Antibiotics Resistance in Haemolytic Bacterial pathogens from Hospital Wastewaters

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Abstract: Burden of morbidities and mortalities originating from bacterial pathogens and antibiotics resistance is on the rise, and hospital wastewaters are possible reservoirs of these pathogens. Infectious diseases and antibiotic resistance from Hospital Wastewaters could be spread into the environment. This study aimed at isolation, identification and antibiotic susceptibility characterization of hemolytic bacterial pathogens from hospitals wastewaters in Benin City, Edo State, Nigeria**.** Hospital wastewaters (HWWs) were aseptically collected from four major Hospitals in Benin City, and selective media were used for bacterial isolation while identification was based on standard procedures. Sheep blood agar was used for hemolytic bacterial isolation, and isolates were subsequently assessed for Antibiotic resistance to common antibiotics using Kirby-Bauer disc diffusion method. Hemolytic bacterial pathogens recovered were *Pseudomonas aeruginosa* (23 %), *Staphylococcus aureus* (19.2 %)*, Salmonella* spp (30.7 %), *Escherichia coli* (19.2 %) and *Escherichia coli* O157: H7 (7.6 %). High resistance against Augumentin (25 µg) was observed in *Salmonella* spp (90.6 %), *Pseudomonas aeruginosa* (79.2 %), *Escherichia coli* (50 %) and *Escherichia coli* O157: H7 (37.5 %). Conversely, *Staphylococcus aureus* (55 %) isolates were resistant to Amoxicillin (30 µg). All Gram negative hemolytic pathogens showed low resistance to Sparfloxacin (10 µg) and Ofloxacin (30 µg). Multiple antibiotics resistance index (MARI) greater than 0.2 was observed in 50 % of the hemolytic pathogens. Antibiotics resistance in hemolytic bacterial pathogens obtained in this study suggests their potential as sources of environmental and public health risks.
Key words: Antibiotics, Bacterial pathogens, Hemolytic bacteria, Hospital wastewaters

INTRODUCTION

Hospital wastewaters are sources of bacterial pathogens that are of environmental and public health risks (Carraro *et al.,* 2016). Environmental sources of antibiotics bacterial resistance, and its proliferation has been recognized as growing public health threats (Williams *et al.,* 2016). Due to high consumption of antibiotics in the hospitals, and subsequent wastewaters containing potentially infectious organisms, antibiotic resistance (AR) acquisition and dissemination of resistant genes are common in these environments (Korzeniewska *et al.,* 2013).
Wastewaters from hospitals could increase the total number of bacteria that are resistant in receiving environment or sewers by the processes of introduction and selective pressure (Beyene and Redaie, 2011; Stalder *et al.,* 2014). These resistant bacteria could be carrying genes that are transmissible and acting as a vector or reservoir of resistant gene (Nunez and Morretton, 2007; Keen and Patrick, 2013). Antibiotics resistance in Streptococcus species, *Pseudomonas aeruginosa, Escherichia coli,* Salmonella spp*,* and Klebsiella spp have led to several diseases outbreaks (Diab *et al.,* 2008; Julien *et al.,* 2014; Youghgho and Choi, 2016).
Hemolysins are important virulence determinants of a disease outcome and pathogenicity of Streptococcus spp and *Staphylococcus aureus* is dependent on their hemolysins (Leclercq *et al*., 2016; Divyakolu *et al*., 2019). Although is a rare trait in Salmonella (Singh *et al*., 2004). Hence the need to assess antibiotics resistant hemolytic bacteria in hospital wastewaters since they pose risks to public health and the environment. Therefore, this study was aimed at isolating hemolytic pathogenic bacteria from hospital wastewaters in Benin City, Nigeria and assessing their antibiotics resistance.

MATERIAL AND METHODS

Sample collection

Hospital wastewater samples were collected from four units (main surgical/Post-operative unit, Laboratory, Laundry and Pediatric ward) of four major hospitals in Benin City, Nigeria. Sampling was done once a week for four (4) consecutive weeks at the point they drain to the sewer/receiving environment, totaling 64 wastewater samples. Samples were collected in triplicates and were carefully labelled and transported in ice-cooler to the laboratory for analysis within 4 h of collection (Tsegahun *et al.,* 2017).

