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Isolation, Identification and Characterization of *Leptospira* Spp and Other Pathogenic Gram Negative Bacteria from the Surfaces of Canned and Bottled Drinks Sold In Ihiala Town

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ABSTRACT

The isolation and identification of microorganisms from the surfaces of Can and Bottled drinks was investigated in the Project laboratory of Microbiology Department, Uli, Anambra State. This study was conducted to isolate, identify and characterize *Leptospira* species and other pathogenic bacteria from the surfaces of can and bottled drinks sold in Ihiala Town as a result of being exposed to rat urine and faeces. A total of 74 samples (Can and bottled drinks) were randomly collected from selected markets in Ihiala. These samples were swabbed using a sterile swab stick for each of the samples, but ten samples were thoroughly washed before been swabbed, to serve as a control, after which, they were incubated in these media: Ellinghausen McCullough Johnson Harris medium (EMJH), MacConkey agar, SSA, centrimide agar, Eosin methylene blue agar. 28 samples were found to contain *Leptospira* spp, by the formation of circular haze or disc formation known as Dinger's ring. After which biochemical tests were carried out on the isolates to identify them. Other pathogenic bacteria were also isolated and identify using biochemical tests. Coliform bacteria such as *E. coli, Klebsiella, Enterobacter* were seen to have this percentage of occurrence 71.8%, 43.6% and 30.9% respectively, while pathogens like *Salmonella* and *Shigella* species have their percentage of occurrence to be 39.4% and 22.3% respectively. There were no blue-green colonies on the centrimide agar, indicating that *Pseudomonas species* are absent. The ten samples used as control had an infinidecimal number of colonies. This indicates that the surfaces of most drinks sold are contaminated. This could result to some very serious health issues; therefore this study is of public health importance.

Keyword: *Leptospira* species, Ellinghausen McCullough Johnson Harris medium (EMJH), Eosin methylene blue agar, *E. coli, Salmonella* and *Shigella* species, public health.

INTRODUCTION

Bacteria are microscopic, single-celled organisms that thrive in diverse environments. Bacteria are classified into five groups according to their basic shapes, which include Spherical (*cocci*), Rod (*bacilli*), Spiral (*spirilla*), Comma (*Vibrios*) or Corkscrew (*spirochaetes*). They can exist as single cells, in pairs, chains or clusters [1]. These organisms can live in /on both animate and inanimate surfaces like soil, the ocean, plastic containers, aluminum containers, skin, bottles, inside the human gut etc[2] Human's relationship with bacteria is complex. Sometimes bacteria play a beneficial role in the association, such as production of vitamins (B complex), help with food digestion [3]. In other cases, bacteria are very destructive, causing diseases like salmonellosis, shigellosis, etc. The ability of a bacterium to cause disease is known as virulence. Pathogenic bacteria are virulent, and pathogenesis sets in when they come in contact with their hosts. There are various sources through which pathogenic bacteria are transmitted, one of which includes the ingestion of food or drinks containing the

cells of pathogenic bacteria. The surfaces of Can and Bottled drinks serve as a medium through which these organisms can be transmitted [4].

There are many different container types and materials used to package beverages, such as glass bottles, aluminum cans and plastic bottles, all of which have the potential to become contaminated. With the popularity of Alcohols and soft drinks, beverages stored in cans are extremely common. These cans are often packed and displayed with the tops uncovered. During storage and transportation, microorganisms may contaminate cans. Thus when drinking from the can, or removing the cover of the bottled drinks with one's teeth, one's mouth comes in direct contact with the cover lid, allowing possible transfer of microorganisms.

[5] conducted a study both food and beverages cans, and found out that there is no correlation between the visual appearance of Cleanliness on the tops of aluminum cans and the level of microbial contamination. The implication of this, is that the surfaces of Can and Bottled drinks are always contaminated with bacteria, whether you can see it or not, because they are always exposed, and also always in contact with contaminants from humans and animals (rats).

MATERIALS AND METHODS

Background to Study Area

Ihiala is the largest City in Ihiala Local Government Area (LGA), which is one of the twenty one LGA in Anambra State. It covers an Area of 304sqkm. In the southern part of Anambra State. The Local Government Area has a population of about 87,796, It also consists of towns like Amorka, Azia, Lilu, Okija, Mbosi, Isseke, Orsumoghu, Ubuluisuzor and Uli. This area was chosen because it's a Rural Area (has a tendency of hosting rodents), with storage houses being exposed to rodents pest. Also, it is known for hosting numerous ceremonious activities (Wedding, Burial, Birthday and Naming ceremonies), where items like Can drinks are always served to invitees, thereby facilitating the transmission of this pathogenic bacteria.

