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Antibacterial Susceptibility Evaluationof Enteric Bacteria from Stool Samples of Diarrheal Patients in Awka metropolis.

Chidi-OnuorahLilianC[1].,OkaforUgochukwuC[1].*,OkoroNnennaC.N[1].,AnyaohaVictoriaI[1].,Kyrian-OgbonnaEvelynA[1].,OnuorahMargaretE[2].,ChiemelaVictorO[1].

[1]DepartmentofAppliedMicrobiologyandBrewing, NnamdiAzikiweUniversityPMB5025Awka,Anambra-Nigeria.
[2]Postprimaryschoolservicecommission,Awka.
Corresponding Author:Okafor Ugochukwu C.

Abstract:*This study was carried out in order to isolate enteric bacteria from stool of diarrheal patients and determine their antibacterial susceptibility profile. Fresh diarrheal stool samples were collected from four Hospitals in Awka, Anambra state, transported to the laboratory, and maintained in the refrigerator at 4^{o}c for microbial examinations. The samples were inoculated onto Eosin Methylene Blue agar and MacConkey agar at room temperature for 24hours. Seven (7) isolates of six genera were isolated and identified as Salmonella spp., Shigella spp., Campylobacter spp., Esherichia spp., Staphylococcus spp. and Vibrio spp. Salmonellaspp. was the most frequently isolated bacteria. Antibacterial susceptibility test of the isolates to conventional antibiotics and aqueous extract of Thymus vulgaris leaves were performed. The results showed that the isolates were susceptible to the antibiotics at various concentrations. Septrin (30ug) had the highest zone of inhibition of 35mm on Salmonella spp. whereas Gentamycin (10ug) had the lowest zone of inhibition of 10mm on Vibrio spp. The antibacterial susceptibility test of the isolates to aqueous extract of leaves of Thymus vulgaris at 1000, 500 and 250mg/ml concentrations showed that the higher the concentration, the greater the zone of inhibition. The zones of inhibition ranged from 14 – 32mm.Though the extracts were in the same range of effectiveness on the isolates as the conventional antibiotics at higher concentrations, it is still suggested that more research be done on the use of natural agents like extracts of Thymus vulgaris in the treatment of diarrhea to discourage the use of synthetic antibiotics.*

Keywords*: Enteric-Bacteria, Susceptibility, Antibiotics, Diarrhea, Stool samples*

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I. Introduction

Diarrhea is defined as having loose or watery stools at least three times per day, or more frequently than normal for an individual. Though most episodes of childhood diarrhea are mild, acute, cases can lead to significant fluid loss and dehydration, which may result in death acute gastroenteritis or infectious diarrhea, is one of the leading causes of illnesses and death in infants and children throughout the world, especially in developing countries. The individuals that are at high risk of contracting infectious diarrhea are the young, the elderly and immunocompromised persons [1]. Diarrheal diseases are the cause of almost three million deaths annually mainly among children younger than five years of age [2].The main aetiology of the diarrhoea is related to a wide range of bacteria, enteroparasites and viruses. The underlying reasons for the spread of diarrhoeal diseases are found in poor hygiene and sanitation, limited access to safe drinking water as well as in inadequate education of health care providers and recipients [3][4].A knowledge of the pathogens associated with diarrhoea is pertinent in not only allowing the optimum use of available interventions but will also direct efforts aimed at developing specific therapies and preventive vaccines. Unfortunately, due to limited resources the microbiological diagnoses of diarrhoea are not done easily in many settings in Nigeria. This study performed microbiological investigation of some potential pathogens associated with diarrhea, to characterize the isolates, their antibiotic resistance and the epidemiological factors related to the diarrheal disease in patients from Awka, Nigeria. The entero-pathogen enter the faecal-oral route of the transmission of pathway via faeces as a results of inadequate sanitation and hygiene, as well as food and water that may be contaminated with human or animal faecal matter [5][6]. Inadequate sanitation and hygiene as a major contributing factor to diarrhoeal disease burden.

