is more suitable for the studies of the blood flow through narrow arteries. It has been established by Merrill et al. (1965) that Herschel-Bulkley fluid model holds satisfactorily for blood flowing in tubes of diameter 130 – 1300 μm.

Musad and Khan (2010) have developed a mathematical model to study blood flow in stenosed region the fluid in core layer as Casson fluid. Thus in this paper, we study core layer as Herschel-Bulkley fluid. and; compare the results of these models.

II. The Model Developed

The small artery under consideration for the development of model is with an axially symmetric stenosis. The blood flow is assumed to behave like a couple stress fluid. Peripheral layer is plasma with viscosity coefficient μ_p (Newtonian fluid) and core

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suspension layer of erythrocytes is with viscosity coefficient μ_H (Herschel-Bulkley fluid) and δ_p is a uniform thickness of plasma layer

Under the above conditions Kapure 1985 have indicated the equations of velocities for the both layers as follow

$$V_P = \frac{G}{4\mu_P}[R^2(z) - r^2], R_1(z) < r \le R(z) \tag{1}$$

$$V_H = \frac{G}{4\mu_H}[R^2(z) - r^2] + \frac{G}{\mu_H}[R^2(z) - R_1^{\,2}(z)][\frac{\mu_H}{\mu_p} - 1], 0 < r \le R_1(z) \tag{2}$$

And the equation of non-Newtonian fluid flow as

$$\tau = (-\mu\frac{\partial v}{\partial r})^n + \tau_y \tag{3}$$

The boundary of the stenosis is represented by the following equations

$$R(z) = \begin{cases} 1 - \dfrac{\delta_P}{2R_0}(1 + \cos(\dfrac{2\pi}{z_0}z)), & -z_0 \le z \le z_0 \\ 1, & 2z_0 < .z < -z_0 \end{cases}$$

$$R_1(z) = \begin{cases} \beta - \dfrac{\delta_H}{2R_0}(1 + \cos(\dfrac{2\pi}{z_0}z)), & -z_0 \le z \le z_0 \\ \beta, & 2z_0 < z < -z_0 \end{cases} \tag{4}$$

Where $R(z)$, $R_1(z)$, are the radii of stenosis region and R_0, βR_0 are radii of normal region of plasma layer and core layer respectively, μ_p, μ_H are the viscosity of plasma and core layer respectively, $2z_0$ is the length of stenosis region and δ is the height of stenosis.
By taking the derivative of the above equation we get

$$(\frac{\partial v}{\partial r})_{r=R(z)} = \frac{G}{2\mu_H}[(1 + 4(\frac{\mu_H}{\mu_p} - 1))R(z) - 4(\frac{\mu_H}{\mu_p} - 1)R_1(z)] \tag{5}$$

Put $R_1(z) = \beta R(z)$ and $\beta = 0.95$ then equation (5) reduced to

$$(\frac{\partial v}{\partial r})_{r=R(z)} = \frac{G}{10\mu_H}[(4 + \frac{\mu_H}{\mu_p})R(z) \tag{6}$$

Then from equations (3) and (6), wall shear stress along the stenosed artery can be expressed as

$$\tau = [-\frac{G}{10}(4 + \frac{\mu_H}{\mu_p})\int R(z)dz]^n + \tau_y \tag{7}$$

$$\tau = [-\frac{G}{10}(4 + \frac{\mu_H}{\mu_p})\int_{-z_0}^{z_0}(1 - \frac{\delta_p}{2R_0}(1 + \cos(\frac{2\pi}{z_0}z))dz + \int_{2z_0}^{-z_0} dz]^n + \tau_y \tag{8}$$

By integrating equation (8) we get

$$\tau = [\frac{G}{10}z_0(4 + \frac{\mu_H}{\mu_p})(1 + \frac{\delta}{R_0}]^n + \tau_y \tag{9}$$

Equation (9) is the model for Herschel-Bulkley fluid flow, where τ is wall shear stress, μ_p and μ_H are the viscosities of peripheral plasma layer and erythrocytes suspension of core layer respectively, τ_y is yield stress, δ is the height of stenosis, R_0 is the radius of normal part of artery, G is pressure gradient, z_0 is the half length of stenosis region and n is the power law index