Isolation and identification of bacterial pathogens

Isolation and identification of specific bacteria were done from wastewater samples. One (1) ml aliquots representative of dilution samples were plated on selective and differential media including Eosin Methylene blue agar (*Escherichia coli*), Sorbitol-MacConkey agar (*Escherichia coli* O157: H7), Salmonella-shigella agar (Salmonella spp), Cystine-Lactose-Electrolyte-Deficient Agar (*Pseudomonas aeruginosa*) and Mannitol salt agar (*Staphylococcus aureus*). Pure colonies were obtained, and important features from growth media as well as morphology of the isolated organisms were recorded. Further identifications were based on Gram's stain as well and biochemical tests (Cheesebrough, 2006).

Hemolysin production in bacterial pathogens

Different isolates from wastewater samples were cultured on sheep blood agar media as described by Pavlov *et al.* (2004). Plates were incubated at 37˚C for 24 h subsequently, growth and hemolytic activity of the bacteria were observed. Greenish zone around the culture indicated α-hemolysis while clear transparent zone indicated β-hemolysis.

Antibiotics Susceptibility Testing and Multiple Antibiotics Resistance (MAR) Index of hemolytic pathogens

The susceptibility of pathogenic bacteria isolates to antibiotics was tested on Mueller-Hinton agar (Oxoid, UK) by the Kirby - Bauer disc diffusion method (Bauer *et al.*, 1966). The following common antibiotic sensitivity discs with their concentrations were used; Ampiclox (30 µg), Augmentin (25 µg), Gentymicin (10 µg), Amoxicillin (30 µg), Chloramphenicol (10 µg), Streptomycin (30 µg), Zinacef (Cefuroxime) (20 µg), Rocephin (Ceftriaxone) (25 µg), Erythromycin (10 µg), Septrin (co-trimoxazole) (30 µg), Tarivid (Ofloxacin) (30 µg), Perfloxacin (10 µg), Ciprofloxacin (10 µg) and Sparfloxacin (10 µg). Standardized inocula were prepared by the direct colony method. Pure colonies of pathogenic bacterial isolates growing on each selective agar plate were picked to make a suspension in 1ml sterile normal saline and this was adjusted to an equivalent of a 0.5 McFarland standard. Sterile Mueller-Hinton agar plates were inoculated by spreading 0.1 ml of each inoculum suspension on the entire surface of the plate. The plates were allowed to air-dry and within 15 minutes antibiotic sensitivity discs containing the above mentioned antibiotics were placed to the surface of the agar at 15 mm equidistance to one another. Incubation was done at 37 ^{o} C for 24 hours. Thereafter, zone of growth inhibitions were measured and interpreted according to recommended standard (CLSI, 2017).

The multiple antibiotic resistance index (MARI) was determined for each pathogenic bacterial isolate by dividing the number of antibiotics the isolates were resistant to the total number of antibiotics tested (Adenaike *et al.,* 2016). Multi-Drug Resistant (MDR) bacterial isolates were determined based on resistance to two antibiotics from different classes (Mandal *et al*., 2011).

RESULTS

A total of sixty-four (64) wastewater samples were processed from four major hospitals in Benin City, Nigeria for four (4) consecutive weeks. Specific bacterial pathogens were assessed using selective and differential media. Isolated bacterial pathogens were identified as *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* spp, *Escherichia coli* O157:H7 and *Escherichia coli* in percentage distribution frequencies of 37.5 %, 31.3, %, 75 %, 12.5 % and 43.8 % respectively (Tables 1 and 2).

Table 1: Identities of potential bacterial pathogens isolated from Hospital wastewaters in Benin City.