Two main types of antidiarrhoeal medicines are used to treat diarrhea. These are called antimotility medicines and bulk-forming agents. Antimotility medicines are used to treat acute diarrhoea. They include codeine phosphate, co-phenotrope, loperamide, and kaolin and morphine mixture. The most commonly used antimotility medicine is loperamide, kaolin and morphine mixture is very rarely used to treat diarrhea nowadays. Antimotility medicines are not advised for children under the age of 12.

Bulk-forming agents are used for people who have diarrhoea because they have irritable bowel syndrome. They include ispaghula husk, methylcellulose and sterculia. It has been confirmed that different probiotic strains including Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus casei, and Saccharomyces cerevisiae (boulardii) are useful in reducing the severity and duration of acute infectious diarrhea in children. The oral administration of probiotics shortens the duration of acute diarrheal illness in children by approximately1 day. Several meta-analyses of controlled clinical trials have been published that show consistent results in systematic reviews, suggesting that probiotics are safe and effective. For treating children, the roots of the Cyperus rotundus mixed with honey are used.In 1725, a German apothecary discovered that the plant's essential oil contains a powerful disinfectant called thymol that is effective against bacteria and fungi. Thymol also acts as an expectorant, loosening phlegm in the respiratory tract so it can be coughed up. Later herbalists listed thyme for these uses and as remedy for numerous other complaints, including diarrhea and fever. They prescribed the oil externally as an antiseptic for fungal infections such as athlete's foot. Thymol (also known as 2-isopropyl-5-methylphenol, IPMP) is a natural monoterpene phenol derivative of cymene, C10H14O, isomeric with carvacrol, found in oil of thyme, and extracted from T. vulgaris (common thyme) and various other kinds of plants as a white crystalline substance of a pleasant aromatic odor and strong antiseptic properties. Thymol also provides the distinctive, strong flavor of the culinary herb thyme, also produced from T. vulgaris. Thymol is part of a naturally occurring class of compounds known as biocides, with strong antimicrobial attributes when used alone or with other biocides such as carvacrol, thymol has been shown to be an effective fungicide [7]. Thymol has microbial activity because of its phenolic structure.

Acute diarrhea due to bacterial infections is an important cause of morbidity and mortality in infants and young children in most developing countries including Nigeria. Though the synthetic antibiotics are effective in the treatment of diarrhea infections, there is still need for the use of natural agents from plant materials like aqueous extracts of leaves ofThymus vulgaris in treatment of diarrhea so as to discourage the use of synthetic antibiotics.

The main aim of this study is to isolate bacteria from stool of diarrhoeal patients and evaluate their susceptibility to antibacterial agents**.**

II. Materials And Methods

Samplecollection

Stool samples were collected from four different Hospitals in Awka, Anambra state. A total of four samples, two from each hospital were collected aseptically in sterile specimen bottles. They were conveyed to Microbiology laboratory at Nnamdi Azikiwe University and the faecal samples were examined with the naked eye for consistency, colour and atypical components such as mucous, blood and parasites and microbiologically analyzed.

Sampleprocessing

2gram of the sample was weighed and transferred into test-tubes containing normal saline and a 10fold serial dilution was carried out under aseptic condition to reduce the microbial load. After the serial dilution 0.1ml of 10^5 and 10^6 of diluted sample was plated out in two separate petri-dishes containing MacConkey agar and Eosin methylene blue agar for 24 to 48hours under room temperature. After, it was sub cultured into different petri- dishes according to their morphological appearance for biochemical analysis.

Identificationoftheisolates

The bacterial isolates were identified on the basis of their morphological and biochemical characterization. Gram staining, motility, catalase, coagulase, oxidase, spore, indole, methlyl red, voges proskaur, and citrate utilization, antibiotics sensitivity test was carried out as done by [8]. The isolates were identified according to the scheme of [9].