Study Population

Study population were Can drinks sold in some selected markets in Ihiala Town in Ihiala LGA which include, Ubahudara market, Afiaegbu-market, Ihiala market and School-front market.

Study Design

A cross-sectional study was undertaken from October to December, 2020 with the objective of Isolation, Identification and Characterization of *Leptospira spp* and other pathogenic gram negative bacteria from the urine deposited on Can and bottled drinks sold in Ihiala LGA and to also assess the role of inadequate Storage houses (Storage Houses without Rodent proof) in the spread of the pathogens

Sample Method

There are about eight markets in Ihiala town from which four markets were purposely selected, which are Ubahudara market, Afiaegbu market, Ihiala market and School-front market. A total of 74 Canned and bottled drinks were swabbed (the covers in particular, i.e. the Top). These Can and bottled drinks were collected randomly from these four different markets (Ubahudara, Afiaegbu, Ihiala and School-front markets). These samples were chosen to be collected from these markets to know whether these Areas were serving as sources of the selected bacterium for the Can and bottled drinks stored and sold to different individuals for consumption.

Study Methodology

Sample Collection

Samples were collected using sterile swabs, masking tape, sterile bag, water-proof markers, and gloves, during the beginning of the biggest festival in Nigeria (Christmas). This period was chosen because many Can drinks were brought to these markets from different Areas of the Country during this period.

Collection of Samples was done by using the sterile swab stick soaked or immersed in saline solution to swab the Top surface of the Can and bottled drinks (ie, the Cover of the Can and bottled drinks) and

immediately placed in its tube. Then, the collected samples were labeled using the water-proof marker with accordance to the sites of collection.

Inoculation of Samples

A total of 74 samples (Can and bottled drinks) were randomly collected, 8 Can drinks and 8 bottled drinks randomly selected from different shops in these 4 different markets were swabbed and inoculated in various media for the isolation of the desired organisms, these swab sticks were directly dipped in EMJH medium (broth culture) for the isolation of *Leptospira* spp, they also directly streaked on MacConkeg agar for the isolation of Coliforms and gram -ve Organism. Ten samples were washed thoroughly with distilled water and then swabbed and inoculated, this serves as a control.

Isolation of *Leptospira* spp

Materials: Sample Organism *Leptospira* spp and coliform bacteria, isolated from the cover or top of Can and bottled drinks sold in Ihiala Town (Fig. 1).

Medium: Ellinghausen McCullough Johnson Harris medium (EMJH medium) which is a selective medium for the Cultivation of *L. interrogans*.

Method of Medium preparation

Preparation of the Medium was according to the formulation which was described by Ellinghausen and McCoullough (1965), and was modified by Johnson and Harris (1967), by replacing rabbit serum medium with polysorbate 80- albumin.

Principles of the Procedure

EMJH medium contains Ammnium Chloride as a nitrogen source, Thiamine as a growth factor, Sodium phosphate dibasic and Potassium Phosphate monobasic as buffering agents, and Sodium chloride which maintains the Osmotic balance of the formula.

This medium was enriched with albumin, polysorbate 80 and additional growth factors.

Constituents of the Medium

Approximate formula per liter

Disodium phosphate..... .....1.0g
Monopotassium phosphate..... .....0.3g
Sodium chloride..... 1.0g
Ammonium chloride..... 0.25g
Thiamine..... 0.005g
Final pH 7.5 +/- 0.2

The medium was prepared according to the Approximate formular per liter asceptically, and then sterilize.

This medium was reconstituted (prepared) using the aforementioned quantities of substances per a liter of distilled water (1000ml) and then, it was sterilized using autoclave. This medium was enriched with Tween 80, by adding 100ml of Tween 80 into 900ml of the medium (EMJH medium). An antibiotic (Ciprofloxacin 200úg) was added to inhibit the growth of contaminants.

