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First Detection of Augmentin and Colistin Resistant *Cronobacter Sakazakii* from a Pharmaceutical Wastewater in South-Western Nigeria

Avemaria Obasi*, Simon Cyril Nwachukwu, Esther Ugoji

Department of Microbiology, University of Lagos, Lagos, Nigeria

Email address:
avemariaobasi@yahoo.com (A. Obasi), simoncyril@yahoo.om (S. C. Nwachukwu), ugojie@gmail.com (E. Ugoji)

*Corresponding author

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Abstract: *Cronobacter sakazakii* formerly known as *Enterobacter sakazakii*, is a bacterium with a rare cause but often fatal infection of the bloodstream and central nervous system. Infants with weakened immune systems, particularly premature infants, are most likely to contact a Cronobacter infection, although the bacteria have caused illnesses in all age groups. Most cases of *C. sakazakii* infection come from powdered infant formula (PIF) contaminated with the bacterium. Although relatively little information is known about the existence of Cronobacter in the environment, more reservoirs are being identified, such as water, soil and plant material. Wastewaters from 6 pharmaceutical industries located in a south-western state in Nigeria were sampled and analyzed. Bacteria were isolated using standard methods and species identification was determined by Gram staining, lactose fermentation, oxidase, catalase and Vitek 2. Antibacterial susceptibility to 25 antimicrobial agents was tested by the disc diffusion method and Vitek 2. Fifty nine Gram-negative bacteria were isolated and identified; one was identified as *C. sakazakii.* The bacterium was susceptible to all antibiotic mentioned but resistant to augmentin (amoxicillin/clavulanate) and colistin which are high potent drugs for the treatment of very stubborn infections. The public health implication of this fact is that this bacterium could be harbouring resistant genes that can be transferred through water ways such as the pharmaceutical wastewaters to bacteria of the same or different species of clinical importance. Therefore, continuous surveillance of the environmental reservoirs of antibiotic resistant bacteria is necessary to prevent their further spread.

Keywords: *C. Sakazakii*, Colistin, Augmentin, Pharmaceutical Wastewater

1. Introduction

Cronobacter is a genus of Gram-negative, facultatively anaerobic, oxidase-negative, catalase-positive, rod-shaped bacteria of the family Enterobactereiaceae. The genus Cronobacter has been divided into seven species: Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter turicensis, Cronobacter muytjensii, Cronobacter dublinensis, Cronobacter universalis and Cronobacter condimenti [1-3]. Among them, C. sakazakii is considered as the predominant species associated with neonatal infections [4].

Clinical manifestation of Cronobacter infections have been associated most frequently with sporadic cases of life-threatening illness, in particular meningitis, necrotizing enterocolitis (NEC) and septicemia in infants [5]. Low birth-weight neonates (i.e. <2.5 kg) and infants <28 days of age are at heightened risk compared to more mature infants [6-9]. Symptoms include meningitis leading to ventriculitis, brain abscess, hydrocephalus and cyst formation as well as NEC characterized by intestinal necrosis and pneumatosis intestinalis; pulmonary, urinary and blood stream infections [10, 11]. In infants, meningital Cronobacter infection is established between the fourth and fifth days post birth and can be fatal within hours to several days following the onset of first clinical symptoms [12]. The mortality rate for neonatal infections has been reported to be as high as 80% [5, 10] and survivors often suffer from severe irreversible neurological disorders. Reports of invasive infections with Cronobacter in adults are rare, although there have been a

few accounts of illness among the immunocompromised [5].

In previous reports, C. sakazakii isolates were genotyped into 14 STs by multilocus sequence typing (MLST), among them, C. sakazakii ST4 was the main sequence type of Cronobacter species, and was associated with neonatal meningitis [4, 13, 14]. Meanwhile, C. sakazakii isolates belonging to ST4 had a stronger ability to resistance to desiccation than ST1, ST8, ST12, ST21, ST64, ST201, and ST258, which may be one of reasons that ST4 was the main sequence type recovered from PIF [15]. C. sakazakii ST83 is another major sequence type with a strong capacity to resistance to desiccation in PIF factories [16]. C. sakazakii ST1 is reported to be a major sequence type of strains from PIF, while C. sakazakii ST8 strains are primarily isolated from clinical sources [17]. In addition, C. sakazakii ST12 can infect neonates and infants to suffer from necrotizing enterocolitis (4). The C. sakazakii strains with these STs have been isolated from commercial PIF, which suggests that ST4, ST1, ST8, ST12, and ST83 should be more risk for neonates and infants.