III. Results and Conclusion

The results based on the numerical solution of equation (9) using mathematical software program called (Microsoft Mathematics 3.0) for $R_0 = 0.2$mm, $z_0 = 4R_0$ and height of stenosis ranging from 0.08 to 0.1mm, indicates that wall shear stress increases with the increase of stenosis height δ. Further wall shear stress also increases with the increase of yield stress, while the other parameters are constant. It is observed that wall shear stress increases with the increase of viscosity core layer μ_H when the other parameters are invariable.

It is of interest to note that the wall shear stress is directly proportional relation with the length of stenosis and inversely proportional relation with the value of power law index n.

It is found also that, for a given values of the parameters wall shear stress of H-B fluid for $n=1.05$ is lower than that of $n=0.95$.

Musad and Khan (2010) have developed a mathematical model to study blood flow through stenosed artery were the blood in core layer considered as Casson fluid.

Estimate of wall shear stress of the both H-B fluid and Casson fluid for different values of height stenosis, viscosity and yield stress are computed in the tables below.

In table 1, it is observed that for the range 0.08 to 0.10 mm of the height stenosis the range of wall shear stress in (case of H-B Fluid) for $n=1.05$ is 0.080 to 0.124 Pascal, and 0.091 to 0.148 Pascal for $n=0.95$. While the range of wall shears stress (in case of Casson fluid) is 0.167 to 0.240 Pascal.

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In table 2, it is found that for the range of the both yield stress and viscosity 0.01 to 0.03 Pascal, and 0.004 to 0.009 Pascal*s respectively, the corresponding range of wall shear stress in (case of H-B Fluid) for n=1.05 is 0.08 to 0.12 Pascal and 0.102 to 0.144 Pascal for n=0.95. While the range of wall shears stress (in case of Casson fluid) is 0.147 to 0.240 Pascal.

It is clear that the wall shear stress in case of Casson fluid is considerably higher than these in Herschel-Bulkley fluid case, while the viscosity and yield stress are the same. And it is observed that for the range of the height stenosis 8×10^{-5} to 10×10^{-5}, the wall shear stress in case of Herschel-Bulkley fluid is considerably lower than these in case of Casson fluid.

Table 1 : Data on wall shear stress for Herschel-Bulkley fluid and Cossan fluid in stenosis artery for values of radius R_0=0.2 mm, half length $z_0=4R_0$

Height of stenosis *(10 ^ (-5))	Gradient Pressure	Wall shear stress For H-B fluid		Wall Shear tress For Casson fluid
		n=1.05	n=0.95	
8.000	150.000	0.075	0.091	0.167
8.222	164.444	0.080	0.097	0.175
8.444	178.888	0.085	0.103	0.183
8.666	193.333	0.091	0.109	0.190
8.888	207.777	0.096	0.115	0.198
9.111	222.222	0.101	0.112	0.200
9.333	236.666	0.107	0.128	0.213
9.555	251.111	0.112	0.134	0.221
9.777	265.555	0.118	0.141	0.230
10.00	280.000	0.124	0.148	0.240

Table 2 : Data on wall shear stress, yield stress and viscosity for Herschel-Bulkley fluid and Casson fluid where radius R_0=0.2 mm, half length $z_0=4R_0$, height of stenosis 0.10 mm, at max. Pressure gradient 267pa/m

Viscosity	Yield Stress	Wall Shear Stress For H-B fluid		Wall Shear Stress For Casson fluid
		n=1.05	n=0.95	
0.0040	0.010	0.081	0.102	0.147
0.0046	0.012	0.086	0.106	0.158
0.0051	0.014	0.090	0.111	0.169
0.0057	0.017	0.094	0.116	0.179
0.0062	0.019	0.099	0.120	0.190
00.0068	0.021	0.103	0.125	0.200
0.0073	0.023	0.107	0.130	0.210
0.0080	0.026	0.112	0.134	0.220
0.0084	0.028	0.116	0.139	0.230
0.0090	0.030	0.120	0.144	0.240