Colony morphology

The colony morphology of the isolates was identified based on their size, colour, shape, elevation, appearance and arrangement with the use of Bergy's manual of bacteriology

Gram staining

This is the most important and widely used procedure for characterizing bacteria. It was first described by Christian Gram. This method divides the bacteria into two groups. Gram positive which is purple/blue in colour and Gram negative which is pink/red in colour. This technique is based on the ability of bacteria to retain primary stain (crystal violet dye) during decolourization with alcohol or acetone. Gram positive bacteria retain the primary stain while Gram negative bacteria are decolourized by alcohol and takes up the red colour of

counter stain. A smear of an isolate was made on a clean slide and allowed to dry. It was then fixed by passing the smear through Bunsen burner. This is done to enhance the sticking of the organisms on the microscope slide. The smear was flooded with crystal violet left for 60 seconds before being washed off with water. lugols iodine was added and allowed to stand for 60secs before being washed off and decolourised with alcohol for 10 seconds. The slide was then washed off, stained with safranin by 30secs, washed off and allowed to air- dry. A drop of immersion oil was added to the slide which was then viewed under the microscope using the 100 objective.

Motility test

Each bacteria isolated was separated inoculated into semi-solid medium using sterile straight wire and incubated at 37^{o}c for 24 hours. Migration of the isolates away from the line of inoculation was a positive result while lack of migration away from the line of inoculation indicated a negative result.

Catalase test

This test is used to detect the enzyme catalase which protects bacteria from hydrogen peroxide accumulation which can occur during aerobic metabolism. Catalase breaks the hydrogen peroxide into oxygen and water. The organism was picked and emulsified on a clean slide. A drop of $3^{0}/_{0}$ hydrogen peroxidewas added to the slide. The presence of sustained bubbles indicated a positive result while their absence indicated a negative result.

Coagulase test

This is used to distinguish pathogenic Staphylococcus which produces the enzyme Coagulase from Streptococcus which does not produce Coagulase. Coagulase causes plasma to clot by converting fibrinogen to fibrin. 0.1 ml of plasma was dropped on a clean slide and the organism emulsified in it. The clumping of the organism within 10 minutes indicated a positive result.

Sugar Fermentation Test

This test is used to detect organisms which utilize different sugar as sources of energy with the production of acid and/or gas. These sugars used were glucose, lactose and maltose. Peptone water broth was prepared. Bromothymol blue indicator was added to the broth. In three separate conical flasks containing glucose, maltose and lactose, the above solution (peptone water broth + indicator) was added at equal proportions. Five millilitre of the mixture was then dispensed into test tubes. Durham tubes were added and the test tubes were sterilized in the autoclave at 121^{o}C for 15 mins. After cooling, the test organism was inoculated into each of the test tubes and incubated for 24hours.

Indoletest

This test was carried out to determine the organism that break down the amino acid tryptophan into indole. The test organism was inoculated in sterile test tubes containing peptone water and incubated for 48 hours at room temperature. 0.5 ml of kovac's reagent was added and mixed to stand for 10 minutes. The development of a red-ring colour indicated a positive result. Escherichiacoli is an example of organism that tested positive for indole test.

Oxidase test

This test is used to detect the ability of bacteria to produce the enzyme oxidase. The presence of this enzyme was tested by mixing 2 drops of $1^{0}/_{0}$ aqueous solution of tetramethyl-p-phenylene diamine hydrochloride with the organism on a filter paper. The development of a colour changes to pink through maroon and then to black within 10 seconds-30 seconds a positive result. Vibrio cholerae indicated positive.

Spore test

A bacterial film was made on a slide and heat fixed with minimal flaming. The slide was placed in the rim of boiling water, with the bacterial film uppermost. The film was flooded with a $0.05^{0}/_{0}$ aqueous solution of malachite green when large droplets have been condensed on the underside of the slide and left to act for 60seconds while the water continued to boil. The slide was washed in cold water and treated with 0.5% safranin solution and left for 30 seconds, the slide was washed with clean water, dried and viewed under the microscope. This method coloured the spores green blue and the negative bacilli red. Bacillus indicated positive for spore test.