Currently, antibiotic therapy is the most common and effective method to treat Cronobacter infections [18]. Antibiotics are extremely important in medicine, but more and more bacteria develop resistance to various antimicrobial substances. Drug-resistant bacteria existed long before humans began using antibiotics therapeutically, but the extensive use or misuse of antibiotics selects resistant strains that spread worldwide. Exposure of environmental bacteria to antibiotics as well as to large numbers of resistant bacteria may fast-track the evolution of resistance, increase the profusion and dissemination of resistance genes within the resistome that is precarious to the development of clinical resistance, and increase exchange of antibiotic resistance genes between bacteria [19, 20]. Because most Enterobacter species are either very resistant to many agents or can develop resistance during antimicrobial therapy, the choice of appropriate antimicrobial agents is complicated. A majority of Cronobacter species strains are reported to be susceptible to frequently-used antibiotics, however, long-term use or abuse of antibiotics is likely to lead to the development of Cronobacter antibiotic resistance [21, 22].

Despite the ubiquity of Cronobacter in the food supply, there is a close association with PIF [23]. In epidemiological studies, [24] examined 141 different breast milk substitute powders from 35 countries and enumerated Cronobacter from 20 samples; another study in Canada also isolated Cronobacter from eight of 120 samples of PIF from five different manufacturers [25]. In the United Kingdom, [26] reported that, out of 84 PIF samples from retailers, 2 were positive for Cronobacter. In addition, [27] applied pulsed field gel electrophoresis to trace the prevalence of Cronobacter in an infant formula processing facility and found that the manufacturing environment serves as a primary route for sporadic contamination of PIF [23]. It is therefore perceived that there is limited information on the prevalence of Cronobacter in water resources.

In recent times, concerns on the effects of pharmaceutical wastewaters on the environment are becoming worrisome, as studies by [28] showed that the physico-chemical analysis revealed the presence of constituents capable of inducing mutations in biological systems, and suggested that the tested pharmaceutical effluent is a potent clastrogenic and mutagenic agent and hence are potential adverse health risk to exposed living organisms. One of the ways in which bacteria acquire resistance to antibiotics is due to selective pressure as a result of human activities, and water plays an important role in the dissemination of these organisms among humans, animals and the environment. Antibiotic-resistant pathogens are profoundly important to human health, but the environmental reservoirs of resistance determinants are poorly understood. There are strict regulations in developed countries on issues concerning treatment of wastewaters before discharge. However, due to ignorance, poverty and lack of monitoring by regulatory bodies on environmental issues in most African countries, such as Nigeria, these are not adequate. Reports by [29] on the lack of treatment measures of pharmaceutical wastewaters before disposal into lakes and rivers in Nigeria could result in the prevalence of antibiotic-resistance bacteria and the dissemination of ARG in wastewaters emanating from pharmaceutical industries in Nigeria. What needs to be done is not to try to defeat the resistance itself, but to try to minimize the spread of it. This means that constant monitoring of the usage and possible emissions of antibiotics is needed in order to reduce environmental risk as much as possible. Therefore, there is need to investigate antibiotic resistant bacteria such as *C. sakazakii* present in wastewaters from pharmaceutical industries in Nigeria as a surveillance strategy for epidemiological studies. This study was aimed at isolating and identifying *C. sakazakii* from pharmaceutical wastewaters in Nigeria and to determine the antibiotic resistance profile of the bacteria.

