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Phenotypic Identification of *Citrobacter* Isolates from Cloacal Swabs of Apparently Healthy Turtles at the River Banks in Lokoja, Kogi State, Nigeria

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A B S T R A C T

Citrobacter species are opportunistic pathogens in humans that can lead to invasive disease, including infections of the urinary tract, respiratory tract, Central Nervous System, skin, and soft tissues. A total of 245 cloacal swab samples were collected from the cloaca of apparently healthy turtles and subjected to conventional biochemical tests. Presumptive identification on MacConkey Agar (MAC) and Xylose lysine deoxycholate agar (XLD) yielded 42.0% (103) on each medium, on Eosin Methylene Blue agar (EMB) yielded 21.6 % (53), and on *Salmonella-Shigella* agar yielded 9.8% (24) isolates while Indole test, Methyl Red, VogesProskauer, Citrate utilization test (IMVIC) and Aesculin hydrolysis tests conformed with the conventional biochemical reactions for *Citrobacter*. The Microbact^{TM} 24 E identification test performed on the 23 isolates yielded a prevalence rate of 47.8 % for *Citrobacter*. However, this study is therefore the first phenotypic study to confirm the presence of *Citrobacter* species in turtles from the River banks in Lokoja, the study location. Humans can contract the infection through exposure, handling and consumption of poorly cooked turtle meat. Control measures are therefore necessary especially during the handling of apparently healthy turtle, to ensure that proper hygienic measures are observed.

Introduction

Citrobacter species are straight, facultative anaerobic, Gram-negative, non-spore forming bacilli belonging to the family *Enterobacteriaceae* and are typically motile by means of peritrichous flagellae. *Citrobacter* usually utilize citrate as a sole carbon source. They ferment glucose and other carbohydrates with the production of acid and gas (Harrigan, 1998). *Citrobacter* species grow optimally at a temperature of 37 °C (Pathogen Safety Data Sheet, 2012). The bacteria has a long

rod shape with a typical length of 1–5 µm (Wang *et al.*, 2000).

Brenner *et al.*, (1999) classified *Citrobacter* into 11 genomospecies by DNA hybridization: *C.freundii*, *C.koseri*, *C. amalonaticus*, *C. farmeri*, *C. youngae*, *C. braakii*, *C. werkmanii*, *C. sedlakii*, *C. rodentium*, *C. gillenii*, and *C. murliniae* (Schauer *et al.*, 1995; Brenner, *et al.*, 1999). The type species, *C. freundii*, is the one that is most frequently isolated. *Citrobacter* species are primarily inhabitants of the intestinal tract of mammals and other vertebrates. When associated with significant human infection, *Citrobacter* can be recovered from blood, cerebrospinal fluid (CSF), urine, respiratory tract secretions, and wounds (Murray *et al.*, 2010). *Citrobacter* species are opportunistic pathogens in humans that can lead to invasive disease, including infections of the urinary tract, respiratory tract, CNS, skin, and soft tissue (Murray *et al.*, 2010). The bacteria can cause osteomyelitis, suppurative arthritis, bacteremia, endocarditis, endophthalmitis, and intra-abdominal infections, particularly in neonates and immunocompromised hosts.

Turtles harbor *Citrobacter* species and this may serve as a source of infection for man (Sabrina *et al.*, 2017). Prevention of *Citrobacter freundii* infection from captive turtles could be achieved through hygienic handling of the turtles and proper cage management (Sabrina *et al.*, 2017). Currently, turtle rearing has become more popular throughout the world. *Citrobacter* species can cause human infections if pet owners and turtle sellers do not maintain proper hygiene when touching their turtles. Bacteria can thereby be transferred through direct physical contact with infected turtles or through contaminated environments, such as the soil and watering turtle cages. Sea turtles have been proposed to play a significant role in the transmission of infection and as carriers of Antibiotic Resistant (ABR) genes in marine habitats (Alduina *et al.*, 2020) because of their migratory lifestyle (Casale *et al.*, 2008), tendency to bio-accumulate contaminants, toxins and different microbial pathogens (Oros *et al.*, 2005). The bank of the River as a study location appears to have the highest concentration of turtles in Lokoja, yet there is paucity of information/research on these species. Turtle meat is a preferred delicacy in the area. Therefore the possibility of acquiring *Citrobacter* infections associated with turtles in the study area is high due to unhygienic handling of turtles and consumption of improperly cooked turtle meat (Luiselli and Akani, 2003).