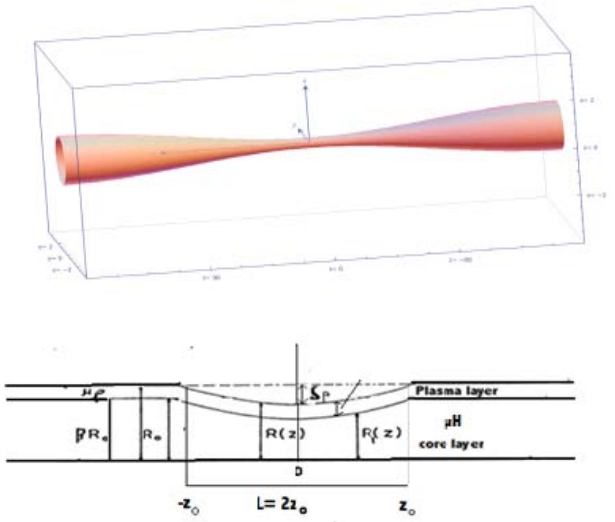


Figure 1 : physical model and coordinate system of two-layer flow in vessels of stenosis

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Degradation of Crude Oil by Bacteria: A Role for Plasmid-Borne Genes

By Akpe, Azuka Romanus, Ekundayo, Afe Omolola & Esumeh, Frederick Ikechukwu

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Abstract - The role of plasmid-borne genes in the biodegradation of Chevron Escravos Crude Oil by bacteria was determined. Plasmid extraction and curing, transformation experiments and biodegradation studies were carried out using standard procedures. Plasmid extraction studies showed that two of the six selected crude oil degrading bacterial isolates had two plasmids each. The isolates were *Klebsiella pneumoniae* from ripe pawpaw fruit and *Serratia marscencens* from oil palm mill effluent. The plasmids were of small (300bp) and large (>1.5kbp) sizes. The results also showed that the isolates were successfully cured of plasmids using 1% Sodium Dodecyl Sulphate (SDS). The transformation experiment using the extracted plasmid DNA and competent *Escherichia coli* K12 DH1 cells was successful. The percentage degradation of crude oil at 37oC by *E. coli* K12 DH1 transformed with the plasmid DNA from *Klebsiella pneumoniae* was 93.03% while that transformed with the plasmid DNA from *Serratia marscencens* degraded 76.97% of the crude oil. It was observed that loss of plasmids by *Klebsiella pneumoniae* and *Serratia marscencens* did not lead to complete loss of their degradative abilities. It only resulted in reduction in their degradation potential.

Keywords : *plasmid DNA, crude oil, biodegradation, bacterial isolates, transformation, plasmid curing.*

GJSFR-C Classification : *FOR Code: 279999p*



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Keywords : plasmid DNA, crude oil, biodegradation, bacterial isolates, transformation, plasmid curing.

I. Introduction

The increase in petroleum exploration and production has brought with it an ever increasing rate of environmental pollution involving both terrestrial and aquatic habitat (Mandri and Lin, 2007). Oil spillage in an oil producing country is inevitable. The impact of these wastes in the Niger Delta ecosystems of Nigeria is an obvious environmental concern particularly with regards to the persistence and ecotoxicity of these wastes (Benka-Coker and Olumagin, 1995). Soil and ground water contamination by crude oil are becoming increasingly sensitive issues in Nigeria, since most of her potable water supply is derived from shallow and unconified aquifers. It is therefore important to assess all remediation options on the basis of their ability to remove organic contaminants successfully. This is because most of these product especially the polycyclic aromatic hydrocarbons (PAHs) are toxic, mutagenic and carcinogenic (Clemente *et al*., 2001).