2. Methods

2.1. Wastewaters Sampling Sites

Wastewater samples (n = 18) were collected from discharge points from 6 pharmaceutical industries located in South-western Nigeria. The Global Positioning System (GPS) readings were taken for every sample location (FDO - N06° 421 33.1”, E003° 141 19.8”; NGO - N06° 431 08.7”, E003° 131 21.4”; DFO - N06° 421 19.7”, E003° 131 44.3”; FAO - N06° 431 18.4”, E003° 131 21.3”; UNO - N06° 431 11.5”, E003° 131 22.9”; WTO - N06° 421 33.1”, E003° 141 19.8”). These chosen sampling sites are known basically for the manufacturing of antibiotics in addition to few other drugs. During and after the manufacturing process, the generated wastewaters are discharged directly (without pretreatment) into local rivers and lakes where human activities take place. This act of lack of consideration for risk assessment strategies and public health implications is worrisome.

2.2. Wastewaters Sampling Technique

The wastewater samples were each initially introduced directly into a 1.5L sterile plastic bottle and rinsed twice with the wastewater sample before finally introducing it into the plastic bottle. The wastewaters were filled to about three quarter of the sterile plastic containers to allow space for oxygen, so that microaerophilic organisms can survive before capping up the bottle. The wastewater samples were preserved on ice packs contained in a flask and then taken immediately to the laboratory for routine microbiological analysis. Samples were processed immediately or stored at 4°C until use. Visit to each pharmaceutical industry for the collection of wastewaters was done three times during the period of sampling. The sampling period was for one year, May 2011 to May 2012.

2.3. Isolation and Identification of Bacteria

Bacteria were isolated on MacConkey agar and Gram-staining performed using standard methods according to the method of [30] as preliminary identification test. Motility test was demonstrated by the method described by [31]. Biochemical tests such as lactose fermentation, catalase and oxidase tests were also carried out to further confirm the identity of the bacteria [32]. Final confirmatory species identification was carried out using the Vitek 2 automated method [33].

2.4. Antimicrobial Susceptibility Testing

Antibiotic susceptibility was done with the use of Vitek 2 automated method with cards AST-N223 (Ref. 413110) and AST-N248 (Ref. 413397) including 25 antibiotics: ampicillin, ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, aztreonam, cefotaxime, cefpodoxime, ceftazidime, cefuroxime, cefuroxime/axetil, cefepim, ertapenem, imipenem, meropenem, ciprofloxacin, levofloxacin, moxifloxacin, gentamicin, augmentin (amoxicillin-clavulanate), tobramycin, amikacin, tigecycline, colistin, fosfomycin and trimethroprim/sulfamethoxazole belonging to 10 classes [33]. The disc diffusion method by Kirby Bauer was also utilized for the susceptibility test [34]. Two control strains (Escherichia coli ATCC 25922 and Klebsiella pneumoniae ATCC BAA-1705) were used to validate the measurement. Results were interpreted according to recommendations of the European Committee on Antimicrobial Susceptibility Testing [35].

3. Results

3.1. Isolation and Identification of Bacteria

Gram-negative bacteria were identified by the pinkish coloration of the bacterial cells when viewed under the microscope. A total number of 59 Gram-negative bacterial isolates from the wastewater samples were obtained and cellular morphology showed that they were all bacilli (rod shaped bacteria). *C. sakazakii* identified was motile as it spreads throughout the medium in a swarming movement most probably achieved due to it being peritritously flagellated, further confirming its identity. Its colonial morphologies on columbia agar appeared as tiny round with mucoid consistency and creamy to light yellow in colour; ferment lactose on macConkey as shown by the pink colouration of colonies on plate, catalase positive and oxidase negative. Analysis of Gram-negative bacterial diversity of the various pharmaceutical industries wastewater samples revealed presence of 17 different bacterial spp. A single *C. sakazakii* was identified from an industry among other bacteria as well identified in the same industry such as *Klebsiella pneumoniae, Burkholderia cepacia group, Pseudomonas aeruginosa, Serratia marcescens and Alcaligenes faecalis.* The Vitek 2 biochemical details for the identification of *C. sakazakii* is shown in Table 1.

