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The Assessment of Antibiotic Resistant Bacteria in Pharmaceutical Effluents from Major Pharmaceutical Companies in Accra, Ghana

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ABSTRACT

Antibiotic resistance is a threat to global health. The study isolated clinically relevant Enterobacteriaceae bacteria strains from pharmaceutical effluent samples coded as EC, KP and EPRC facilities (company names not to be disclosed) *and assessed their antibiotic resistance profile. The study compared the sensitivity of MALDI-TOF MS and API 20E in identification of bacteria isolated and evaluated the presence of β-lactam resistance. The methods used are the pour plate, bacteria isolation, gram-staining, identification by MALDI-TOF MS and API 20E, antibiotic susceptibility testing, and Extended Spectrum β-Lactamase. The results of the total viable count and total coliform count (cfu/ml) of pharmaceutical effluent samples were $>10^6$. The Enterobacteriaceae isolates tested for antibiotic sensitivity showed 50%, 66.67%, 100%, 66.67%, 83.33%, 83.33%, 91.66%, 66.67%, 75% and 58.33% resistance to Cefepime, Cefoxitin, Ciprofloxacin, Cefotaxime, Cefuroxime, Tetracycline, Sulfamethoxazole, Norfloxacin, Ampicillin and Chloramphenicol, respectively. Ceftazidime, Gentamycin, Amikacin and Meropenem were sensitive up to 75%, 66.67%, 75% and 50% of bacteria isolates, respectively. Multiple antibiotic resistance from 3 up to 12 antibiotics occurred in 100% of isolates. E. cloacae isolates were resistant to 9-11 out of a total of 14 antibiotics tested and sensitive to only 2-5 of the antibiotics. Klebsiella spp. isolate was resistant to 4 antibiotics and sensitive to 9 antibiotics. C. freundii was resistant to 12 (85.71%) and sensitive to 2 antibiotics. K. pneumoniae strains were resistant to 9-10 antibiotics and sensitive to 3 antibiotics. A. hydrophila and A. caviae isolates were resistant to 12 antibiotics and sensitive to 2 antibiotics. P. otitidis isolate was resistant to 5 antibiotics and sensitive to 6 antibiotics. The results of the study reveal that pharmaceutical industrial effluents are flashpoints for antibiotic resistant bacteria. To reduce or prevent pharmaceutical industrial activity effect on antibiotic resistance in the environment, a multi-part one-health approach, multi-sectorial commitment, and national action plan are recommended.*

Keywords: *Pharmaceutical Effluents, Bacteria isolates, Resistance, Antibiotic, Gram-negative.*

INTRODUCTION

In Africa, antibiotics are among the most prescribed medicines. Pharmaceuticals play a major role in modern medicine. They are now indispensable tools to diagnose, cure, treat, and prevent diseases in humans (Wess, Schmidt and Höger, 2020). Pharmaceuticals production sites are known to be pollution hotspots for specialized pharmaceuticals and therefore pose an environmental risk as pharmaceutical residues can be released into the environment through their life cycle and cause adverse effects on human health, animal health, and environmental health, as well as antibiotic resistance (Wess, Schmidt and Höger, 2020), as several antibiotic resistance occurrences have been reported. The fact is that these pharmaceuticals place the environment at high risk when they are released into freshwater such as surface water and groundwater and even terrestrial ecosystems (Jírová et al., 2018). Effluents, liquid, and solid discharges from pharmaceutical industries threaten the environment and public health and are now a central concern of all stakeholders, regulators in the health system and environment protection agencies (Kraupner et al., 2021). There is a need to reduce pharmaceutical pollution of the environment and the problem of antibiotic resistance (Kotwani, Joshi and Kaloni, 2021). The management of effluents presents a major problem in Accra, the whole Ghana, and many developing countries in general (Kodom, Attiogbe and Kuranchie, 2021). The pharmaceutical manufacturing industries lack the initiative to reduce environmental toxicities and antibiotic resistance (Marselle et al., 2021), in their effluents. Pharmaceutical discharges have again and again been shown to provide conditions where antibiotics reach concentrations which can be selective for resistance enrichment. According to the European Commission (2019), quite a few antimicrobials had been discovered in soil and water and their presence may additionally play a role in accelerating the improvement, conservation and spread of resistant bacteria and fungi. As a result, antimicrobial resistance has become a matter of global health concern (Kotwani, Joshi and Kaloni, 2021). The emergence of antibiotic-resistant microorganisms and antibiotic-resistant genes within the wastewater environment has turned out to be a critical environmental fitness trouble. The pharmaceutical industry has been reported to be a major influencer on the environment due to growth in the industry globally to satisfy the ever-growing demand for antibiotics and healthcare, especially in low- and middle-earnings countries (Kotwani, Joshi and Kaloni, 2021). Antibiotic resistance is an extreme global threat inflicting about seven hundred thousand deaths a year and is projected to increase to 10 million deaths every 12 months by the year 2050 (May, 2021). The presence of antibiotic pharmaceutical residues in effluents may additionally play a crucial function in accelerating the improvement, renovation and spread of resistant bacteria and fungi (Kraupner et al., 2021). The absence of in-situ treatment processes in most pharmaceutical manufacturing facilities within Accra, Ghana, is principal for the negligent disposal of pharmaceutical effluents into the environment and contributing to unprecedented antibiotic contamination inside the environment (Mackull'ak et al., 2021). These wastes are mostly channeled directly into the rivers which contribute to our fresh and underground water systems. The discharged effluents contain a mixture of substantial concentrations of chemicals,

antibiotic resistance genes and antibiotics of different kinds (Ulvi, Aydın and Aydın, 2022). Some pharmaceutical companies, within Accra, Ghana, and other developing countries, often discharge pharmaceutical effluents into the environment without any treatment and knowing very well the relationship among the pharmaceutical enterprise, surroundings, human beings, and animals (Kotwani, Joshi and Kaloni, 2021). In contrast, the Western nations, where effluents regularly go to municipal wastewater treatment plants, with or without pretreatment, or are treated by the pharmaceutical industry wastewater treatment plants. Pharmaceutical manufacturing facilities, therefore, stand out as potential sites harboring high antibiotic resistant genes which consequently results in prolonged illness, as well as increased burden on Ghana's health systems and insurance schemes. Prolonged ill-health may result in loss of working hours and productivity and hence loss of income to the family and the nation. Likewise, there is a rising threats from antibiotic resistance on public health as antibiotic resistance could lead to the end of modern medicine (Aus der Beek et al., 2016). Antibiotic resistance presents an escalating global crisis, causing a substantial number of deaths annually and jeopardizing the effectiveness of modern medicine.