Methyl-RedTest

The test organism was introduced into glucose phosphate peptone water and incubated at 37^{o}C for 48 hours. Five dropsof methyl red reagent were added, mixed and the result read. A red colouration indicated a positive result while a yellow colouration indicated a negative result.

CitrateUtilizationTest

The medium used was Simmon Citrate Broth. The test is used to identify which of the organism can utilize citrate as the sole of carbon for metabolism. It is used in the differentiation of the organism in the Enterobacteriaceae and other genera. Test tubes were used for the test. A saline preparation of the organism was inoculated into citrate medium and incubated at 37^{0}C for 24 hours. A change in colour from green to blue indicated a positive result.

VogesProskaeurTest

The test organism was introduced into glucose phosphate peptone water and was incubated at 37^0c for 48 hours. Five drops of alpha naphtol and potassium hydroxide reagents were added, mixed and the result read. A pink colouration indicated a positive result

Antibacterial Susceptibility Test of isolates to Conventional Antibiotic.

Susceptibility tests were carried out by the disc diffusion method, [10]. using Sensitivity-disc. The discs (6 mm in diameter) impregnated with different antibiotics (Ofloxacin 10 µg, Ciprofloxacin 10 µg, Septrin 30ug, Augmentin 30ug, Gentamycin 10ug, Tetracycline 30ug, Chloramphenicol 30ug, were placed on the inoculated agar. The inoculated plates were incubated at 30°C for 24 h for bacteria [11]. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Aqueous extraction of powdered leaves of Thymus vulgaris

50g of the powdered plant material was extracted using percolation process in 500ml of water at room temperature overnight with intermittent shaking according [12]with little modification. The mixture was first filtered with a mesh sieve and evaporated in a hot air oven t 100°C the % yield was calculated as grams of extract divided by grams of original powdered multiplied by 100.

$$\% \text{ yield} = \frac{\text{Weight of extract}}{\text{Weight of initial plant material}} \times 100$$

Antibacterial Susceptibility Test of Isolates to Aqueous Extracts of leaves of Thymus vulgaris.

Two (2) fold serial dilutions was carried out with the aqueous extract, where 5g of extract obtained was added to 5ml of the diluents in a test tube, 5ml of the solution in the test tube was transferred to a second test tube containing 5ml of water as the diluents. Finally 5ml of the solution in the second test tube was transferred into a third test tube containing 5ml of the diluents. Different concentrations such as 1000, 500, and 250mg/ml were obtained; sensitivity disc was immersed into each concentration and allowed for 30 minutes. 0.1ml of the isolates prepared using Mc Farland turbidity Standard was spread on a solid agar and the disc placed at the center, the plate was incubated at 30^0c for 24- 48hours and the zone of inhibition determined. All experiments were done in duplicate.

III. Results

The colony morphology of the bacterial isolate from the stool samples are shown in table 1. The organisms were identified as Vibrio spp, Shigella spp, Salmonella spp, Escherichia spp, Staphylococcusspp and Campylobacterspp. Salmonellaspp was the most frequently isolated bacteria in all the four stool samples. Table 3 shows the sugar fermentation of bacterial isolates from stool sample. Antimicrobial susceptibility tests were performed on all identified isolates. Susceptibility test of the isolates to antibiotics is in table 4 indicates that SXT (30ug) (Septin) had the highest inhibition (35mm) to the growth of Salmonella spp while Gentamycin CN (10ug) had the lowest zone of inhibition (10mm) on Vibrio spp. The antibacterial susceptibility test of bacteria to aqueous extract of leaves of thymus vulgaris is also determined at 1000, 500 and 250mg/ml concentrations and it was observed that the higher the concentration of the extract, the greater the zone of inhibition. 1000mg/ml of the concentration had the highest zone of inhibition of 32mm to staphylococcus spp whereas 250mg/ml of the concentration had the lowest zone of inhibition of 14 mm to Salmonella spp as shown in Table 5.