Bacterial isolates	colour of colonies on growth Medium	Gram rxn	morphology	Arrangement	Mot	Ind	MR/VP	Cit	Cat	Oxi	Ure	Lac	Sor	Coa	Glu
E. coli O157	Colourless and transparent	_	Rod	Single	+	+	+/ -	-	+	_	_	+	+	-	+
E. coli	green with metallic sheen	_	Rod	Single	+	+	+/ -	-	+	_	_	+	_	-	+
Salmonella spp	colourless with black eye	_	Rod	Single	+	_	+/-	+	+	_	_	_	+	-	+
P. aeruginosa	Green, matte surface	_	Rod	Single	+	_	-/-	+	+	+	_	_	_	-	+
S. aureus	Golden yellow, shinny surface	+	Cocci	Irregular cluster	_	_	+/+	+	+	+	+	+	ND	+	+

Legend: rxn: reaction, ND: Not determined, +: Positive, - : Negative, rxn: Reaction, MR: Methyl Red, VP;_Voges- Proskeur
Mot = Motility, Ind = Indole, MR = Methyl red, VP = Voges proskaeur, Cit = Citrate, Cat = Catalase, Oxi = Oxidase, Ure = Urease, Lac = Lactose, Sor = Sorbitol, Coa = Coagulase, Glu = Glucose

Table 2: Frequency of distribution of isolated potential pathogens from Hospital wastewaters

Bacterial Isolates	Examined samples	Positive samples	Distribution (%)
Pseudomonas aeruginosa	64	24	37.5 %
Staphylococcus aureus	64	20	31.3 %
Salmonella spp	64	48	75 %
Escherichia coli O157	64	8	12.5 %
Escherichia coli	64	28	43.8 %

The total number of these pathogens were one hundred and twenty eight (128) and each of their percentage occurrence is shown in Table 3.

Table 3: Percentage occurrence of potential bacterial pathogens and frequency of distribution of hemolytic pathogens from hospital wastewaters

Bacterial Isolates	N/%	hemolysin +ve
Pseudomonas aeruginosa	24 (18.8 %)	24 (100 %)
Staphylococcus aureus	20 (15.6 %)	20 (100 %)
Salmonella spp	48 (37.5 %)	32 (66.7 %)
Escherichia coli O157	8 (6.25 %)	8 (100%)
Escherichia coli	28 (21.9 %)	20 (71.4 %)
Total	128 (100 %)	104 (81.25 %)

Legend: N/%: Total number of bacteria/Percentage of occurrence; hemolysin +ve: percentage number of bacterial positive for hemolysin production

These isolates were further tested for hemolysin production, and a hundred and four (104) (81.25 %) out of the 128 isolated pathogens were positive for hemolysin production (Table 3). All *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E. coli* O157:H7 isolates were positive for hemolysin. However, thirty two (32) out of forty eight (48) Salmonella spp isolates and twenty (20) out of the twenty eight (28) *E.coli* isolates were positive for hemolysin production (Table 3). The frequency of distribution and percentage occurrence of these hemolytic pathogens are shown in Tables 3 and 4 respectively.

Table 4: Percentage occurrence of hemolytic pathogenic bacterial isolates from Hospital Wastewaters (HWWs) in Benin City.

Bacterial isolates	Total (%)
Pseudomonas aeruginosa	24 (23)
Staphylococcus aureus	20 (19.2)
Salmonella spp.	32 (30.7)
Escherichia coli O157	8 (7.6)
Escherichia coli	20 (19.2)
Total	104 (100)

Isolated hemolytic bacterial pathogens from hospital wastewaters were subsequently subjected to common antibiotics to assess their susceptibility patterns to them. The resistance profiles of isolated hemolytic pathogens are shown in Figures 1, 2 and 3.