A total of 64 test tubes containing 5ml of the enriched EMJH medium containing an antibiotic (bacteriostatic Agent) were inoculated by directly dipping the swab sticks used to swab the samples into it. The ten controls were also Inoculated in ten test tubes containing the medium. These inoculated test tubes were then covered with cotton wool and then incubated at temperature of 25°C to 28°C in a dark place, and it was examined for growth of *Leptospires* at approximately 5 days, 10 days, 2 weeks, 3 weeks, 5 weeks, and 7 weeks.

Isolation and Characterization of Coliform bacteria

All the samples were streaked on macConkey agar, and was left for 24 hours, the ten control samples were also inoculated in MacConkey agar, after which shades of pink to dark pinkish colonies were observed for the samples, these colonies were subcultured in EMB medium, which is a selective and a differential medium. It's selective for gram negative organisms against gram positive organisms. This also differentiates coliforms.

Fig. 1: Samples for experiment

After 7 days

After 3 weeks

After 5 weeks

Fig 2: Formation of Dinger's ring

RESULTS

When *Leptospires* were detected, the cultures were sub cultured in the same medium, to get sufficient growth. *Leptospira* spp reaches a maximum density in a discrete zone beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. This is known as a Dinger's ring or disk as shown in Figure 2. Biochemical tests (Grams staining, Citrate utilization test, Indole test, Oxidase test, Catalase test, VogesProkeur test, Motility test) were also carried out on the isolate to identify it, [7].

The following results were recorded and presented as follows.

Table 1, shows the number of samples that were contaminated, total of 68 samples, 28 samples were contaminated with *Leptospira* spp. when streaked on macConkey agar, and was left for 24 hours. with Can obtained from Ubahudara market having the highest percentage of contamination 87% while that Bottle drinks gotten from center Market was Nil.

Table 2, reveals the market with larger number of contaminated samples with *Leptospira* spp, from the four markets. Samples obtained from Ubahudara market are the most contaminated, followed by School-front Market. Least was obtained from Center market.

The biochemical characteristics of *Leptospira* spp were reviewed in Table 3.

In Table 4, the morphological characteristics of the *Leptospira* spp and other bacterial isolate were stated, from the size to the texture and color of each colony in different selective and differential media.

Figure 3 and 4 shows the Frequency of samples contamination with pathogenic bacteria and their percentage of occurrence on the samples.

At the end of this study, *Leptospira* spp and coliform bacteria were isolated and identified, Table 5 gave a detailed biochemical test for the identification of the bacteria isolates. *Klebsiella pneumonia* formed pink to purple colonies on EMB agar. *E coli* formed green metallic sheen on a dark purple medium (EMB agar), this is because of the rapid fermentation of lactose and production of strong acids. *Enterobacter* spp produced large, mucoid, pink to purple colonies with no metallic green sheen on EMB agar. *Salmonella* and *Shigella* spp colonies were translucent or colourless, which were subcultered in Salmonella Shigella Agar (SSA), where *Salmonella* spp formed dark centered colonies, while Shigella spp form colourless colonies. For the samples that served as control, an infinidecimal number of colonies were (TLTC) observed in MacConkey agar. Other biochemical test were shown in the diagram (Fig. 5 and 6).

Table 1: Percentage of Contaminated samples obtained from the four markets
(Number of samples inoculated from each markets were 8 Cans and 8 bottles each)

Samples from different markets	No. of contaminated samples	Percentage of contamination
AC	6	75%
AB	2	25%
BC	7	87.5%
BB	3	37.5%
CC	3	37.5%
CB	0	-
DC	5	62.5%
DB	2	25%

AC: Can drinks gotten from school-front market
AB: Bottle drinks gotten from school-front market
BC: Can drinks gotten from Ubahudara market
BB: Bottle drinks gotten from Ubahudara market
CC: Can drinks gotten from Center market
CB: Bottle drinks gotten from Center market
DC: Can drinks gotten from Ihiala market
DB: Bottle drinks gotten from Ihiala market.