Materials and Methods

Equipment and materials used

The equipment used for this study include

Autoclave (LDZX-30FBS, England), Incubator (Genlab, UK), Oven (Gulfex Medical and Scientific, England), Refrigerator (Haier Thermocool, China), Weighing balance, Hot plate, Bunsen burner, and Laminar flow hood.

The materials used for this study include

Test tubes, Beakers, Conical flasks, Inoculating loop, Sterile needles and syringes, McCartney bottles, Hand gloves, non-absorbent cotton wool, Aluminum foil, Alcohol (95%), Potassium hypochlorite solution, Paper tape, Petri dishes, Universal sterile bottles, Sterile distilled water, Mueller Hinton agar (Oxoid, UK), Microbact™ 12A and 24E identification Kits (Oxoid, UK).

Study Location

Kogi State is one of the 36 States of Nigeria, located in the Middle Belt. Created in 1991 from parts of Kwara State and Benue State. Kogi State is noted as being the only state in Nigeria to border ten other states. Kogi State is nicknamed the "Confluence State" due the fact that the confluence of the River Niger and the River Benue occurs in its capital, Lokoja.

Lokoja can be found between Latitude $7^{\circ}\ 45^{1}\ 27.56^{\circ}$ – $7^{\circ}\ 51^{1}\ 04.34^{11}$N and Longitude $6^{\circ}\ 41^{1}\ 55.64^{11}$ – 6°

$45^{1}36.58^{11}$ E of the equator with a total land coverage of about 63.82 sq.km. Agriculture is the mainstay of the economy.

Experimental design and sampling

A cross sectional study was conducted based on convenience observation of a selected sample of individuals from a larger population. Thereafter, each individual sample was determined by simultaneous presence or absence of disease or a causative agent of a disease and hypothesized risk factor. A purposive sampling technique was used based on the availability of the turtles in the study community.

Sample Collection and Bacterial Isolation

A total of 245 samples of cloacal swabs from the cloaca of healthy turtles were collected for this study. About 49 samples were collected from the households for every sampling visit once the turtles were available, and the samples were collected once in a month for a period of 5 months, from January to May 2021. Following the proper restraint of the turtle, samples (about 1g) were collected aseptically by cloacal swab using a sterile swab stick (gently rotating the swab stick inside the cloaca of the turtles) into a disposable plastic sampling jar containing 5.0 ml of Amies Transport Medium (ATM). The samples were taken to the Veterinary Microbiology Laboratory at University of Abuja, Federal Capital Territory, ice-cooled in a vacuum flask.

Selective Plating of the Samples

All samples and media were prepared based on standard bacteriological method as indicated by the manufacturer's instructions (Oxoid, UK). The bacterial isolation, biochemical identification and confirmation of the isolated colonies were done according to Barrow and Feltham (1993). Each of the swabbed samples (1.0 g) were enriched in 9 ml of tetrathionate broth (MB cell Ltd., Seoul, Korea) and incubated at 37° C for 24 hours. After incubation, the broth were mixed for 5 seconds with a vortex agitator. A loopful from each tube were streaked onto a plate of MacConkey agar (Oxoid, UK) and incubated at 37° C for 24 hours, appearance of tiny, pinkish colonies with a dried surface which was suggestive of *Citrobacter* species was smeared onto a plate of Xylose Lysine Deoxycholate (XLD) agar, Eosin Methylene Blue (EMB) agar and *Salmonella Shigella agar* (Oxoid, UK) and incubated at 37° C for 24 hours. Appearance of smooth yellow colonies on (XLD), tiny brown and dried colonies on (EMB) and orange colonies with black center on (SSA) were suggestive of *C. freundii*. The harvested isolates were stored in the refrigerator at 4 ° C for further biochemical characterization.

Preliminary Identification

Stocked isolates suspected to be *Citrobacter* species were further verified using conventional biochemical tests as described by Harrigan (1998) on the basis of indole production and motility with SIM medium (Merch, Germany), citrate utilization with simmons citrate agar (Merck, Germany), Methyl Red and Vogues-Proskauer using MR-VP medium (Merk, Germany), Acid and gas production using triple sugar iron agar, and urease production using urea agar (Oxoid, UK).