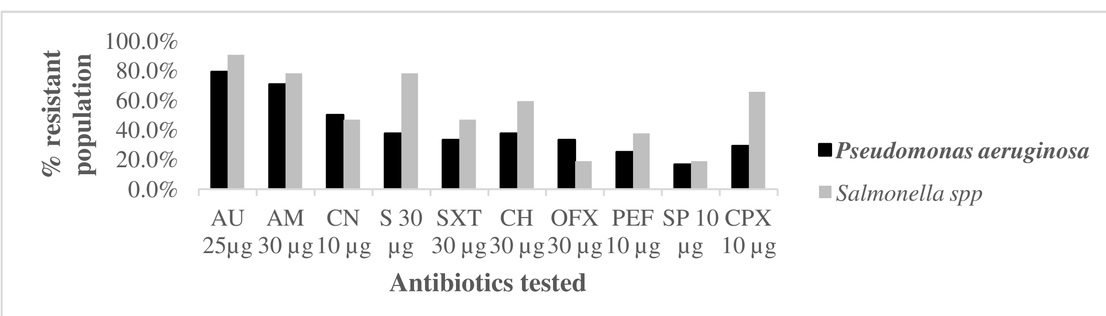

Figure 1: Percentage Resistant pattern of hemolytic *Pseudomonas aeruginosa* and Salmonella spp isolates from hospital wastewaters in Benin City

Legend: AU: Augmentin; AM: Amoxacillin; CN: Gentamycin; S: Streptomycin; SXT: Septrin (Co-trimoxazole); CH: Chloramphenicol; OFX: Tarivid (Ofloxacin); PEF Perfloxacin; SP: Sparfloxacin; CPX: Ciprofloxacin

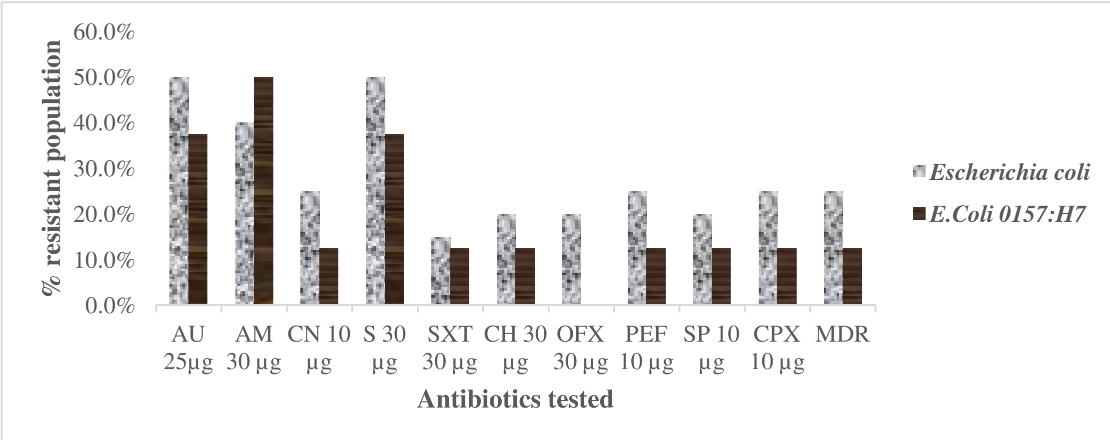

Figure 2: Percentage Resistant pattern of hemolytic *Escherichia coli* and *E.coli* O157: H7 from hospital wastewaters in Benin City

Legend: AU: Augmentin; AM: Amoxacillin; CN: Gentamycin; S: Streptomycin; SXT: Septrin (Co-trimoxazole); CH: Chloramphenicol; OFX: Tarivid (Ofloxacin); PEF Perfloxacin; SP: Sparfloxacin; CPX: Ciprofloxacin