Table 1. *Biochemical Details for Vitek 2 Identification for C. sakazakii.*

Test (Abbreviation)	Test (sugars)	Qty (mg)	*Cronobacter sakazaki* group (94%)
APPA	Ala-Phe-Pro-ARYLAMIDASE	0.0384	-
ADO	ADONITOL	0.1875	-
PyrA	L-Pyrroolydonyl-ARYLAMIDASE	0.018	-
IARL	L-ARABITOL	0.3	-
dCEL	D-CELLOBIOSE	0.3	+
BGAL	BETA-GALACTOSIDASE	0.036	+
H_2S	H2S-BILDUNG	0.0024	+
BNAG	BETA-N-ACETYL-GLUCOSAMIDASE	0.0408	+
AGLTp	Glutamyl-Arylamidase-pNA	0.0324	-
dGLU	D-GLUCOSE	0.3	+
GGT	GAMMA-GLUTAMYL-TRANSFERASE	0.0228	-
OFF	FERMENTIERUNG/GLUCOSE	0.45	-
BGLU	BETA-GLUCOSIDASE	0.036	+
dMAL	D-MALTOSE	0.3	+
dMAN	D-MANNITOL	0.1875	+
dMNE	D-MANNOSE	0.3	+
BXYL	BETA-XYLOSIDAE	0.0324	+
BAlap	BETA-Alanin-Arylamidase-pNA	0.0174	-

Test (Abbreviation)	Test (sugars)	Qty (mg)	*Cronobacter sakazaki* group (94%)
ProA	L-Proline-ARYLAMIDASE	0.0234	-
LIP	LIPASE	0.0192	-
PLE	PALATINOSE	0.3	+
TyrA	Tyrosin-ARYLAMIDASE	0.0276	+
URE	UREASE	0.15	-
dSOR	D-SORBIT	0.1875	-
SAC	SACCHAROSE/SUCROSE	0.3	+
dTAG	D-TAGATOSE	0.3	-
dTRE	D-TREHALOSE	0.3	+
CIT	CITRAT (NATRIUM)	0.054	-
MNT	MALONAT	0.15	-
5KG	5-KETO-D-GLUCONAT	0.3	-
ILATk	L-LACTATE	0.15	-
AGLU	ALPHA-GLUCOSIDASE	0.036	+
SUCT	SUCCINATE	0.15	-
NAGA	Beta-N-ACETYL-GALACTOSAMINIDASE	0.0306	-
AGAL	ALPHA-GALACTOSIDASE	0.036	+
PHOS	PHOSPHATASE	0.0504	-
GlyA	Glycin-ARYLAMIDASE	0.012	-
ODC	ORNITHIN-DECARBOXYLASE	0.3	-
LDC	DECARBOXYLASE-BASIS	0.15	-
IHISa	L-HISTIDINE-Assimilation	0.087	-
CMT	COURMARAT	0.126	-
BGUR	BETA-GLUCORONIDASE	0.0378	(+)
O129R	0/129-RESISTANCE (Comp. vibrio)	0.0105	-
GGAA	Glu-Gly-Arg-ARYLAMIDASE	0.0576	-
IMLTa	L-MALATE-Assimilation	0.042	-
ELLM	ELLMAN	0.03	+
ILATa	L-LACTATE-Assimilation	0.186	-

3.2. Antimicrobial Susceptibility Testing

The analysis of the pattern of resistance to the 25 antibiotics observed in *C. sakazakii* showed that this bacterium was susceptible to ampicillin, ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, aztreonam, cefotaxime, cefpodoxime, ceftazidime, cefuroxime, cefuroxime/axetil, cefepim, ertapenem, imipenem, meropenem, ciprofloxacin, levofloxacin, moxifloxacin, gentamicin, tobramycin, amikacin, tigecycline, fosfomycin and trimethroprim/sulfamethoxazole. However, in contrast to earlier observation, this same *C. sakazakii* isolate was resistant to augmentin (amoxicillin/clavulanate) and colistin (Table 2), which are high potent antibiotics used for the treatment of clinical infections or in combination therapy sometimes as last line of drugs in the treatment of very stubborn infections. The MIC values for the breakpoint for resistance in *C. sakazakii* ranges from ≤1 for gentamycin to ≥320 for trimethroprim/sulfamethoxazole while the value for susceptibility ranges from ≤0.5 for meropenem to 4 for cefuroxime. The disc diffusion method as shown on plate presents the susceptibility pattern of the bacterial isolate with few antibiotics for a clear demonstration of a phenotypic representation of resistance and susceptible as the case may be (Figure 1).