Table 2: Percentage of Samples contamination with *Leptospira* spp from the four markets

Markets	No. of contaminated samples	Percentage of contamination
School-front Market	8	50%
Ubahudara Market	10	62.5%
Center market	3	18.75%
Ihiala Market	7	43.75%

Table 3: Biochemical Characterization of *Leptospira* spp

Test tube number	Oxidase test	Catalase test	Motility test	EMJH medium containing antibiotics
AC 1	+	+	+	+

Table 4: Morphological Characterization of isolated *Leptospira* spp and coliform bacteria

Isolated Microorganism	Colony description	Medium inoculated
Leptospira spp	Creamy colored circular haze or disk colony after some weeks	Ellinghausen McCullough Johnson Harris medium (EMJH) broth
E. coli	Dried, donut-shaped and dark pink in color surrounded by a zone of precipitated bile Formed green metallic sheen on a dark purple medium (EMB)	MacConkey agar EMB agar
Salmonella spp	Translucent or colourless colonies Formed dark centered colonise	EMB agar SSA
Klebsiella pneumonia	Pink to purple colonies Pink to purple colonies	MacConkey agar EMB agar
Enterobacter spp	Pink to purple colonies Produced large, mucoid, pink to purple colonies with no metallic green sheen	MacConkey agar EMB agar
Shigella spp	Translucent or colorless colonies	MacConkey and EMB agar

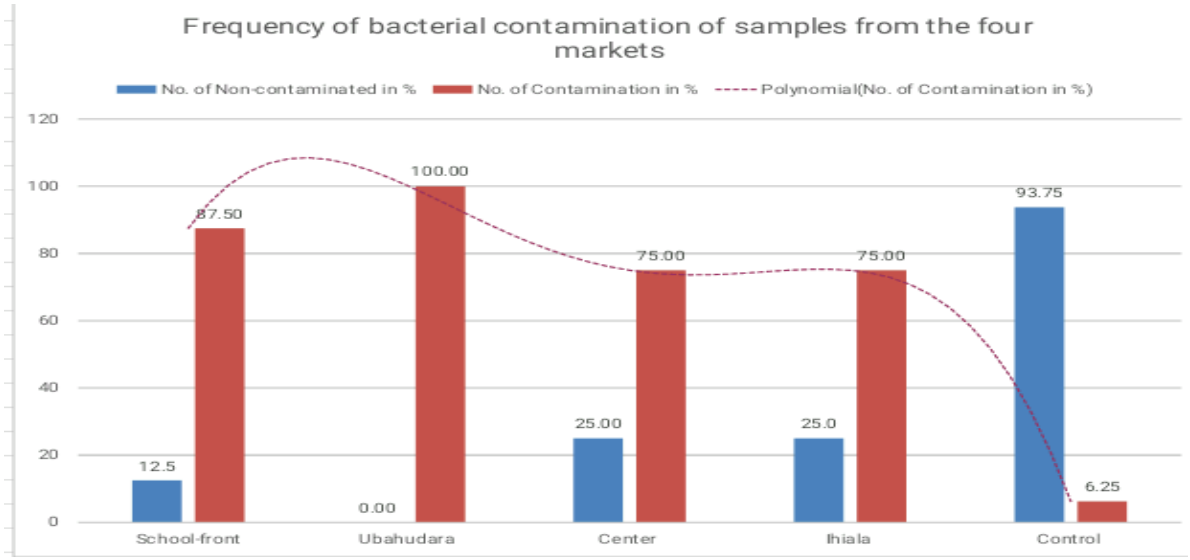

Figure 3: Frequency of bacterial contamination of samples from the four markets

Table 5: Biochemical test for the Isolated bacteria

Organism isolated	Gram staining	Catalase test	Oxidase test	Citrate test	Indole test	Motility test	Shape	V. P Test
E. coli	-	+	-	-	+	+	Rod	-
Salmonella spp	-	+	-	-	-	+	Rod	-
Klebsiella pneumonia	-	+	-	+	-	+	Rod	+
Enterobacter spp	-	+	-	+	-	+	Rod	+
Shigella spp	-	+	-	-	Variable	-	Rod	-