Confirmation of *Citrobacter* to Specie Level Using Microbact GNB 24 E Identification Kit

A suspension of the overnight culture of the organism was emulsified in 5ml sterile saline solution and then adjusted to 0.5 McFarland turbidity standards. The wells of the individual substrate sets was exposed by cutting the end tag of the sealing strip and slowly peeling it back. The strip/plate was placed in the holding tray, using a sterile Pasteur pipette, about 4 drops (approximately 100µl) of the bacterial suspension was inoculated into the MICROBACT^{TM} GNB containing the dehydrated substrates. Reactions involving different colours (yellow, red, tan, green, blue) was observed. Using a dropper bottle, the underlined substrates on

the holding tray were overlaid with sterile mineral oil (wells 1, 2 and 3) for 12A or 24E and wells 8 and 12 for 12B or wells 20 and 24E. The inoculated rows were resealed with the adhesive seal and the specimen identification number was written on the end tag with a marker pen. The strip/plate was incubated at 37° C for 18 to 24 hours. On reading the test strip (results), the reactions were evaluated as positive or negative by comparing them with the colour chart. The following reagents were added to well 8 (Indole production) - 2 drops of Kovacs reagent, well 10 (Vogues- Proskauer reaction) - 1 drop each of VP 1 and VP 11 reagents, and well 12 (Tryptophan Deaminase) – 1 drop of TDA reagent was added. The interpretation of the result was based on an octal coding system which was adopted for Microbact. Each group of 3 reactions produced a single digit of the code. Using the results obtained, the indices of the positive reactions were circled. The sum of these indices in each group of three reactions formed the code number. This code was entered into the computer package where the percentage figure shown against the organism name was the percentage share of the probability for that organism (Balows, 1991).

Data analysis

The results were presented using simple descriptive statistics involving percentages, tables and charts (CDC, 2012).

Results and Discussion

Various reactions to confirm *Citrobacter* species on growth media are shown on plates I to IV. Of the 245 cloacal swab samples analysed, reactions on MacConkey and Xylose Lysine Deoxycholate agar gave 103 (42%) on both media, while further evaluation using these isolates gave 53 (21.6%) and 24 (9.8%) on Eosin Methylene Blue (EMB) and *Salmonella Shigella* Agar (SSA) respectively (Table 1). Table 2 showed the results of the biochemical tests (IMVIC), which conformed to the conventional biochemical reactions of *Citrobacter*. After biochemical characterization, using Microbact™ 24E Gram negative identification kit (Oxoid, UK), 11 out of the 23 presumptive *Citrobacter* isolates evaluated, 4 (17.4%) were confirmed to be *Citrobacter freundii*, 2 (8.7%) were *Citrobacter werkmanii*, 2 (8.7%) were *Citrobacter braakii*, and 2 (8.7%) were *Citrobacter youngae* respectively, and 1 (4.3%) was *Citrobacter gillenii*, thereby making the isolation rate of *Citrobacter* specie for this study to be 47.8% (Fig. 1, and Table 3).

Citrobacter species are the common causes of infections in neonates, young children, immuno compromised adults and older children (Schwartz *et al.,* 1997). Neonates may acquire the organisms horizontally as nosocomial infections or vertically from the mother at the time of delivery. They are normal inhabitants of the gut and have been clubbed with the coliforms but when host defenses are weak other factors may favour their establishment in other tissues thereby causing serious infections. Turtles have been documented as the reservoirs of the organism (Sabrina *et al.,* 2017). A total of 245 cloacal swabs was collected from turtles in Lokoja for this study on different months. The reactions on MacConkey agar (MCA) and Xylose Lysine Deoxycholate (XLD) agar gave 42% on both media while further evaluation using these isolates gave 21.6 % and 9.8% respectively on Eosin Methylene Blue (EMB) and *Salmonella Shigella* Agar (SSA) (Table 1). This result has buttressed the point that SSA and EMB have both selective and differential property which selectively promotes the growth of Gram negative bacteria and aids in the differentiation of lactose fermenters and non-lactose fermenting organisms (Madigan and Martink, 2005).

The inclusion of bile salts, citrate and brilliant green in SS agar serve to inhibit gram- positive, coliform organism and swarming *Proteus* spp., while allowing *Citrobacter* species to grow. This may have accounted for the specificity and inhibitory effects of the SS agar on the isolates. Therefore, SS agar tends to be the best medium for the isolation and identification of *Citrobacter* species when compared to MAC and XLD agar as observed in this study.

Table.1 Reactions of *Citrobacter* Species Isolated from Cloacal Swab of Turtles on Various Growth Media.