Table 2. *Antimicrobial Susceptibility pattern of C. sakazakii from a pharmaceutical Wastewater.*

Antimicrobial agent	Susceptibility
Ampicillin (AMP)	S
Ampicillin/sulbactam (SAM)	S
Piperacillin (PIP)	S
Piperacillin/tazobactam (TZP)	S
Cefuroxime (CXM)	S
Cefuroxime/axetil (ACE)	S
Cefpodoxime (CPD)	S
Cefotaxime (CTX)	S
Ceftazidime (CAZ)	S
Ertapenem (ERT)	S
Imipenem (IPM)	S
Meropenem (MEM)	S
Gentamicin (GEN)	S
Ciprofloxacin (CIP)	S
Moxifloxacin (MXF)	S
Levofloxacine (LEV)	S

Antimicrobial agent	Susceptibility
Tigecycline (TGC)	S
Cefepim (FEP)	S
Aztreonam (ATM)	S
Amikacin (AMK)	S
Tobramycin (TOB)	S
Fosfomycin (FOS)	S
Colistin (COL)	R
Sulfamethoxazole/Trimethoprim (SXT)	S
Augmentin {Amoxicilin/Clavulanate}(AMC)	R

S, Susceptible; R, Resistant.

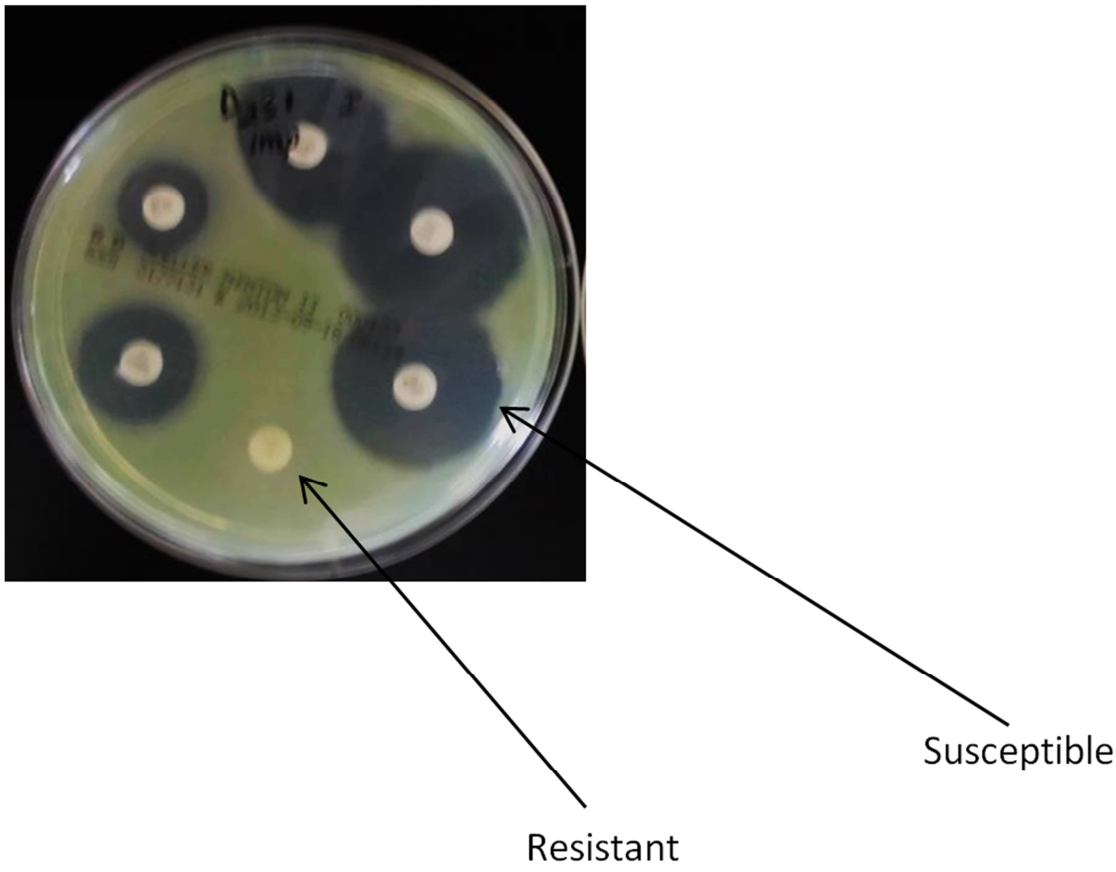

Figure 1. *C. sakazakii susceptibility to antibiotic displayed on Mueller-hington agar plate.*

4. Discussion

Wastewaters in mega cities are notorious reservoirs of microbial pathogens; through the production of biofilms. Wastewater effluent systems represent a protective niche for commensals and pathogens favouring the horizontal transfer of genes encoding for resistance factors [36]. In order to analyse the bacterial contamination and presence of clinically relevant antibiotic resistance, wastewaters from 6 pharmaceutical industries were investigated.

Isolation and identification of bacteria from these pharmaceutical wastewaters apparently revealed the diversity of different bacteria in the wastewater environment. Wastewater samples from one industry had appreciable representation of diverse organisms identified, including antibiotic resistant C. sakazakii. Although much has been said about their presence in PIF [23-27]. The results obtained in the identification technique for this bacterium conformed to that obtained by [37] in their study as well, where the cultural, cellular and biochemical tests examined in their report on 'the documentation of a new species of E. sakazakii now called C. sakazakii' are in tune with our result also (Table 1).