According to Kotwani, Joshi and Kaloni (2021), the trouble of antibiotic resistance is expected to push approximately twenty-four million humans into intense poverty by using 2030, globally and affords an excessive risk to the sustainable development goals (SDGs) two (zero hunger), three (good fitness and nicely-being), six (clean water and sanitation), nine (industrial innovation and infrastructure), twelve (accountable production and consumption), and partnership for the goals if not addressed immediately. In Ghana, there is low enforcement of interventions such as a country wide coverage on antimicrobial use and resistance. As a result, law enforcement tools specifically aimed at limiting antibiotics in pharmaceutical effluents, increase good manufacturing practice (GMP) compliance and other regulations on antibiotic resistance are facing several challenges. Data on specific environments which are likely sources for resistant microorganism are vital (Larsson and Flach, 2022). The assessment of pharmaceutical effluents for antibiotic resistant bacteria is fundamental to understanding gaps. It is vital to identify high-risk environments that cause the evolution of resistance and spread of antibiotic resistance around the world, should international mitigation techniques be made effective.

MATERIALS AND METHODS

Study Area

This study was conducted within the Greater Accra metropolitan assembly of Ghana. Environmental pharmaceutical effluent samples were collected from three (3) major pharmaceutical industries within Accra and coded as EPRC, EC and KP, (and names are not to be disclosed), for all microbial analysis.

Sampling Procedure and Processing

Three effluent samples from different sampling points were collected from three (3) pharmaceutical manufacturing companies within Accra (Nine samples in all) for all analyses. The samples were coded based on the pharmaceutical industry. Samples were kept in sterilized bottles and containers and transported in the cold chain using an ice chest to the Noguchi Memorial Institute for Medical Research (NMIMR), Advanced Research Laboratories (ARL) at the Bacteriology Department for all analysis.

Media Preparation Procedures
Brilliance *E. coli*/coliform selective agar (Oxoid CM1046) and Plate Count Agar (PCA; Oxoid, UK) were prepared by following the manufacturer's instructions.

Isolation of Bacteria Species from Environmental Samples

Pour Plate Method (Standard Plate Count Procedure)
The quantitative analysis involved the enumeration of bacteria using the pour plate method. A ten-fold dilution series (10^{-1} to 10^{-5}) of the pharmaceutical effluent samples were prepared by adding 1 ml to 9 ml of sterile Buffered Saline Peptone Water (BSPW; Oxoid) to obtain a 10ml volume of each dilution. Prepared Plate Count Agar (PCA; Oxoid CM0325, ISO, UK) and Brilliance *E. coli*/coliform selective agar (Oxoid CM1046) were inoculated into appropriately labelled Petri dishes with 1 ml of 10^{-1} to 10^{-5} dilutions in duplicates (APHA, 2017). The plates were swirled and incubated at 37ºC for 48 hours. After incubation, colonies observed on the plates, were counted.

Microbial detection by streaked plate method
Pharmaceutical effluent samples were vortexed and one (1ml) each was used to streak on Brilliance *E. coli*/coliform selective agar (Oxoid CM1046) and incubated at 37ºC overnight. After overnight incubation, bacteria growth observed were pink in color on the brilliance medium, which were typical of coliforms. The different colonies were sub-cultured to get pure colonies.

Isolation of Specific Pathogens
The different colonies of the pure culture were inoculated on Chromogenic Coliform Agar (UriSelect 4, France) and incubated at 37ºC for 24 hours. Individual colonies were sub-cultured on nutrient agar. These pure colonies from the nutrient agar were subjected to Gram stain, biochemical (API 20E test) and MALDI-TOF MS identification.

Gram Stain Procedure
The individual pure colonies from the nutrient agar were picked using a sterile loop to make a smear on a microscope slide. The smears were air-dried and heat-fixed, with moderate heat using a Bunsen burner. The heat-fixed smears of cells were flooded for one minute with crystal violet staining reagent. The slides were washed in a gentle and indirect stream of tap water for two seconds. The slides were flooded with iodine for one minute. The slides were washed in a gentle and indirect stream of tap water for two seconds. This was followed by flooding the slides with a decolorizing agent (95% ethanol/acetone) for fifteen seconds. Following this, the slides were fully covered with a counterstain, safranin for 30 seconds to 1 minute. The slides were then washed in a gentle and oblique movement of tap water till no coloration appears inside the discharge and then blot dry with absorbent paper. Finally, the outcomes of the staining technique were observed with the x100 lens and under oil immersion. At the completion of the Gram stain, gram-negative bacteria-stained pink/red and Gram-positive bacteria retained the purple color. On the same slides as the test culture, a sample with a known Gram stain reaction was added to serve as a control for success in the Gram stain technique. *Staphylococcus aureus* (ATCC 6538) was used as positive control and *E. coli* (ATCC 8739) was used as negative control. All the colonies stained were pink/red in color indicative of gram-negative organisms.

Confirmatory/secondary identification of bacteria isolates

Identification by MALDI-TOF MSP

A confirmatory or secondary identification of the bacteria species was performed using MALDITOF MS (Bruker Daltonik GmbH, Leipzig, Germany). Here, fresh cultures of pure colonies from the overnight culture on the nutrient agar were used. The organisms that have been stored at 4°C or lower were not used because it has a negative impact on the quality of spectra and reproducibility. Prior to beginning the procedure, it was noted on a sample key spreadsheet the position of the well each specimen will be placed on the 96-spot reusable stainless steel target plate. Briefly, from the pure culture plate, the surface of the colonies to be identified were touched with a sterile loop to make a smear on the 96-spot reusable stainless steel target plate and air–dried for two (2) minutes. The resulting smears were overlaid with 1 µl of 70% formic acid and air-dried for another two (2) minutes.

Following this, they were overlaid again with 1µl of matrix solution (containing cyano-4- hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) and allowed to air dry for two minutes. The steel target plate was placed in the high vacuum source chamber of the Microflex LT benchtop instrument, operated by the FlexControl software (Bruker Daltonik GmbH, Leipzig, Germany). As an internal control, a 0.5L Bacterial Test Standard (Bruker Daltonics Inc Billerica, MA) was used. The spots were subjected to MALDI-TOF analysis in a MALDI-ToF MS assay on the Bruker Biotyper platform. The resulting spectra were interpreted using the Bruker database. The results were saved and printed out. Protein identities were assigned using a CSU-PMF internal library, where a 70% match of total mass-spectral peaks (score of >2.0) identified isolates (Haberecht et al., 2019). To interpret the results, at least one of the duplicates for each control strain must generate a score of ≥ 2 with a correct identification (no conflicting results between the duplicates). When the control strains generate scores < 2 or incorrect identification, the results were not interpretable, and the extraction/submission process was repeated. If no species identification is made, the extraction/submission process was repeated using a new subculture. If no identification was successful after the second submission, the isolate was further identified by the API 20E test procedure.