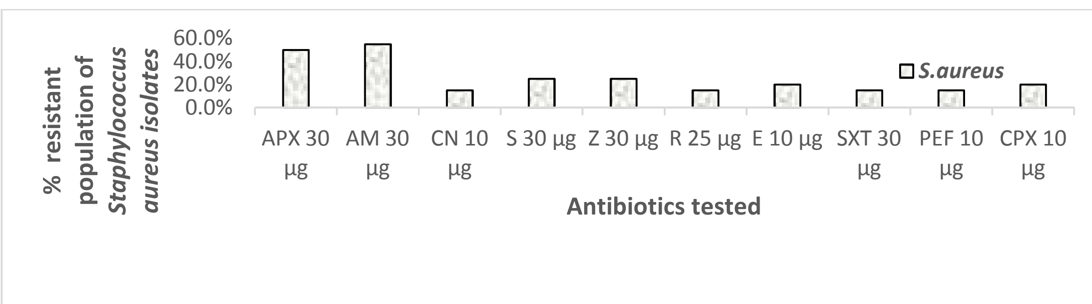

Fig. 3: Percentage Resistant pattern of hemolytic *Staphylococcus aureus* from hospital wastewaters in Benin City.

Legend: APX: Ampiclox; AM: Amoxicillin; CN: Gentamycin; S: Streptomycin; Z: Zinacef (Cefuroxime); R: Rocephin (Ceftriaxone); E: Erythromycin; SXT: Septrin (Co-trimoxazole); PEF Perfloxacin; CPX: Ciprofloxacin

Pseudomonas aeruginosa isolates showed highest resistant to Augmentin (79.2 %), followed by Amoxacillin (70.8 %) however, they showed the least resistant to Sparfloxacin (16.8 %). Similar pattern was also observed with Salmonella spp, these showed highest resistance to Augmentin (90.6 %) followed by amoxacillin (78.1 %) and Streptomycin (78.1%) but were least resistant to Ofloxacin (18.8 %) and Sparfloxacin (18.8 %) (Figure 1).

Escherichia coli isolates were resistant to Augmentin and Septrin (50 %) as compared to other antibiotics tested. However, their resistance to Chloramphenicol, Ofloxacin and Sparfloxacin were low (20 %). Conversely, *Escherichia coli* O157:H7 isolates were resistant to Amoxacillin (50 %) followed by Augmentin and Streptomycin (Figure 2).

These isolates were less resistant to Gentamycin, Septrin (Cotrimoxazole), chloramphenicol, Pefloxacin, Sparfloxacin and ciprofloxacin. However, *E. coli* O157:H7 isolates were susceptible to Ofloxacin (Figure 2).

The highest antibiotic resistance in *Staphylococcus aureus* isolates was observed with Amoxicillin (55 %), followed by Ampiclox (50 %). However, they were least resistant to Gentamycin, Rocephin (ceftriaxone), Septrin (cotrimoxazole) and Pefloxacin (15 %).

Multiple antibiotics indices (MARI) of the hemolytic bacterial pathogens from Hospital wastewaters were also assessed, and the highest percentage multi drug resistant population was observed in Salmonella spp (68.8 %). *Pseudomonas aeruginosa* (50 %) was sequel to this with *E. coli* and *Staphylococcus aureus* following. However, *E. coli* O157: H7 had the least percentage of multi drug resistant population (Table 5).

Table 5: Multiple Antibiotics Resistance indices (MARI) > 0.2 Patterns of pathogenic bacterial isolates from Hospital Wastewaters in Benin City.

Pathogenic Bacterial isolates	Total number of isolates	MARI > 0.2
Pseudomonas aeruginosa	24	16 (66.7%)
Staphylococcus aureus	20	5 (25 %)
Salmonella spp	32	22 (68.8%)
Escherichia coli O157	8	2 (25 %)
Escherichia coli	20	7 (35 %)
Total	104	52 (50 %)

Fifty percent (50 %) of isolated bacterial pathogens had multiple antibiotics index > 0.2 with Salmonella spp having the highest index and *E. coli* O157:H7 the least.

DISCUSSION

Microbial analysis of hospital wastewaters sampled for four (4) weeks from four major hospitals in Benin City, Nigeria showed a total of 128 bacterial pathogens based on the use of differential and selective media. These were *Pseudomonas aeruginosa,* Salmonella spp, *Staphylococcus aureus, Escherichia coli and Escherichia coli* O157: H7. Tsegahum *et al.* (2017) isolated similar organisms (Klebsiella spp, *Staphylococcus aureus* and *Pseudomonas aeruginosa)* from Ayder Referral Hospital wastewater in North Ethiopia.