The antibiotic resistance mechanisms of bacteria are diverse; changes of the antibiotic target molecules lead to resistance or the antibiotics were inactivated or neutralized by different biochemical modifications. Active export of antibiotics or the loss of distinct outer membrane proteins (porins) can also reduce the antimicrobial susceptibility. Many antibiotic resistance genes are transferable and can be spread successfully in various bacterial species. The clinical failure of antimicrobial drugs that were previously effective in controlling infectious disease is a tragedy of increasing

magnitude that gravely affects human health. The emergence and dissemination of antimicrobial resistance are well established as clinical problems that affect human and animal health [38, 39].

The antibiotic susceptibility patterns of the 59 Gram-negative bacterial isolates in this study showed that there were single, double and multiple resistance phenotypes, however the antibiotic susceptibility test results for the only C. sakazakii isolate from the pharmaceutical wastewater showed that it has double resistance phenotype susceptible to all other antibiotics except for amoxicillin-clavulanate and colistin. In a study by [15], antibiotic profile of 56 C. sakazakii strains isolated from PIF in China retail markets showed that all C. sakazakii isolates were susceptible to ampicillin-sulbactam, cefotaxime, ciprofloxacin, meropenem, tetracycline, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole. The majority of C. sakazakii strains were susceptible to chloramphenicol and gentamicin, with sensitive rates of 87.5 and 92.9%, respectively. In contrast, most C. sakazakii strains were resistant to cephalothin, with resistance and intermediate rates of 55.4 and 41.0%, respectively. Other reports also confirmed that Cronobacter strains resistant to amoxicillin-clavulanate, ampicillin, cefazolin, cephalothin, cefotaxime, and streptomycin have been isolated from food samples [15] and [40-44], which was similar to the result obtained for amoxicillin-clavulanate resistant C. sakazakii in our study. In addition, it is observed that colistin resistance in C. sakazakii in this study seems to be the first report of colistin resistant C. sakazakii, as previous reports were not indicative of this fact. Therefore, it was necessary to evaluate the antibiotic resistance profile of Cronobacter species isolated from other environment such as in a pharmaceutical wastewater.