Months	No. of samples collected	MAC (%)	XLD (%)	EMB (%)	SSA (%)
1	49	13 (26.5)	13 (26.5)	10 (20.4)	5 (10.2)
2	49	15 (30.6)	15 (30.6)	9 (18.4)	4 (8.2)
3	49	41 (83.7)	41 (83.7)	8 (16.3)	3 (6.1)
4	49	18 (36.7)	18 (36.7)	10 (20.4)	10 (20.4)
5	49	16 (32.7)	16 (32.7)	16 (32.7)	2 (4.1)
Total	**245**	**103 (42.0)**	**103 (42.0)**	**53 (21.6)**	**24 (9.8)**

Key: MAC = MacConkey agar, XLD = Xylose Lysine Deoxycholate agar, EMB = Eosin Methylene Blue agar, SSA = *Salmonella-Shigella* agar.

Table.2 Biochemical Identification of *Citrobacter* Species Isolated from Turtles in Lokoja using Conventional Methods

Isolates	TSI	Indole	Motility	Citrate	MR	VP	Urease	Aesculin
1B	A/A +gas + H_2S	–ve	+ve	Blue	+ve	-ve	Pink	-ve
2B	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
58B	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
67B	++	+ve	+ve	Blue	+ve	-ve	Pink	-ve
C17	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
C9	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
C6	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
D9a	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
D16a	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
D2a	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve
D9b	++	–ve	+ve	Blue	+ve	-ve	Pink	-ve

KEY: TSI = Triple Sugar Iron agar, MR = Methyl Red, VP = VogesProskauer, ++ = A/A+ gas+H_2S, –ve = Negative, +ve = positive.

Table.3 Biochemical Identification of *Citrobacter* Specie from Cloacal Swab of Healthy Turtles Isolated from Lokoja, Nigeria using Microbact^{TM} 24 E Identification Test Kit.

S/N	Organism	Incidence of Isolates	Percentage identified (%)
1	*Citrobacter freundii*	4	17.4
2	*Citrobacte rwerkmanii*	2	8.7
3	*Citrobacter braakii*	2	8.7
4	*Citrobacter youngae*	2	8.7
5	*Citrobacter gillenii*	1	4.3
6	*E. coli*	2	8.7
7	*Salmonella subsp.*3B	2	8.7
8	*Serratia odoriferabgp-1*	1	4.3
9	*Enterobacter cloacae*	1	4.3
10	*Enterobacter agglomerans* complex	1	4.3
11	*Klebsiella pneumoniae*	4	17.4
12	*E. coli inactive*	1	4.3
	Total	**23**	**100**

47.8% 11

52.2% 12

Fig.1 Confirmation of *Citrobacter* to Specie Level Using Microbact GNB 24 E Identification Kit