Prolonged exposure to high concentration may cause the development of live or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra *et al*., 2001). In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment crude oil pollution of soil and surface water have been prevalent in Nigeria and other oil producing countries since the commencement of soil exploration and development petroleum industry (Ifeadi and Nwankwo 1987; Okoh *et al*., 2001). Of the many remediation methods, currently in use or under development, bioremediation is viewed as one of the most promising technologies. The microbial by-product of oil biodegradation becomes part of the natural food chain with much of the degraded hydrocarbon material further metabolized by marine organism or incorporated into soil humus without accumulation to toxic materials in the environment (Shoemaker, 1989; Ijah *et al.*, 2003).

The ability of microorganisms to degrade an organic compound is the result ultimately of the genetic makeup of the organisms. The chemical reactions involved in metabolism are mediated by enzymes. The range of enzymes which a bacterium has is a reflection of the specific genetic information in the cell. Genetic information in bacteria, as in all organisms, is stored in the form of DNA. The information is physically present in bacterial cells in two forms – the chromosome and the plasmids. The bacterial chromosome is a single circular, highly folded double-strand of DNA. In addition to chromosomal DNA, a larger number of bacteria also have extra-chromosomal DNA in the form of plasmids (Zylstra and Gibson, 1991).

Many plasmids contain genes which code for the enzymes necessary for the derivative pathways important to bioremediation. Enzymes involved in the degradation of toluene, naphthalene, salicylate, octane etc. have been shown to be plasmid encoded (Barbly and Barbour, 1984; Nelson, 1990).

Plasmids are also important in the development (transformation) of new organisms with enhanced degradative capability. Using molecular biology techniques, it is possible to slice pieces of DNA containing genes for specific degradative pathways into plasmids. These plasmids can then be introduced into a host organisms resulting in a recombinant or genetically engineered microorganisms (GEM) with new degradative capabilities (Brook and Madigan, 1991; Brand *et al.* 1992). These are used to bioremediate contaminated sites mainly as organisms for

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bioaugumentation (McClune *et al.,* 1989; Philips *et al.,* 1989; Focht, 1998). Appropriate environmental factors are essential for the performance of these organisms.

Many bacteria of diverse types have extra chromosomal DNA, suggesting the widespread presence of plasmids in prokaryotic cells. Thus the ability to identify plasmids in bacterial systems is important. Both physical agents and chemical agents like surfactants, DNA intercalating agents, antibiotics and metals may increase the frequency of elimination of plasmids in bacteria (Caro *et al.,* 1984). These agents interfere with DNA replication or affect a particular organelle or enzyme of a bacterial cell (Stanisich, 1984).

The goal of this current study is to determine the role of plasmid- borne genes in the crude oil degrading capabilities some selected crude oil degrading bacterial isolates. The possibility of transforming a control organism (*E. coli* K12 DH1) with any isolated plasmid DNA will also be examined.

II. Materials and Methods

a) Sources/Confirmation of Isolates

Six (6) crude oil degrading bacteria previously isolated from different environmental sources (Akpe, 2011) were used for this study. The isolates used were *Pseudomonasfluorescence* isolated from crude oil contaminated soil, *Klebsiella pneumoniae* isolated from ripe paw-paw fruit, *Serratia marscencens* isolated from oil palm mill effluent, *Pseudomonasaeruginosa* isolated from garden soil, *Bacillussubtilis* isolated from rubber effluent and *Enterobacteraerogenes* isolated from cassava mill effluent.

The identities of these isolates were confirmed using morphological and biochemical characteristics. These tests includes gram staring, catalase test, oxidase test urease test, indole test, citrate utilization test and sugar fermentation tests as described Barrow and Feltham, (1986) and Holt, (1994).

b) Plasmid Curing and Isolation

Plasmid Curing was carried out on the isolates using the methods of Winkler *et al*., (1979) and Lexmi*et al*., (1987). The curing agent used was sodium dodecyl/ sulphate (SDS). Physical evidence for the presence or loss of plasmid(s) in curved and non-curved isolates was obtained by alkaline phosphate method of rapid DNA isolation technique of Dillion (1985) and Birnboim and Doly, (1979). This involved four basic steps – cellharvest, lysisdeproteination and decontamination.