API 20E Test procedures

API **(**Analytical Profile Index) 20E is a biochemical test used to identify most gram-negative bacteria, especially the Enterobacteriaceae using the API 20E. It is made up of many sugars sealed in wells. Prepared inoculums are dispensed into the wells and incubated at 37^{o}C for 18-24 hours. The wells are read by following the manufacturer's instructions and compare with a standard chart to indicate positive and negative (presence or absence) of the bacteria type suspected. A secondary identification was performed using pure colonies of bacteria species on Nutrient Agar using the API (Analytical Profile Index) 20E procedures. A single isolated colony from pure cultures was picked using sterile inoculating loops to make a suspension of the pure culture in sterile saline water. Strips of API 20E biochemical test kit which contains dehydrated bacterial media and biochemical reagents in twenty (20) distinct compartments were used to confirm positive and negative isolates for the suspected bacteria species.

The compartments of the various strips were filled (some compartments only filled up to the brim) with bacterial suspensions using sterile pipette. Sterile oil was added into the various compartments with ADH

(decarboxylation of the amino acid arginine by arginine dihydrolase), LDC (decarboxylation of the amino acid lysine by lysine decarboxylase), ODC (decarboxylation of the amino acid ornithine by ornithine decarboxylase), H_2S (production of hydrogen sulfide and URE (test for the enzyme urease). A few drops of distilled water were put in tray and the API test strips put into the trays and closed. The strays were marked with the date, initials, identification numbers, and organism IDs. The trays were then incubated at 37ºC for 24 hours. Some of the color changes of the compartments were read immediately after the 24 hours of incubation of the strips.

One drop of Ferric chloride was added to the TDA (Tryptophan deaminase) compartments, one drop of Kovacs reagent was added to the IND (Indole Test-production of indole from tryptophan by the enzyme tryptophanase) compartments, and to VP (the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway) compartments, one drop of 40 % KOH (VP reagent 1) and one drop of VP Reagent 2 (α-Naphthol) were added to specific compartments. The API color chart (reading scale) was used to determine each test as positive or negative on the Biomerieux labelled paper according to the tray readings. The wells are marked or divided off into triplets, with scores allocated to each well. The scores are added up for the positive wells only in each triplet. Three test reactions are added together at a time to give a seven (7) digit number, which is then looked up in the codebook. The highest score possible for a triplet is seven (7), the sum of 1, 2 and 4 and the lowest being zero (0). Finally, each of the organisms were identified by API catalogue (book) using seven (7) digit number and organism codes.

Antibiotic Susceptibility Testing (AST)

To test antibiotic resistance of selected isolates, various commercially prepared antibiotic discs (Oxoid, UK) such as Tetracycline (TE), Amikacin (AK30), Norfloxacine (NOR) Meropenem (MEM10), Gentamicin (CN), Cefoxitin (FOX), Cefotaxime (CTX30), Ciprofloxacin (CIP5), Ceftazidime (CAZ30), Ampicillin (AMP10), Cefuroxime (CXM30), Chloramphenicol (C30), Cefepime (FEP) and Sulfamethoxazole (SXT1.25/23.75) were used. The antibiotics were chosen based on the Clinical and Laboratory Standards Institute (CLSI) guidelines for antibiotic susceptibility testing (AST) of *Enterobacteriaceae* CLSI M100, 2022 and also because they are frequently used in treatment of Gram-negative infections in human and veterinary medicine in Ghana. Disk diffusion method was employed. The first step in this process was to prepare and pour Mueller-Hinton Agar (Oxoid, UK) into 150 mm sterile Petri dishes (at a depth of 6-8 mm). The nephrometer BD analyzer (Becton, Dickinson & Co., USA) was calibrated with calibration standards (BD Phoenix SpecAP calibrator kit) in the range of 0.1, 0.4, 0.5 and 1 McFarland standards. Following, a blank saline solution was measured to ensure the equipment is working appropriately. Five millimetres of 0.85% sterile saline solution was inoculated with bacteria colonies (between 3 to 5) from overnight cultures on Nutrient Agar (Oxoid, UK) plate with a sterile inoculation loop to attain a 0.5 McFarland suspension. The suspension was vortexed and measured with the nephrometer. Where necessary, the suspension was diluted appropriately with sterile saline solution until a 0.5 McFarland standard was reached. To increase the reliability of the results, a 0.5 McFarland suspension of the positive control organisms was prepared. The suspensions were used within ten minutes of

preparation. The dried surface of a MH agar plate was inoculated with the bacterial suspension on the surface of the MHA with a with a plate inculator (Inculator Retro C80, Biomérieux, SA, France) set to moderate speed, from the edges to the centre. Extra suspension was squeezed from the swab by rotating cotton swab against the side of the tube before seeding the plates. The inoculums were allowed to dry for three to five minutes but no more than 10 minutes, for the surface of the agar plate to dry before proceeding to the next step. A total of fourteen antibiotic discs were placed on the seeded agar plates at 24 mm using flamed forceps. The plates were placed on an antibiotic disk template to ensure antibiotic disks were suitably positioned. The disks were gently pressed down to ensure contact. After placing the disks on the MH plates, the plates were inverted and incubated at 37°C for 12 to 24 hours. Two negative control plates; one inoculated with bacteria species by spreading method and the other not inoculated. The rest of the plates were incubated alongside the test plates. After the overnight incubation, the zone of inhibition in diameters (including the 6 mm disk) were measured using a ruler on the undersurface of the Petri dish. The diameter of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disk. A reading of 6 mm indicates no zone of inhibition. The zones of inhibition diameters were recorded and interpreted as Resistant (R), Susceptible (S), Intermediate (I) and Susceptible Dose Dependent (SDD) using the breakpoint tables for interpretation of minimum inhibitory concentrations (MICs). The zone diameters measured on plates were characterized using CLSI M100, 2022 document for *Enterobacteriaceae*. This procedure was applied to all tested bacteria isolates identified by the MALDI-ToF MSP Bruker biotyper secondary identification method.

Data analysis

All data were analyzed using Microsoft excel 2016 (Microsoft office 2016 suite).

RESULTS

Quantitative enumeration of bacteria using the pour plate method

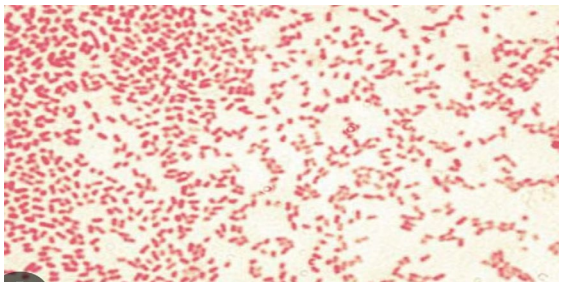
The result of the enumeration of the bacteria colonies for the total viable count and total coliform count (cfu/ml) of pharmaceutical effluent samples were $>10^6$ (too numerous to count) at a ten-fold dilution series (10^{-1} to 10^{-5}) and incubation at 37ºC for 24-48 hours. However, there were no colonies for total coliform count for EC2, EC3 and EPRC1 culture plates as indicated in Table 1.