Treatment options for Cronobacter infection include newer options like tigecycline which has an excellent in vitro activity against these gram-negative bacilli [45]. Although older options which might include intravenous administration of polymyxin B or colistin are drugs that are now rarely used [45]. This statement is justified due to the fact that the bacteria might be fast developing resistance to colistin, like was observed in the strain of *C. sakazakii* isolated from the wastewater from our study. In addition, traditionally, antibiotic therapy with a combination of ampicillin and gentamycin has been successful in treatment of Cronobacter infection [5]. However, optimal antibiotic treatment regimens still need to be determined and the emergence of strains resistant to ampicillin has led to consideration of use of the newer cephalosporins [46] and possible combination therapy. In the light of this, [47] reported that colistin or polymyxin B or tigecycline combined with carbapenem were the most commonly used combination for the treatment of KPC infection when treatment failures were observed in monotherapy as compared with combination therapy, resulting to improved survival. Also a combination therapy with rifampicin and colistin for CRE *Acinetobacter baumannii* infection was also reported [47]. In addition, this could also imply that *C. sakazakii* resistant to colistin isolated from a pharmaceutical wastewater calls for concern as treatment failures to certain infections might be imminent, especially if resistance determinants are widely spread in wastewaters which can be disseminated from non-pathogenic strains into their pathogenic counterparts.

The extent to which discharge of antimicrobial resistant bacteria into the environment contributes to the dissemination of antimicrobial resistance is uncertain because studies are always limited to antimicrobial resistance in bacteria in the environment [39] and [48-50]. Antimicrobial resistant bacteria may be discharged into the environment from human sources (hospital and municipal effluents), agricultural sources and industrial sources (pharmaceutical wastewaters) [51-54]. This study has shown that amoxicillin-clavulanate and colistin resistant C. sakazakii strain are present in the environment in Nigeria. Most previous studies of the antibiotic resistance profile of pathogenic bacteria have been directed towards clinical isolates. There is a possibility that antibiotic resistance genes in C. sakazakii strains in this study could have opportunities for environmental dissemination and possible human exposure and transmission.

At present, antibiotic treatment is a primary preference, and in many cases the only way of treating infectious diseases. More detailed studies of environmental reservoirs of resistance are essential to future ability to combat infections. This is the first report describing amoxicillin-clavulanate and colistin resistant identified *C. sakazakii* strain isolated from industrial wastewaters in Nigeria. Most industries do not have wastewaters treatment facilities, especially in a majority of pharmaceutical industries in Nigeria. Possible remedies could be achieved when treatment measures of the wastewaters are implemented. However urgent mitigation is needed to minimize the effects from the release of pharmaceutical wastewaters to water resources. The potential threat posed by the continued evolution of antibiotic resistance seems sufficiently grave and imminent that reliance upon participant behavioural change should be considered a high-risk strategy. A major drawback to this investigation was haven not gone further to establishing the sequence type of the *C. sakazakii* strain identified in our study, this is necessary in order to categorically attribute its possible link to the sequence types implicated in clinical strains known to be colonizers or causes of Cronobacter infections in unsuspecting individuals; this is important for the purpose of epidemiological studies.

5. Conclusion

In conclusion, this study established the presence of augmentin and colistin resistant *C. sakazakii* in a pharmaceutical wastewater from Nigeria. This bacterium has always been found in PIF, with little information of its presence and susceptibility to antibiotics in wastewaters. The knowledge of the possibility of the existence of this fact reveals possible dissemination of resistant genes into the environment. Therefore, there is the need for further research

on the continuous surveillance of this bacterium with resistant determinants in the environment in other to help reduce their impact on public health; this calls for great concern.

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Competing Financial Interests

None declared.

Conflict of Interest

As this work was unfunded, with respect to government and pharmaceutical industry sources, there were no conflicts of interest or undue influence in the preparation of this manuscript.

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