The plasmids were characterized using agarose gel electrophoresis (Sambrook *et al*., 1989). The molecular weights of plasmids were visualized using UV transilluminator (AlphaImager TM 2200) at 302-365nm.

c) Transformation Studies on E. coli K-12 DHI with Isolated Plasmid using Calcium

i. *Chloride Method*

In the transformation of *E. coli* K-12 DH1, the organism (*E. coli* K-12 DH1) was inoculated into fresh nutrient broth in a tube and incubated overnight at 37°C. Half a milliliter (0.5ml) of this overnight culture was taken into 20ml of fresh nutrient broth in a flask attached to a rotating shaker and incubated at 37°C until the cell density is $5x10^7$ cell per ml with an absorbance reading of 0.55 at 550 nm. The culture was chilled in ice for 10 minutes and centrifuged at 3000xg for 15 minutes at 4°C. The cell pellets were collected and re-suspended in 5 ml of ice cold 0.1M $CaCl_2$. This was incubated on ice for 20 minutes and centrifuged at 3000xg for 15 minutes at 4°C. Thereafter the cell pellets were re-suspended in 5ml of ice cold 0.1m calcium chloride and incubated on ice for 20 minutes, this was centrifuged as before at 3000xg for 15 minutes at 4°C. Then the cells were re-suspended in another 5 ml of ice cold calcium chloride. 200µl of these competent cells were dispensed into labeled tubes. 40 µl of DNA extract was added to it and the control were placed on ice for 30 minutes. The tubes were removed from ice and placed in a water bath already set at 42°C for 2 minutes (heat shocking) and this enabled the cell membrane of the bacterium to be permeable to DNA). The tubes were removed from the water bath after two minutes and placed back on ice, then 1ml of nutrient broth was added into each of the tubes and incubated (don't shake). After one hour of incubation 150µl from each of the tubes were plated in already prepared mineral salt agar with crude oil saturated filter paper on the plate cover and incubated at 37°C for 24 – 72 hours. The plates viz-a-vis the control were examined. The *E. coli* K-12 DH1 cells able to grow on this medium were said to be transformed. Transformed cells were used for crude oil degradation studies in a liquid mineral salt medium with crude oil as only source of carbon (Mervat, 2009).

d) Crude oil degradation studies/Quantitative Determination of Biodegraded Crude Oil

The crude oil used was Chevron Escravos crude oil obtained from chevron tank farm at Escravos. The mineral salt medium used was that previously described by Mills *et al.* (1978) and modified by Okpokwasili and Amanchukwu (1988). Crude oil degradation studies were carried out on the control strain -*E. coli* K12 DH1, *E. coli* K12 DH1 transformed with *Klebsiella pneumoniae* plasmidgene, *E. coli* K12 DH1transformed with *Serratia marscencens* plasmid gene, original *Klebsiella pneumoniae* isolate, original *Serratia marscencens* isolate, plasmid-cured *Klebsie-llaaerogenes* isolate, plasmid-cured *Serratia marscencens* isolate, consortium of the six selected isolates and the isolated DNA. Samples were inoculated onto 100ml of liquid mineral salt medium containing 1ml

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(1%) crude oil and incubated on a shaker at a temperature of 37°C for 21 days. The residual crude oil recovered at the end of the degradation period was analyzed using spectrophotometer (Optima SP-300). The values obtained were expressed as percentage of the original optical density of the crude oil at the onset of the experiment (Oruansi and Ogugbue, 2003).

III. Results

Result of plasmid extraction showed that two of the six selected isolates, had two plasmids each (lanes 1-6 of Plate 1). The isolates were *Klebsiella pneumoniae* (lane 2) from oil palm mill effluent and *Serratia marscencens* (lane 3) from ripe pawpaw fruit. The plasmids as revealed in Figure 7 were of the same size (lanes 2 and 3). The smallest is 300bp while the largest was found to be above 1.5kbp. The results also showed that the plasmids were successfully cured hence the disappearance of the band post plasmid curing as shown in lanes 8 and 9 of Plate 1.