Table 1**:** Enumeration of bacterial load in pharmaceutical effluents samples

Sample ID	Total viable count (cfu/ml) 37°C /24-48 hours	Total coliform count (cfu/ml) 37°C/24-48hours
EC1	$>10^6$	$>10^6$
EC2	$>10^6$	0
EC3	$>10^6$	0
KP1	$>10^6$	$>10^6$
KP2	$>10^6$	$>10^6$
KP3	$>10^6$	$>10^6$
EPRC1	$>10^6$	0
EPRC2	$>10^6$	$>10^6$
EPRC3	$>10^6$	$>10^6$

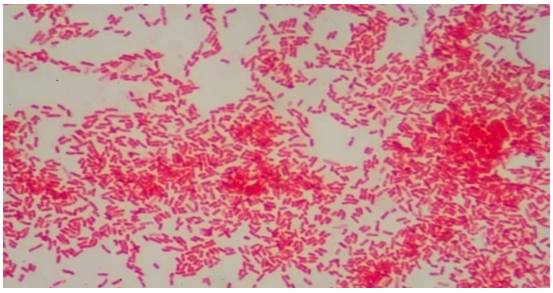
Figure 1: Isolation of pure cultures by streak plate method

Gram stain



Gram stain image 1

The results of the staining observed with the x100 lens and under oil immersion showed bacteria-stained pink/red in colour indicative of gram-negative organisms as showed in Figure 2.



Gram stain image 2

Figure 2**:** Gram-negative bacteria observed with x100 lens and under oil immersion

API 20E Identification of bacteria.

The results of the bacteria species identified by the API 20E identification method for the bacteria isolated from pharmaceutical effluents are shown in Table 2.

Table 2: API 20E interpretation of biochemical test

Organism	Test ID	% Ident	T	Description
Enterobacter sakazaki	3345763	99.8	0.98	Excellent identification
Enterobacter amnigenus 1	2301000	88.3	0.86	Excellent identification
Enterobacter spp	7305573	91.3	0.67	Excellent identification
Salmonella spp.	6331201	88.8	0.68	Excellent identification
Enterobacter spp.	3235573	89.3	0.86	Excellent identification
Enterobacter cloacae	3205573	88.3	0.86	Excellent identification
Pseudomonas fluoresce/putida	22010000	42.1	0.67	Identification acceptable
Enterobacter spp.	7305573	88.6	0.70	Excellent identification
Pseudomonas aeruginosa	2213000	98.1	0.67	Identification acceptable
Salmonella spp.	6301001	99.8	0.70	Excellent identification
Klebsiella.pneum. pneumoniae	5215773	97.8	1	Excellent identification
Flavi oryzihabitans	0001000	87.7	0.83	Identification acceptable
Pseudomonas fluoresce	2203000	74.3	0.83	Excellent identification

Comparison of MALDI-TOF MSP Biotyper and API 20E methods in identification of bacteria isolates.

The results for the secondary identification of bacteria species using the MALDI-TOF MS and API 20E from collected pharmaceutical effluents showed that both methods were sensitive enough to identify the isolates. The MALDI-TOF MS is fast, simple, user friendly and inexpensive technique. However, the API method identified 100% of the isolates to MALDI-TOF MS which identified 93% of the isolates to the genus and species level. Even though there were six isolates which were not in agreement between the two methods, there were generally comparable in terms of sensitivity as indicated below in Table 3.

Table 3: Comparative identification of Enterobacteriaceae by MALDI-TOF MS and API 20E.

Sample ID	API 20E Bacterial Isolate IDs	MALDI-TOF MSP Bacterial Isolate IDs
EC1	*Enterobacter sakazaki*	*Enterobacter cloacae*
	Enterobacter amnigenus 1	No ID
	Enterobacter spp	*Enterobacter cloacae*
KP1	*Salmonella spp.*	*Enterobacter/Klebsiella spp.*
	Enterobacter spp.	*Enterobacter cloacae*
KP2	*Enterobacter cloacae*	*Enterobacter cloacae*
	Pseudomonas fluoresce/putida	*Citrobacter freundii*
	Enterobacter spp.	*Enterobacter cloacae*
KP3	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
	Salmonella spp.	*Klebsiella pneumoniae*
EPRC2	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
EPRC3	*Flavi oryzihabitans*	*Aeromonas hydrophila/caviae*
	Pseudomonas fluoresce	*Pseudomonas otitidis*

Comparison of isolates identification in effluents by MALDI-TOF MSP and API 20E

In comparing the total number of isolates (bacteria species) identified in EC and KP pharmaceutical effluent samples by the API and MALDI-ToF MSP methods, the API method, in terms of the number of bacteria species, identified three (3), seven (7) and three (3) different bacteria species from EC, KP and EPRC effluents samples respectively, while the MALDI-ToF MSP method identified one (1), six (6) and three (3) bacteria species from EC, KP and EPRC effluent samples, respectively as shown in Figure 3. The results were comparable for the two methods used in the secondary identification of isolated bacteria from pharmaceutical effluent.