Table 1: The colony morphology of bacterial isolate from stool sample

Table 1: The colony morphology of bacterial isolate from stool sample

Samples	Colonial Characteristics	Shape	Arrangement	Identified Organisms
A	Shinycolorless and transparent, though they have dark centers	Bacilli	Pairs, singles	*Salmonellaspp*
B	purple/red, umbonate, undulate smooth shining colonies	Rod	Circular	*Shigellaspp*
CGolden	yellow, shiny, rough, flat colonies	Curved rod-shape	flate	*Vibriospp*

D	Translucent, smooth and raised, Short rod colonies	Short rod	Clusters	*Escherichia spp*
E	Large, circular, shiny, viscous and convex	Cocci	Clusters	*Staphyloccusspp*
F	Mucoid, Raised and smooth	S-shape	coccoid	*Campylobacterspp*

Table 2: Microscopic and biochemical characteristics of the bacterial isolate from stool samples

Samples	Shape	Gram	Catalase	motility	Coagulase	Indole	oxidase	MR	identified org
A_1	SP	-	+	+	+	-	-	+	*Salmonellasp*
A_2	RS	-	+	-	+	+	-	+	*Shigellaspp*
B_1	SP	-	+	+	+	-	-	+	*Salmonelaspp*
B_2	CRS	-	+	+	+	+	+	-	*Vibriospp*
B_3	RS	-	+	+	+	+	-	+	*E.coli spp*
C_1	CHS	-	+	+	+	+	+	-	*Campylobacter spp*
C_2	Cocci	+	+	-	+	-	-	-	*Staph. spp*

KEY
+ = POSITIVE
- = NEGATIVE
CHS= CURVED AND HELICAL-SHAPE
SP = SPIRAL ROD SHAPE
RS = ROD SHAPE
CRS = CURVED-ROD SHAPE
MR = METHYL- RED

Table 3: Sugar fermentation of Bacterial Isolates from stool samples

Samples	Glucose	Lactose	Sucrose	Maltose	Organisms
A_1	+	-	-	+	*Salmonella spp*
A_2	+	-	-	+	*Shigella spp*
B_1	+	-	-	+	*Salmonella spp*
B_2	+	-	+	-	*Vibrio spp*
B_3	+	+	+	+	*E*scherichia spp
C_1	+	-	-	-	*Campylobacter*
C_2	+	+	+	+	*Staphylococcus spp*

KEY
+ = POSITIVE
- = NEGATIVE

Table 4: Antibacterial Susceptibility Test Results of isolates to Antibiotics

Sensitivity Disc =6mm

Microbial Isolates	Antibiotics	Zone of Inhibition(mm)
Salmonella spp	SXT(30ug)	35
	CN(10ug)	25
	CPX(10ug)	19
	CH(30ug)	30
Staphylococcus spp	Au (30ug)	12

	CN (10ug)	16
	CPX(10ug)	15
	KF (30ug)	22
Campylobacter spp	OFX (10ug)	21
	CH (30ug)	12
	CPX (10ug)	17
	TE (30ug)	23
Escherichia spp	AU (30ug)	18
	CN (10ug)	14
	CPX (10ug)	26
	OFX (10ug)	22
Vibrio spp	SXT (30ug)	13
	CN (10ug)	10
	CH (30ug)	24
	CPX (10ug)	26

KEY
OFX=Tarivid TE=Tetracycline CPX=Ciprofloxin AU=Augmentin
SP=Sparfloxacin CH= Chloramphenicol SXT= Septrin CN= Gentamycin

Table 5: Antibacterial Susceptibility Test Results of isolates to Aqueous Extracts of leaves of Thymus vulgaris

Microbial isolates	Concentrations in mg/ml of extract	Zone of inhibition in (mm)
Staphylococcus spp	1000	32
	500	23
	250	18
Shigella spp	1000	22
	500	18
	250	16
Salmonella spp	1000	25
	500	19
	250	14
Escherichia spp	1000	28
	500	20
	250	15
Campylobacter spp		
	1000	30
	500	23
	250	19
Vibrio spp		
	1000	20
	500	18
	250	16