The transformation experiment using the extracted plasmid DNA and competent *Escherichia coli* K – 12 DH1 cells was successful. The percentage degradation of crude oil at 37°C by *E. coli* K – 12 transformed with the plasmid DNA from *Klebsiella pneumoniae* was 93.03% while that transformed with the plasmid DNA from *Serratia marscencens* degraded 76.97% of the crude oil.

The optical densities of the degraded crude oil by the non-cured and cured isolates were 0.095 and 0.272 for *Klebsiella pneumoniae,* 0.198 and 0.265 for *Serratia marscencens* and 0.053 and 0.175 for *E. coli* K-12 transformed with plasmids from *Klebsiella pneumonia* and *Serratia marscencens* respectively. The optical densities of the controls (competent *E coli* K-12 and DNA extracts) were approximately the same as the original crude oil (0.76) meaning there were no degradations. The lower the optical density value the higher the percentage degraded. This is shown in figure 1. It was observed that the loss of plasmids by *Klebsiella pneumoniae* and *Serratia marscencens* did not lead to complete loss of degradative ability. It only resulted in reduction in their degradation potential.

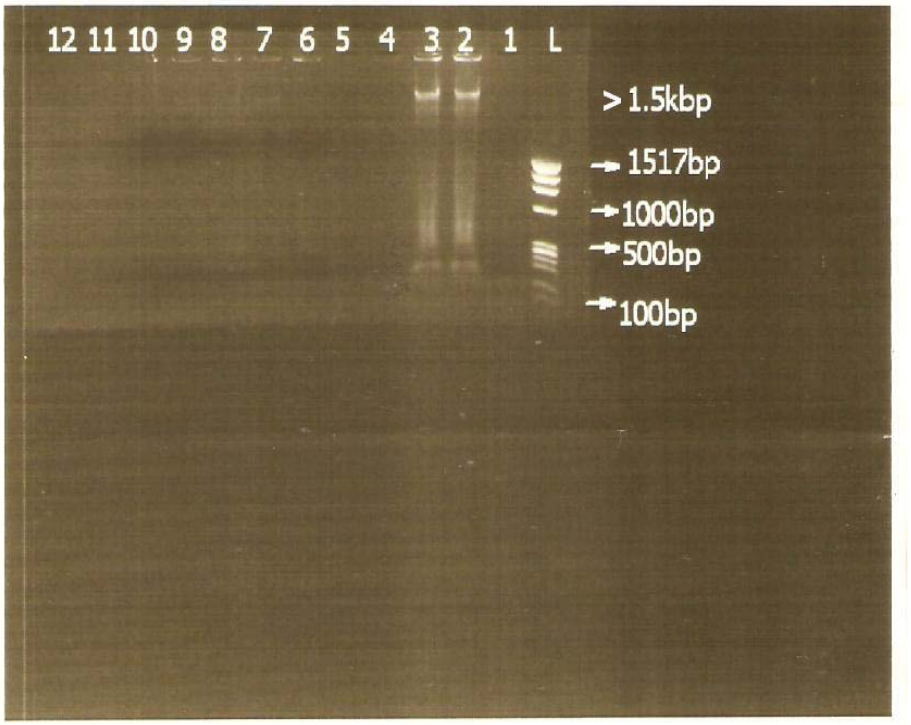


Plate 1: Plasmid profile of cured and non-cured isolates

KEY:

L = 100bp DNA ladder

Lane 1 = Non-cured isolate of *Pseudomonas fluorescens*

Lane 2 = Non-cured isolate of *Klebsiella pneumoniae*

Lane 3 = Non-cured isolate of *Serratia marscescens*

Lane 4 = Non-cured isolate of *Pseudomonas aeruginosa*

Lane 5 = Non-cured isolate of *Bacillus subtilis*

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Lane 6 = Non-cured isolate of *Enterobacter aerogenes*
Lane 7 = Cured isolate of *Pseudomonas fluorescens*
Lane 8 = Cured isolate of *Serratia marscescens*
Lane 9 = Cured isolate of *Klebsiella pneumoniae*
Lane 10 = Cured isolate of *Pseudomonas aeruginosa*
Lane 11 = Cured isolate of *Bacillus subtilis*
Lane 12 = Cured isolate of *Enterobacter aerogenes*