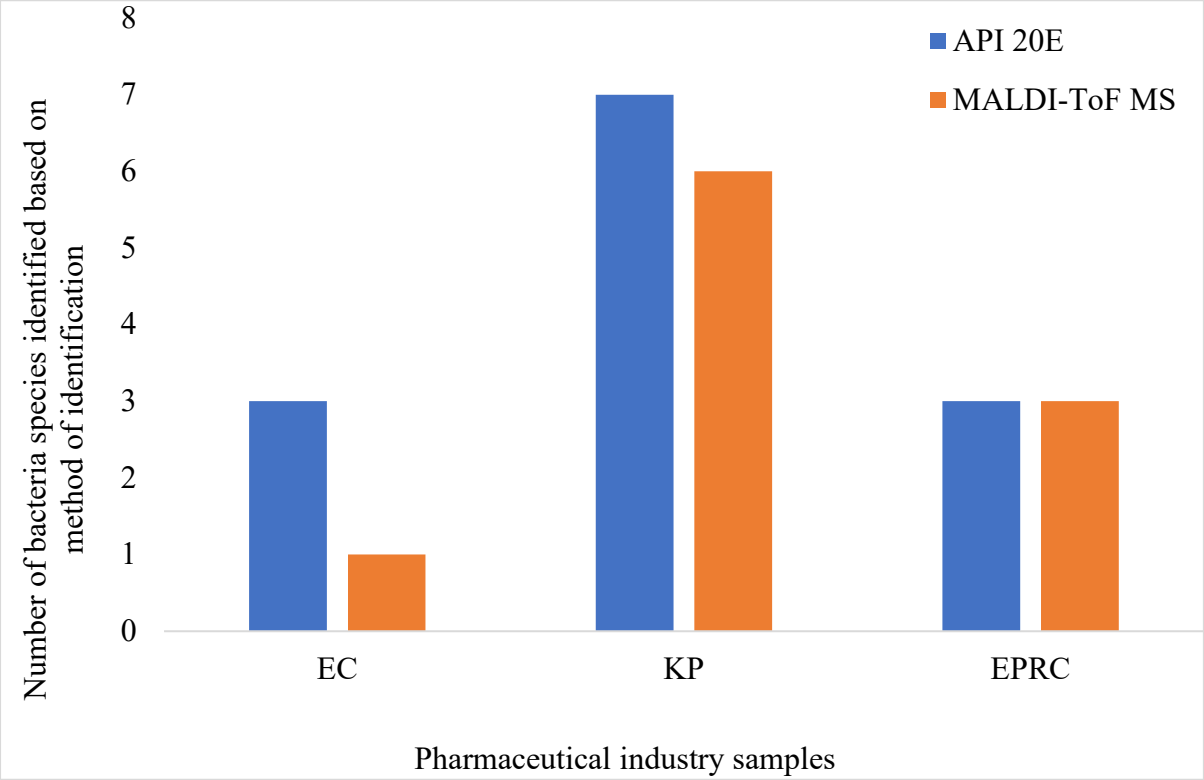


Figure 3: Comparison of strains identification by MALDI-ToF MS and API 20E

Quantitative comparison of isolates based on pharmaceutical industry

Enterobacter cloacae were identified more in KP pharmaceutical effluent samples compared to EC and EPRC effluents samples. However, EC samples identified. **A**s *Enterobacter cloacae* was higher than

EPRC sample isolates. All isolates from the pharmaceutical effluents identified by both API 20E and MALDI-ToF Biotyper MSP standard methods, were clinically relevant as presented in Figure 4.

Antibiotic susceptibility testing

The results of the antibiotic susceptibility test for bacteria isolates identified by MALDI Biotyper MSP from the pharmaceutical industrial effluents revealed multiple resistance to antibiotics tested (Table 4). The same bacteria isolate from different sampling points and pharmaceutical industry, showed different antibiotic resistant profiles. This could be

due to differences in resistant patterns. Identification at the genus and species level by 16S rRNA sequencing methods may decipher the differences. All AST results measured on plates were characterized using CLSI M100, 2022 document for *Enterobacteriaceae*.

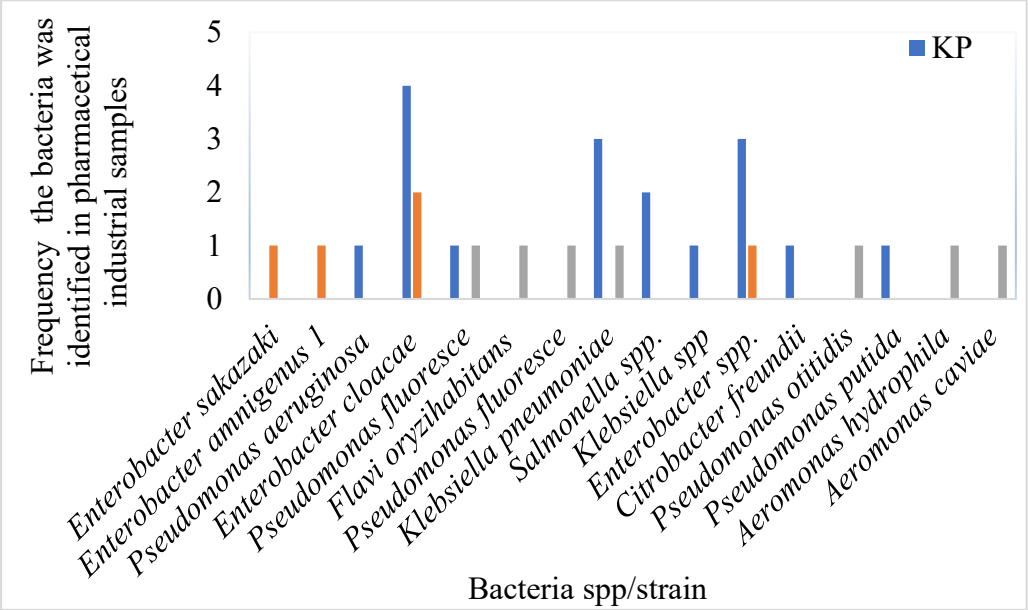


Figure 4**:** Bacteria spp/strains identified based on pharmaceutical industry

Table 4: Antibiotic susceptibility testing of organisms identified by MALDI Biotyper MSP

Sample ID	Bacterial Isolate IDs	Antibiotic Resistance													
		TE	AK	NOR	MEM	CN	FOX	CTX	CIP	CAZ	AMP	CXM	C	FEP	SXT
EC1	*Enterobacter cloacae*	S	S	R	S	S	R	R	R	S	R	R	R	R	R
	Enterobacter cloacae	R	S	R	R	S	R	R	R	I	R	R	R	SDD	R
	Enterobacter cloacae	R	R	R	R	R	S	R	R	S	R	R	S	R	R
	Enterobacter cloacae	R	R	R	R	R	S	R	R	R	S	R	S	S	R
KP1	*Enterobacter/Klebsiella spp.*	R	S	R	S	S	S	S	R	S	S	I	S	S	R
	Enterobacter cloacae	R	S	R	S	S	S	S	R	S	S	R	S	-	R
KP2	*Enterobacter cloacae*	R	S	I	S	S	R	S	R	S	R	S	R	S	R
	Citrobacter freundii	R	R	R	R	S	R	R	R	S	R	R	R	R	R
KP3	*Klebsiella pneumoniae*	R	S	S	I	R	R	R	R	S	R	R	I	R	R
	Klebsiella pneumoniae	R	S	I	R	S	R	R	R	S	R	R	R	R	R
EPRC3	*Aeromonas hydrophila/caviae*	R	S	R	S	R	R	R	R	R	R	R	R	R	R
	Pseudomonas otitidis	S	S	S	S	S	R	I	R	S	R	R	R	SDD	I

KEY: S: Susceptible, **I**: Intermediate, **R**: Resistant and **SDD**: Susceptible Dose Dependent, **ANTIBIOTICS: TE**: Tetracycline, **AK**: Amikacin, **NOR**: Norfloxacin, **MEM**: Meropenem, **CN**: Gentamicin, **FOX**: Cefoxitin, **CTX:** Cefotaxime, **CIP**: Ciprofloxacin, **CAZ**: Ceftazidime, **AMP**: Ampicillin, **CXM**: Cefuroxime, **C**: Chloramphenicol, **FEP**: Cefepime, **SXT**: Sulfamethoxazole.