Isolates

Figure. 1: susceptibility test results of bacteria to aqueous extract of leaves of Thymus vulgaris

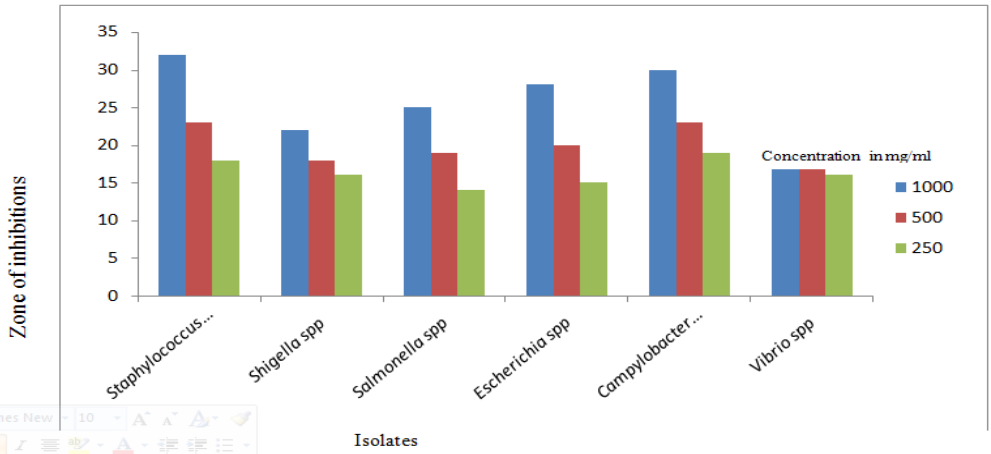

Figure. 1: susceptibility test results of bacteria to aqueous extract of leaves of Thymus vulgaris

IV. Discussion

Sevenisolates of six different genera were isolated and identified on the basis of their morphology and biochemical characteristics such as Salmonella spp, Shigella spp, Campylobacter spp, Esherichia spp, Staphylococcus spp and Vibrio spp. Some microorganisms identified from the study of [13] such as Salmonella spp, Shigella spp, Campylobacter spp, were in accordance with the result of this study.Also according to this result, Salmonella sp, werethe most common bacteria isolated(Table 2) which is in agreement with the findings of [14] who isolated Salmonellaspp as the most dominant organisms in his work. Antibacterial susceptibility test of the isolates to the antibiotics at varying concentrations showed that all the isolate were susceptible to the tested antibiotics. Septrin (SXT) (30ug) had the highest zone of inhibition of 35mm to salmonella spp whereas Gentamycin (CN) (10ug) had the lowest zone of inhibition of 10mm to Vibrio spp. The antibacterial susceptibility test of bacteria to aqueous extract of leaves of Thymus vulgaris was also determined at 1000, 500 and 250mg/ml concentrations and it was observed that the higher the concentration of the extract, the greater the zone of inhibition. 1000mg/ml of the extract had the highest zone of inhibition of 32mm to Staphylococcus spp whereas 250mg/ml of the extract had the lowest zone of inhibition of 14 mm to Salmonella spp. These results were in agreement with the work of [15] who stated that 1000mg/ml of thymusvulgaris extracts gave 32mm zone of inhibition on Staphylococcusspp while 250mg/ml of the same extract gave 14mm on Salmonellaspp Therefore, the antibacterial activity of the plant extracts is wholly attributed to its inhibitory potentials. The inhibitory potentials can also be attributed to the rate of diffusion of the plant extracts microbial load of the test organism, growth rate of the organisms, temperature and time of incubation. These results gave further support to the use of plants in treatment of several infection and disease condition.

V. Conclusion And Recommendations

Acute diarrhoea due to bacterial infections is an important cause of morbidity and mortality in infants and young children in most developing countries including Nigeria. Though synthetic antibiotics are effective in the treatment of diarrhea,it is still recommended that more attention be paid to the use of natural plant in treatment of diarrhoea infections so as to discourage the use of synthetic antibiotics.

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