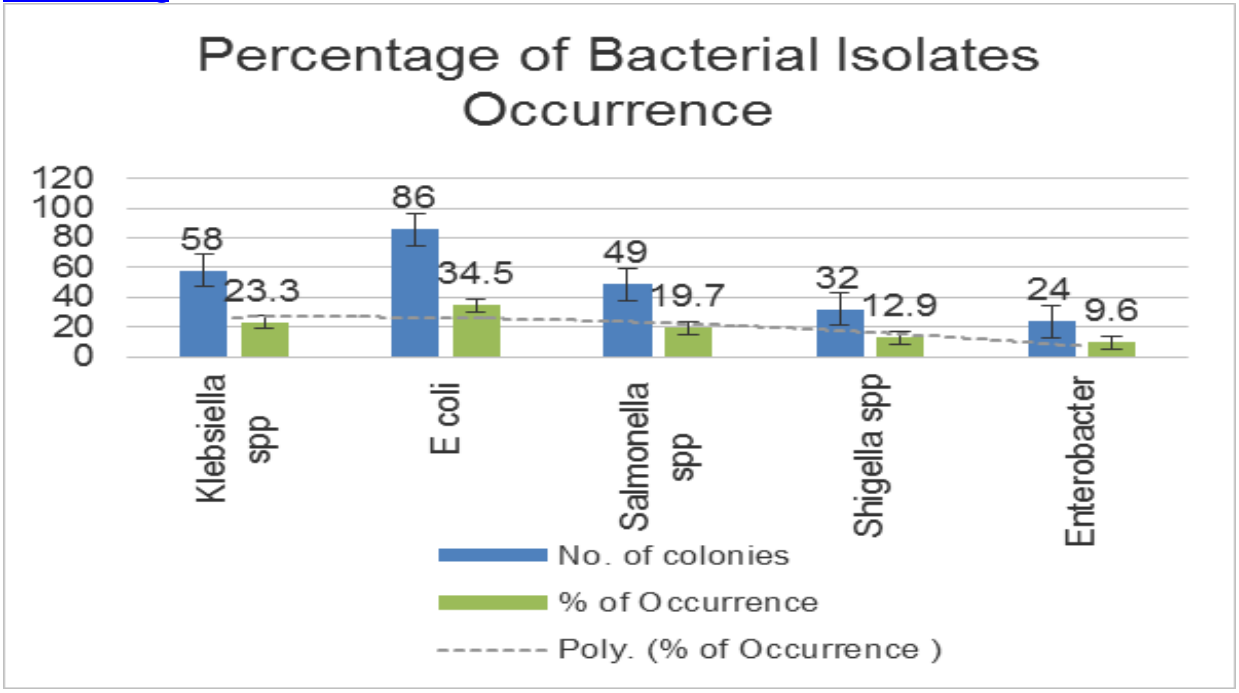

Figure 4:Percentage of Occurrence

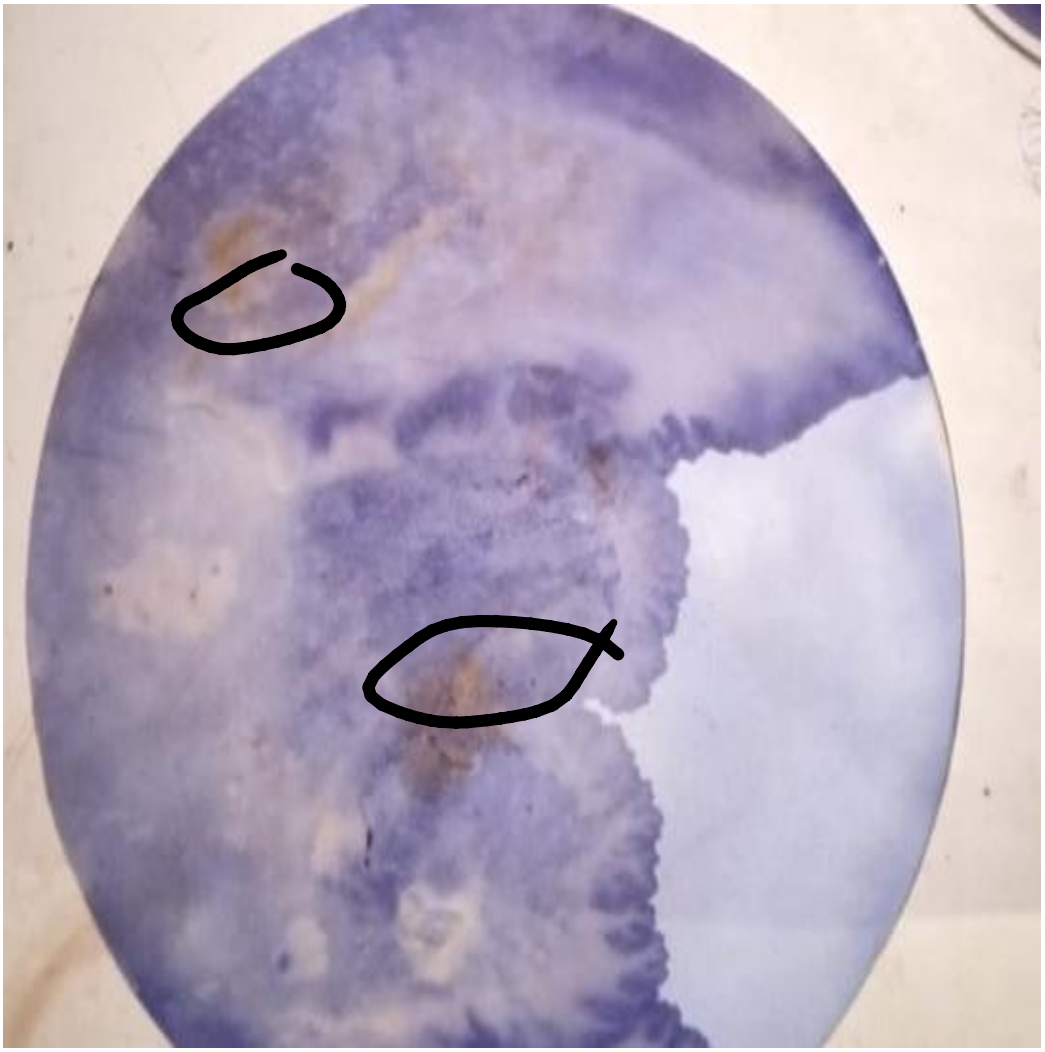

Fig. 5: Oxidase negative organisms

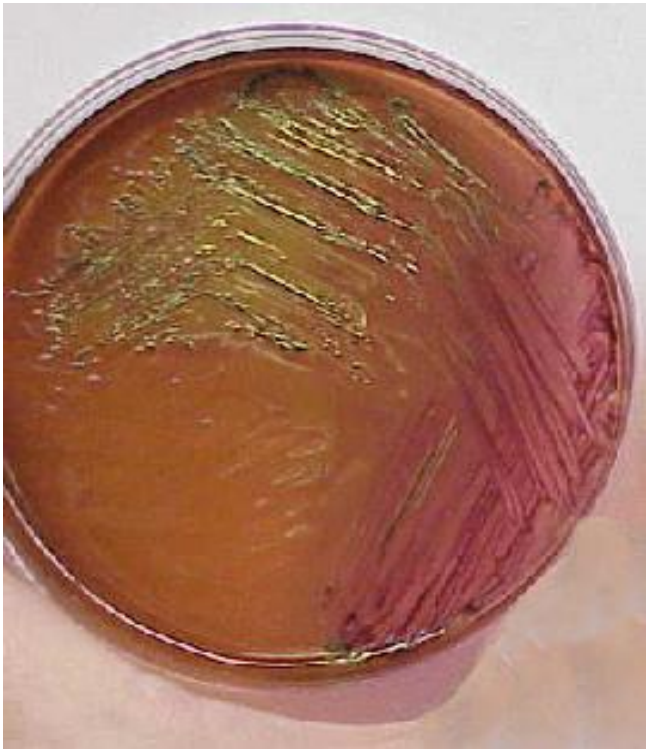

Fig. 6: *E coli* identified by the metallic green sheen formation on EMB agar.

DISCUSSION

A total of 28 samples were contaminated, (Table 1), Can drinks were observed to have greater number of contamination, due to the fact that it has larger surface area when compared with bottles. Bottled drinks are less contaminated than canned drinks because of the reduced surface area. Samples with considerable level of contamination were found to be non-contaminated after washing thoroughly with sterilized distilled water. This suggests the fact that washing surfaces of canned and bottled drinks with portable water can result to a gross reduction in the number of contamination.

From the Table 2, samples obtained from Ubahudara market are the most contaminated, showing that the storage system employed by store owners is very poor, thereby creating access for rodents to come in contact with the stored drinks. When these contaminated drinks are sold, and consumed by the buyers, it results to the manifestation of the disease Leptospirosis. While low % obtained from Center market maybe due to fast rate of distribution to customers and directly consumers for immediate consumption. Also population and economy might also be part of the factors that lead to non-contamination occurrence at center market.