Resistance profile of the strains identified by MALDI-ToF MSP to tested antibiotics

Figure 5 indicates the number of antibiotics that an isolate has developed resistance (R) and or is sensitive (S). It revealed that, *Enterobacter cloacae* isolated from most effluent samples was resistant to multiple antibiotics and only sensitive to a few of the tested antibiotics. It was showed from the data that isolates identified as *Citrobacter freundii, Klebsiella pneumoniae, Klebsiella Pneumoniae, Aeromonas hydrophila* and *Aeromonas caviae* were resistant to multiple antibiotics as high as about 10 to 12 of the 14 antibiotics examined. An isolate identified as *Enterobacter/Klebsiella spp*. from KP1 industrial site was sensitive to 9 out of the 14 antibiotics, resistant to 4, and intermediately resistant to 1 antibiotic.
The *Pseudomonas otitidis* isolate showed sensitivity to 6 antibiotics, resistant to 5, intermediately resistant to 1 and SDD to 1 antibiotic.

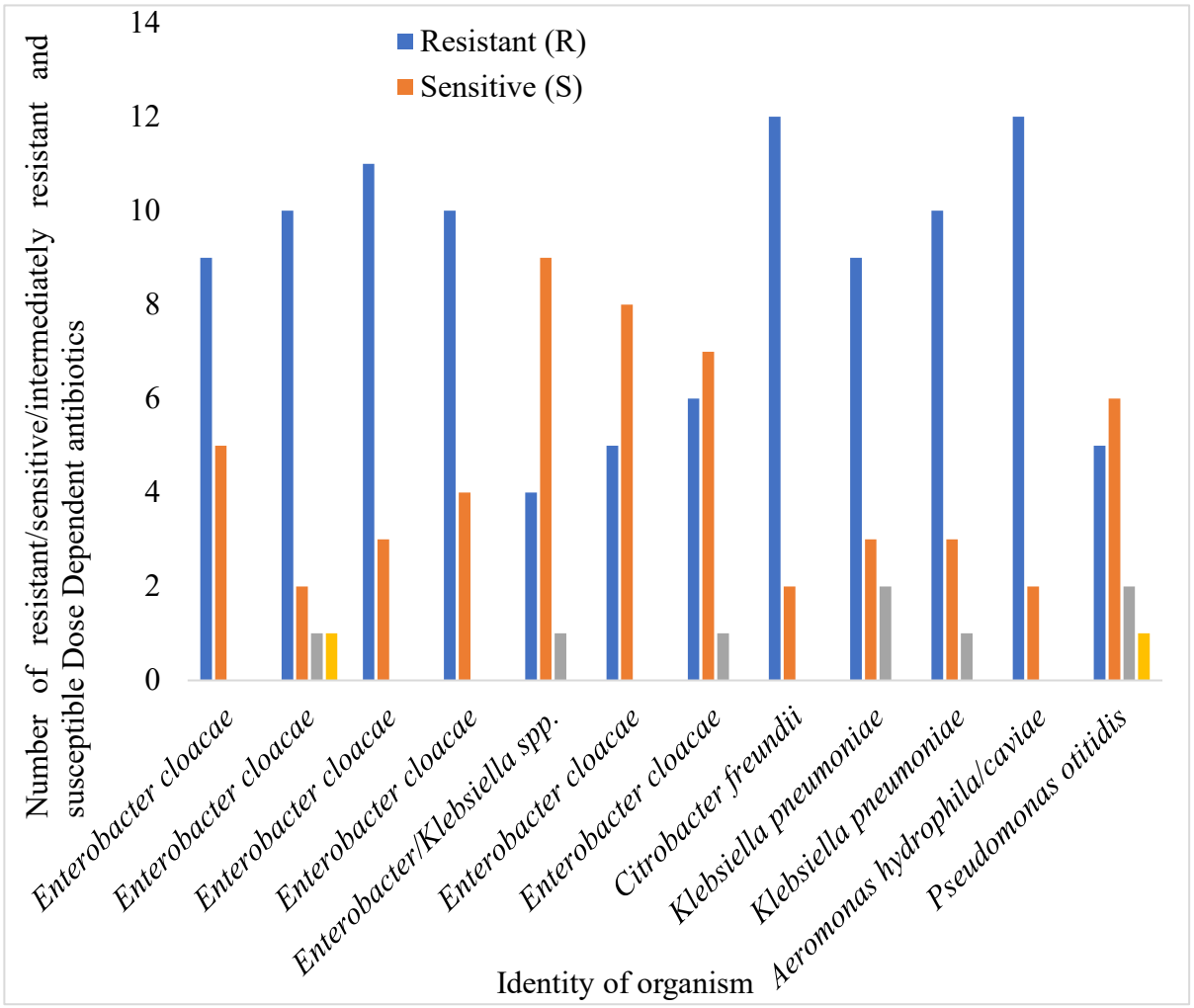


Figure 5: Resistance profile of the strains identified by MALDI-TOF MSP

Resistance distribution of bacteria strains among antibiotic classes

The bacteria isolates showed about 50% - 83% resistance to all cephalosporins antibiotics (Cefeprime (FEP), Cefoxitin (FOX), Cefotaxime (CTX), Ceftazidime (CAZ) and Cefuroxime (CXM)) except for Ceftazidime antibiotics which recorded 75% sensitivity to the isolates. The isolates were 83% resistant to Tetracycline. The Aminoglycocides, Amikan and Gentamycin were sensitive against 75% and 67% of the isolates, respectively. The Carbapenems (Meropenem) were sensitive against 50% of isolates. The percentage of isolates resistant to Sulfonamides (Sulfamethoxazoles) antibiotic were 92%. The resistance of isolates to Ciprofloxacin and Norfloxacine (Fluoro-quinolones) were 100% and 67%, respectively. The isolates acquired 75% resistance to the Penicillins (Ampicillin) and 58% resistance to Chlorophenicol antibiotics as showed in Figure 6.