In this study, Salmonella spp had the highest frequency of distribution and percentage occurrence, followed by *E.coli* and *Pseudomonas aeruginosa* while *E.coli* O157:H7 was least. Conversely, Onuoha. (2017) reported low percentage occurrence of Salmonella spp (21.3 %) in hospital wastewater from South Eastern, Nigeria. However, the percentage occurrence of *Staphylococcus aureus* (15.5 %) from this study is similar to that reported from North Ethiopian hospital wastewater (Tsegahum *et al.,* 2017). Similarly, Moges *et al.* (2014) reported 16.8 % occurrence of *Pseudomonas aeruginosa* from hospital wastewater in Northwest Ethiopia which is close to that of this study. However, that of *E.coli* (11.5 %) from same study is much lower than our findings.

Eighty-one percent (104) out of these bacterial pathogens were positive for hemolysin production. The presence of these hemolytic pathogenic bacteria in the sampled hospital wastewaters from this study portend potential risks to the environment, and could suggest sources of pathogen spread to the Salmonella spp were the most resistant isolates, having 90.6 % resistance to augmentin and 78.1 % to Amoxicillin and Streptomycin. This is in contrast with the report of Akubuenyi *et al.* (2011) who reported 100% resistant *Salmonella* spp from hospital wastewater in Calabar, Nigeria to Augmentin, Amoxicillin and streptomycin. This suggests that Salmonella spp isolated from hospital wastewaters in Benin City, Nigeria are less resistant to antibiotics as compared to the findings of Akubuenyi *et al.* (2011).

Conversely, the resistance pattern of *Pseudomonas aeruginosa* isolates to Gentamycin and Ciprofloxacin from this study is similar to the report of Tsegahum *et al.* (2017). Although higher resistance (70%) to gentamycin and Ciprofloxacin was reported by Bolaji *et al.* (2011). Low percentage (15%) resistance to gentamycin was seen in *Staphylococcus aureus* isolates from this study which is similar to the report from a referral Hospital wastewater in North Ethiopia (Tsegahum *et al.,* 2017). In this present study, Sparfloxacin, Gentamycin, Rocephin, Tarivid (Ofloxacin) and Septrin proved more effective against pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Salmonella species and *Escherichia coli* as compared to other antibiotics studied. Amechi *et al*. (2016) and Abakpa *et al*. (2015) also reported better activity of Tarivid (Ofloxacin), Gentamycin, ciprofloxacin, and Environment (Amores *et al*., 2013). Antibiotic resistant profile of hemolytic bacteria isolated from this study showed varied patterns. sparfloxacin against pathogenic bacterial isolates from wastewater samples.

Multidrug resistance (MDR) occurs when strains are resistant to four or more antimicrobial agents but sometimes as low as two antibiotics from different classes (Mandal *et al*., 2011; Imanah *et al*., 2017). Gram negative isolates from this study showed high multidrug resistance with Salmonella spp having the highest multidrug resistance (68.8%) to the antibiotics analysed. MDR patterns have been reported in Gram negative rods such as Klebsiella spp., Enterobacter spp., *E. coli* and Pseudomonas spp. (Bolaji *et al.,* 2011; Moges *et al.,* 2014). In this study, fifty (50 %) of the pathogenic bacterial isolates showed multiple antibiotic resistance index (> 0.2). The high MARI values obtained in this study may suggest the exposure of the isolates to antibiotics pressure thus furthering the development of multidrug resistance in the Environment (Adekunle *et al*., 2011, Fair and Tor, 2014).

CONCLUSION

Hospital wastewaters contain bacterial pathogens that are hemolytic in nature. Further, their increased antibiotic resistance are of great threat to public health because of dissemination of antibiotics resistant genes. A call is made therefore, for proper regulatory monitoring and treatment of hospital wastewaters before disposal in order to safeguard our health and sustain the Environment

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