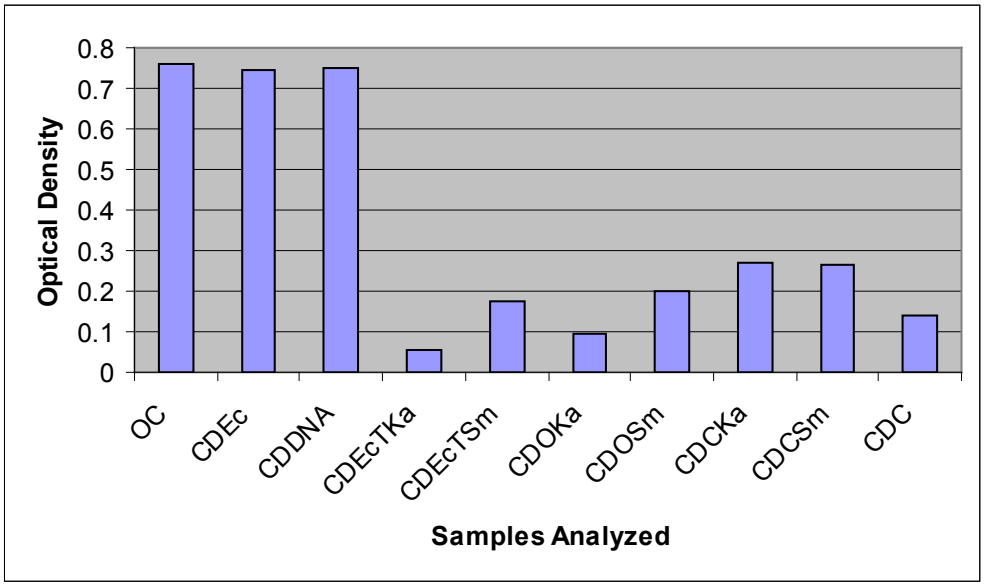


Figure 1: Optical Densities of Original and Degraded Crude Oil at 37°C by Cured and Non-Cured Isolates of *Klebsiella pneumoniae* and *Serratia marscescens* and Original and Transformed *E coli* K12DH1

KEY

OC = Original Crude Oil
CDEc = Crude degraded by *E. coli* K12 DH1
CDDNA = Crude degraded by isolated DNA
CDEcTKa = Crude degraded by *E. coli* transformed with *Klebsiella pneumoniae* plasmidgene
CDEcTSm = Crude degraded by *E. coli* transformed with *Serratia marscescens* plasmid gene
CDOKa = Crude degraded by Original *Klebsiella pneumoniae* isolate
CDOSm = Crude degraded by Original *Serratia marscenscens* isolate
CDCKa = Crude degraded by plasmid-cured *Klebsiella pneumoniae* isolate
CDCSm = Crude oil degraded by plasmid-cured *Serratia marscenscens* isolate
CDC = Crude degraded by consortium of six selected isolates

Note that the lower the OD value, the higher the quantity of crude oil degraded

IV. Discussion

Two of the isolates in this study were found to harbour two plasmids each (Plate 1). This supports the claims and works of Summers and Silver (1978) who reported that in some cases the hydrocarbon degrading capabilities of some bacterial strains are plasmid borne. The presence of plasmids in bacteria has been widely reported. The plasmid that bear genes encoding for enzymes capable of degradation have been a great attraction. These plasmids, known as catabolic plasmids can give the organisms harbouring them the ability to degrade certain compounds. Sayer *et al.* (1990) reported the presence of catabolic plasmids in species of *Pseudomonas, Alcaligenes, Actinobacter, Flavobacterium, Klebsiella, Moraxella* and *Arthrobacter.*

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The two plasmids in the two isolates in this study have the same band and their approximate molecular size were 300bp and above 1.5kbp.