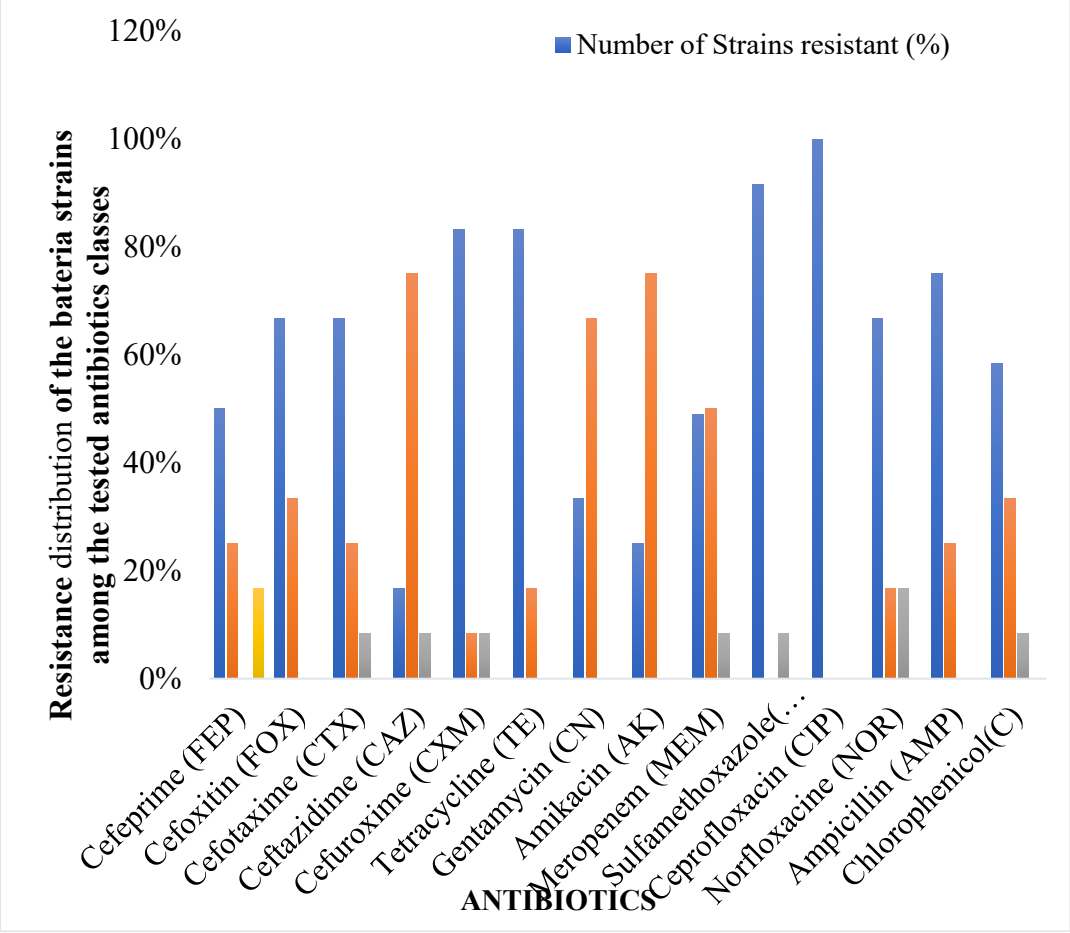


Figure 6: Resistance distribution of the isolated bacteria strains among antibiotic classes

DISCUSSION

There is an increasing problem that resistant bacteria can be generated in pharmaceutical manufacturing spots which can also journey into soil, ground, and surface waters with the release of pharmaceutical effluents into the environment without biological treatment. The presence of antibiotic resistant Enterobacteriaceae in the pharmaceutical effluent samples in this study point to the fact that pharmaceutical industries contribute greatly to emergence and spread of antibiotic resistance in the environment and have an influence on the increase in

global spread of antibiotic resistant genes (Kotwani, Joshi and Kaloni, 2021).

The situation of antibiotic pharmaceutical residues in effluents may additionally play a crucial function in accelerating the improvement, renovation and spread of resistant bacteria and fungi. The absence of in-situ treatment processes in most pharmaceutical manufacturing facilities within Accra, Ghana, is principal for the negligent disposal of pharmaceutical effluents into the environment and contributing to unprecedented antibiotic contamination inside the environment.
These wastes are mostly channelled directly into the rivers which contribute to our fresh and underground water systems. Pharmaceutical industrial effluents, therefore, stand out as potential sites harbouring high antibiotic resistant genes which consequently results in prolonged illness, as well as increased burden on Ghana's health systems and insurance schemes. Prolonged ill-health may result in loss of working hours and productivity and hence loss of income to the family and the nation.

From this study, *Enterobacter sakazaki, Enterobacter amnigenus* 1, *Pseudomonas aeruginosa, Enterobacter cloacae, Pseudomonas fluoresce, Flavi oryzihabitans, Pseudomonas fluoresce, Klebsiella pneumoniae, Salmonella spp, Klebsiella spp, Enterobacter spp., Citrobacter freundii, Pseudomonas otitidis, Pseudomonas putida, Aeromonas hydrophila* and *Aeromonas caviae* were found in pharmaceutical effluents as identified by MALDI-TOF, Biotyper MSP and API 20E methods. The API 20E and MALDI-TOF identification of bacteria species were both sensitive enough to identify the various isolates. However, there were some disagreements in isolate identity by the API 20E and MALDI-TOF secondary identification. Nonetheless, the identification of clinically relevant isolates of Enterobacteriaceae by MALDI-TOF MSP was fast and shortened the time required to identify an organism. Comparing API20E and MALDI-TOF MSP methods in identification of bacteria isolates showed that the MALDI-TOF MS protocol does not require more reagents like the API 20E and aligns with previous report by Jamal, Shahin and Rotimi (2013).

As presented (Figure 3), the API 20E method identified 3, 7 and 3 bacteria isolates from EC, KP and EPRC pharmaceutical effluent samples, while the MALDI-TOF MSP identified 1, 6 and 3 bacteria isolates from EC, KP and EPRC pharmaceutical effluent samples. In view of that, more organisms were identified by API 20E method than the MALDI-TOF MSP. The identification of clinically relevant isolates of Enterobacteriaceae by MALDI-TOF MSP was fast to identify an organism and therefore improves the clinical testing outcome (Cherkaoui et al., 2010).

The testing of twelve (12) Enterobacterales isolates revealed the following resistance patterns: 50% were resistant to Cefepime, 66.67% to Cefoxitin, 66.67% to Cefotaxime, 16.70% to Ceftazidime, 83.33% to Cefuroxime, and 75% were sensitive to Ceftazidime. Among the cephalosporins, Ceftazidime was the only one to show a higher sensitivity among the isolates. Additionally, 83.33% of the isolates were resistant to Tetracycline and 33.33% to Gentamicin, with 66.67% being sensitive to Gentamicin. Regarding Amikacin, 25% of the isolates were resistant, indicating that 75% were sensitive to it. For Meropenem, 49% of the isolates were resistant and 50% were sensitive. Sulfamethoxazole resistance was observed in 91.66% of the isolates, with 8.33% showing intermediate resistance. All isolates (100%) were resistant to Ciprofloxacin, while 66.67% were resistant to Norfloxacin, 16.70% were sensitive, and 16.70% exhibited intermediate resistance. Lastly, 75% of the isolates were resistant to Ampicillin, with 25% being sensitive, and 58.33% were resistant to Chloramphenicol, 33.33% were sensitive, and 8.33% showed intermediate resistance to Chloramphenicol as shown in Figure 6. These results agreed with previous report on antibiotic resistance of *Enterobacteriaceae* isolates by Karlowsky et al. (2023).