The biochemical characteristics of *Leptospira* spp were reviewed in Table 3.whereby the samples were first inoculated in MacConkey agar, and incubated for 24hrs, after which the colonies were observed macroscopically, for their morphology. Coliforms form pinkish colonies on MacConkey agar. The colonies obtained were shades of pink to purple colonies. Each representative colonies were sub cultured in Eosin Methylene Blue agar. Here, E. coli formed metallic green sheen colonies, *Enterobacter spp.* produced large, mucoid, pink to purple colonies with no metallic green sheen, *Klebsiella pneumonia* just formed pinkish colonies, Salmonella and Shigella spp colonies were translucent or colorless on MacConkey agar, when they were subcultured in Salmonella-Shigella Agar, *Salmonella* spp. produced dark centered colonies, while *Shigella* spp. formed colorless colonies. After this biochemical tests were carried out on the pure cultures to identify them, and the results were recorded as shown in Table 4. This is in conformity with the study conducted by [8], on the isolation of microorganisms from the surfaces of both food and beverages Cans, where it was established that there is no correlation between visual appearance of cleanliness on tops of Aluminum Cans and the level of microbial contamination. This is a risk factor for the On-set of diseases. Pathogenic bacteria have been ingested without knowing, which has resulted to some serious health issues like Leptospirosis, Typhoid fever, Abdominal pains, etc. Disease like Leptospirosis is becoming a global challenge, as more cases of Leptospirosis are being reported. This is due to the regular contacts (ingestion) with the causative agents: *Leptospira* spp, which fatality could result to liver and kidney damage and could also lead to death.

Fig. 3 and 4, which shows the frequency of samples contamination with pathogenic bacteria, observed the effectiveness of thoroughly washing of Can and bottled drinks in reducing pathogenic bacteria on the surfaces of the samples is required and must be advised.

The overall morphological and biochemical characteristics of *Leptospira* spp and other bacteria Table 5 and 6, was in agreement with the works of [9] (Table 3) thus confirming the isolate as a fastidious organism that does not utilize glucose as an energy source, but utilizes beta oxidation of long-chain fatty acids as the major energy and carbon source instead, thereby growing on a selective medium Ellinghausen McCullough Johnson Harris medium (EMJH medium) which was enriched with Tween 80. The results obtained for the utilization of EMJH medium agreed with those obtained by [10]; the formation of Dinger's ring. Other pathogenic bacteria are constantly being isolated from Can and bottled drinks. Coliform bacteria like *E. coli* have been associated with diarrhea and could

get to the surfaces of Canned and bottled drinks either from direct contact with the stool of rats or as a result of vendor's poor personal hygiene practices. The presence of *Salmonella* and *Shigella* spp could be attributed to direct contact with rat stool, or contaminated water or poor handling (poor hygiene) by the vendors and distributors, while Table 7, shows their percentage of occurrence on the samples.

In summary, from the series of experiments carried out on this study (Fig. 1-4 and Table 1-5), it has revealed that pathogenic bacteria can be isolated from the surfaces of Canned and bottled drinks irrespective of how clean it may look.

CONCLUSION

This present study suggests that it is of paramount importance to ensure that canned and bottled drinks are kept in storage houses free from rodents like rats, and it is also necessary to wash thoroughly the surfaces of Canned and bottled drinks before consuming them, or better still avoid using your mouth to open them. Also employ the use of straw when consuming the content, in other to avoid direct contact with the mouth, since the surfaces of canned and bottled drinks have been found to habour pathogenic bacteria. So therefore, measures earlier stated should be employed to avoid the risk of infection, since the pathogenic bacteria isolated could result to serious health issues. This study is of public health importance.

RECOMMENDATION

This study emphasizes the need to investigate the genotypes, molecular characteristics, pathogenicity and plasmids in pathogenic bacteria like *Leptospira* spp*, Salmonella, Shigella* etc. as further markers that useful in describing the epidemiology of some severe diseases like Leptospirosis (which is usually confused with liver failure etc.). Thus as a next step of this study, it is recommended to carryout molecular detection of genes carried by these multidrug resistant gram negative pathogens.

Agencies should be established by the Federal Government to monitor storage houses, in other to reduce to rate of contamination of canned and bottled drinks, thereby controlling the transmission of pathogenic bacteria.

It is necessary and important that molecular or nano-technology in employed in the therapeutic approach of the diseases caused by these organisms.

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