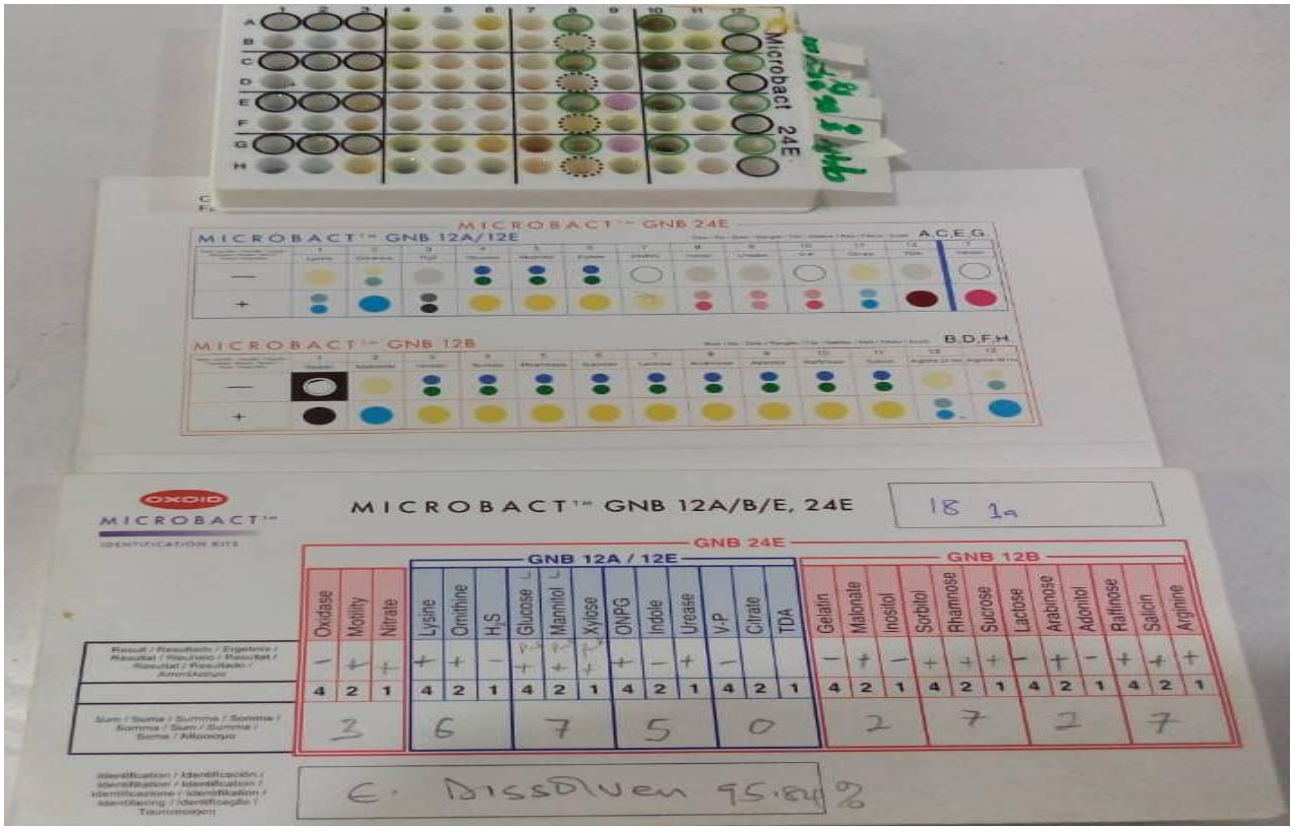

Plate.1 Reaction of *Citrobacter* on MacConkey agar plate.

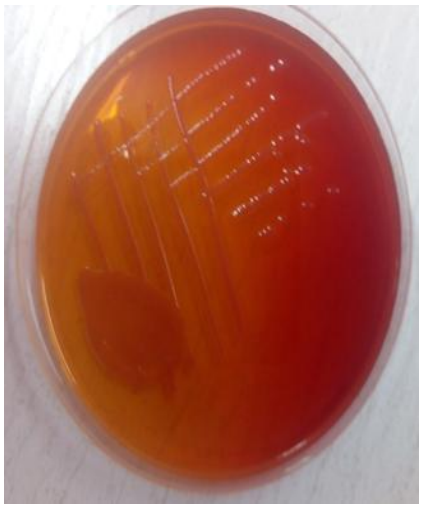

Plate.2 Reaction of *Citrobacter* on Eosin methylene blue agar plate.

Plate.3 Reaction of *Citrobacter* on Xylose deoxycholate agar plate.

Plate.4 Reaction of *Citrobacter* on lysine *Salmonella Shigella* agar plate.

A prevalence rate or isolation rate of 47.8.9% was obtained from this study for *Citrobacter* species (Table 3). This is much higher than that reported by Mahmood and Atyah, (2021), Adegun *et al.,* (2019) and Osuntokun *et al.,* (2018) who reported a prevalence rate of 30.5%, 10.4% and 20% in Iraq, Ile-Ife, Osun state, and Ibadan, Nigeria respectively, but lower with the findings of Sabrina *et al.,* (2017) who reported a prevalence rate of 79.7% in healthy turtles and their environment in Seoul, South Korea.

The difference observed in the isolation rates as reported by many authors could be due to the difference in the sampling locations, the size of the sample collected, and the media used for the isolation and identification of the isolates.

However, the prevalence reported in this study might be due to the fact that healthy turtles are the reservoirs for *Citrobacter* species. However, this result validates the findings of Di Ianni *et al.,* (2015)

who isolated *Citrobacter freundii* from healthy pond sliders (*Trachemys scripta*).

The isolation of these organisms from the healthy turtles in this study suggests that healthy turtles are a silent carrier of pathogenic *Citrobacter* species because they may shed bacteria in their faeces (FDA, 2016). The turtles are a potential public health risk due to the *Citrobacter* species and other bacteria which they harbour. The findings from this study has proven the possibility of isolating *Citrobacter* species from Lokoja, Nigeria. *Citrobacter* may spread from turtles to humans. Therefore, turtle keepers, sellers, consumers, and children who handle turtles should be properly educated/enlightened based on the fact that turtles harbor many zoonotic and pathogenic bacteria/ organisms including *C. freundii* which can be transferred to man.

Conflict of Interests

The authors wish to declare that they do not have any conflict of interests.

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