Generally, there was higher level of antibiotic resistance among the isolates except for Ceftazidime, Amikan and Gentamycin antibiotics which exhibited high sensitivity to the bacteria isolates from the pharmaceutical effluent samples analyzed. Resistance to more than two antibiotics and antibiotic classes was observed in all strains. Multiple antibiotic resistance from 3 up to 12 antibiotics occurred in 100% of the isolates (Figure 5). The higher incidence of multiple antibiotic resistance among the bacterial strains or isolates from the pharmaceutical effluents (EC, KP and EPRC industrial effluents) were in accordance with previous report by Marathe et al. (2013). Their report showed multiple drug resistant bacteria isolated from pharmaceutical wastewater treatment plants with about 80% of the strains resistant to more than 20 antibiotics (Marathe et al., 2013). This is highly comparable to the findings of this research. Moreover, multiple drug resistant bacteria have also been detected in final pharmaceutical effluents (Tahrani et al., 2018; Leu et al., 2018). The multiple antibiotic resistance occurrence among the bacteria isolates examined did not change the difference in the resistance profiles of these strains. The development of antibiotic-resistant bacteria in pharmaceutical effluents calls for an important environmental health issue.

Based on resistance to specific bacteria isolate, the *Enterobacter cloacae* isolated from EC pharmaceutical manufacturing facility was resistant to nine (9) to eleven (11) antibiotics and antibiotic classes (Cephalosporins, Tetracyclines, Aminoglycocides, Carbapenems, Sulfonamides, Fluoro-quinolones, Penicillins and Chlorophenicol) and sensitive to only 2 to 5 of the antibiotics.

The *Enterobacter/Klebsiella spp*. (KP1 sample) were resistant to 4 and sensitive 9 and 1 intermediate resistant antibiotics, the *Enterobacter cloacae* (from KP1 and KP2 samples) were resistant to 5-6 and sensitive to 8-7 antibiotics with 1 intermediate resistant antibiotic (KP2 sample).

Citrobacter freundii was resistant to 12 (85.71%) of the antibiotics, sensitive to only 2 out of the 14 antibiotics tested and conforms with previously report by Marathe et al. (2013), indicating that *Citrobacter freundii* from multiple bulk-drug manufacturing sites showed high resistance up to 21 out of 39 antibiotics tested. Also, the *Klebsiella pneumoniae*

isolates from KP3 pharmaceutical effluent samples were resistant to 9-10 antibiotics, sensitive to 3 antibiotics and 1 intermediate resistant antibiotic. Taking into consideration EPRC pharmaceutical effluent bacteria isolates, the *Aeromonas hydrophila* and *Aeromonas caviae* isolate were resistant to 12 antibiotics and sensitive to 2 antibiotics. *Pseudomonas otitidis* isolated from EPRC was also resistant to 5, sensitive to 6, 2 intermediate resistant and 1 susceptible dose dependent antibiotics and are supported by literature where Gyesi et al. (2022), also reported the presence of antibiotic resistant bacteria (*Enterobacter, Pseudomonas, and Klebsiella*) isolated from reservoirs of Owabi and Barekese Dams in Ghana.

All the strains in this study were found to be resistant to Cefepime, Cefoxitin, Cefotaxime, Cefuroxime, Tetracycline, Sulfamethoxazole, Ciprofloxacin, Norfloxacin, Ampicillin and Chloramphenicol antibiotics and comparable to clinical isolates of *Enterobacteriaceae* reported by Karlowsky et al. (2023). Moreover, the study is in accordance with Marathe et al. (2013), who reported that 77.42% and 97.85% of bacteria strains from pharmaceutical manufacturing sites were resistant to Ampicillin and Sulfamethoxazole, respectively. The findings of the antibiotic resistance profiles align with the report by Milakovi (2018), who indicated the presence of genes that confer resistance to sulfonamides, tetracyclines and ampicillin in a functional metagenomics analysis for resistome in bacteria isolates from pharmaceutical effluent samples. The antibiotic resistant bacteria from pharma effluents are a great threat to treatment of bacterial infections and a sign of non-compliance with Good Manufacturing Practices (GMP) by the pharmaceutical industries. The possible reasons for the higher resistance of the isolated bacteria could be due to lack of effective pharmaceutical wastewater treatment plants (PWWTPs) in the studied pharmaceutical industries. Also, continuous exposure of bacteria to sub-inhibitory concentrations or doses in the pharmaceutical effluents can lead to the formation of biofilm and cause resistance. For instance, *P. aeruginosa* strains exposed to inhibitory concentrations of sulfamethoxazole resulted in biofirm formation and antibiotic resistance due to the impact of this low-inhibitory doses on bacterial ecology, growth and replication that activates the selection of antibiotic resistant bacterial cells (Bruchmann, Kirchen and Schwartz, 2013). It has been reported by Gyesi et al. (2022), that the presence of antibiotic resistant isolates notably *Pseudomonas, Klebsiella* and *Enterobacter* in samples from reservoirs of Dams in Ghana is due to high concentrations of pharmaceutical residues that was detected in these dams. The isolation of antibiotic resistant *Pseudomonas, Klebsiella* and *Enterobacter* and other bacteria from this research greatly provide a link between biologically untreated pharmaceutical effluents released to the environment, pollution of surface waters as a result and the serious widespread of those organisms in the country.

CONCLUSION

The data from this study established the isolation of clinically relevant Enterobacteriaceae from pharmaceutical effluents and their resistance to the tested antibiotics. It was also found that the bacteria isolates were resistant to Cephalosporins, Tetracyclines, Aminoglycocides, Carbapenems, Sulfonamides, Fluoro-quinolones, Penicillins and Chlorophenicol antibiotics. Lastly, all the isolates found in this study

exhibited multi-drug resistance. The results from this study show that pharmaceutical industrial sites are hotspots for antibiotic resistance. To the best of our knowledge, this is the first study that directly demonstrates antibiotic resistance in pharmaceutical effluents from selected pharmaceutical industries in Accra through controlled experiments.

RECOMMENDATIONS

Further research should be conducted to access the concentrations of antibiotic residues in the pharmaceutical effluent which leads to rise in development of resistance to several antibiotic classes. Also, a multi-part "One Health" approach needs to be adopted by policymakers in the country to curb antibiotic resistance and ensure effectiveness in bacterial infections treatment. Thus, an urgent call for enforcement of mandatory treatment of the pharmaceutical effluent and their proper disposal to curb antibiotic contamination in the environment as their persistent presence in the environment significantly modulates the bacterial genomes' expression that is responsible for increase spread of resistance among antibiotic classes.

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