These isolates were successfully cured of plasmids using 1% sodium dodecyl sulphate (SDS). The success of this curing as revealed in Plate 1 agrees with the works of Lakshmi *et al.* (1988), Corah *et al.* (2006), Enabulele and Orikpete (2009), Esumeh, *et al.* (2009) and Chu-Hui *et al.* (2011) who independently reported the elimination of plasmids using DNA intercalating agents - SDS, acridine orange, ethidium bromide and other agents. Esumeh *et al.* (2009) for instance isolated *Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa* from ripe pawpaw fruits and *Serratia marscencens* from ripe orange fruits. All these isolates had one plasmid of 23.1 kbp each except *Enterobacter cloacae,* which possessed an additional plasmid of 12.0 kbp. Plasmids obtained in this current study are of smaller molecular weight of 300bp and a little above 1.5kbp, this shows that plasmids borne by bacteria can be of different molecular sizes. These supported the claims by Vahaboglon *et al.* (1996) that microorganisms capable of degrading petroleum hydrocarbon could possess one or more plasmids.

Unlike the findings of Corah *et al.* 2006) whose plasmid-cured isolates lost resistance to tellurite, and Esumeh *et al.* (2009) whose plasmid-cured isolates lost the ability to grow in crude oil medium, the plasmid-cured isolates in this present study retained their ability to degrade crude oil although non-cured isolate recorded higher percentage degradation. However there is a correlation between this present finding and that of Ajayi and Ebeigbe (2009) whose *Staphylococcus aureus* isolate haboured plasmid gene that did not code for antibiotic resistance. This observation suggest that the chromosomal DNA of these organisms also carry genes for crude oil degradation and the ability of the plasmid cured isolates to degrade crude oil was due to the chromosomal genes. The higher or enhanced degradative potential of the non-cured isolates of *Klebsiella pneumoniae* and *Serratia marscencens* suggest that there is a complementary action by the chromosomal and plasmid DNA.

The crude oil degrading potentials of the extracted plasmids were confirmed by using them to transform *E. coli* K12 DH1. The results were successful as the transformed cells became good degraders (Figure 1). Similar findings was reported by Mervat (2009) who successfully transformed *E. coli* K – 12 DH5α with plasmid encoding for methomyl degradation from *Stenotrophomonasmaltophilia* M♀ strains and the transformed cells were able to grow on the contaminant. Also Chu-Hui *et al.* (2011) reported that the isolated ESBLs gene from *E. coli* in their study can be transferred to other organisms.

This study have clearly indicated that the crude oil degrading capability of some microorganisms is as a result of their possession of plasmids and that there was complementary action between the plasmid and chromosomal DNA because bacterial isolates that were cured of their plasmid could not degrade as much crude oil as they degraded when they had the plasmid. It is believed that plasmid isolated can be manipulated to enhance their degradation potentials.

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2. Ethical Guidelines,

3. Submission of Manuscripts,

4. Manuscript's Category,

5. Structure and Format of Manuscript,

6. After Acceptance.

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Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
- As always, give awareness to spelling, simplicity and correctness of sentences and phrases.

Procedures (Methods and Materials):

This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

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The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.



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Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

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- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

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- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

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Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

INDEX

A

Acclimiatization · 14
Achinewhu · 14, 21
Artherosclerosis · 19

B

Bacillussubtilis · 32
Borontrifluoride · 16

C

Cellharvest · 32

E

Enterocolitica · 1, 3, 5, 6, 7, 8, 9, 11, 12, 13
Eukaryotic · 3, 9
Extrachromosomal · 9

G

Gastroentistenal · 1
Glucose · 14, 16, 17, 19, 21, 23

H

Hyperglycaemic · 14, 17, 20, 21

L

Leucocytes · 1
Lysisdeproteination · 32

N

Njasang · 14, 15, 16, 17, 19, 21
Nucleobond · 4

P

Pentachlorophenol · 39
Pseudomonasfluorescence · 32

R

Randox · 16

S

Serogrouped · 1
Staphylococcus · 38

T

Transilluminator · 32
Triglycerols · 14

U

Ugochukwu · 14, 23

V

Vahaboglon · 38, 41

Y

Yersiniabactin · 1, 2, 11



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