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Antibiogram and Detection of Metallo-Beta-Lactamase Producing *Klebsiella* Species Isolated from Poultry Feaces in Owo Metropolis with its Legal Critics.

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ABSTRACTS

Beta-lactamase enzymes e.g. metallo β-lactamases (MBLs) that produces Gram-negative bacteria is no doubt one of the widely spread resistance strategies which was geared by these group of bacteria towards β-lactam drugs. MBLs hydrolyzes bacteria and resist carbapenems. This study aimed at determining the antibiogram and the production of MBLs by *Klebsiella* species from poultry feaces in Owo metropolis. From Poultry feaces aseptically collected *Klebsiella* species was isolated using MacConkey agar (MAC) as well as Eosin Methylene Blue (EMB) Agar followed by conventionally characterization. Antimicrobial susceptibility test of the isolates were by disc diffusion method against ceftazidime (30µg), cefuroxime (30µg), gentamicin (10 µg), cefixime (5 µg), ofloxacin (5 µg), augmentin (30 µg), nitrofurantoin (300 µg) and ciprofloxacin (5 µg). The isolated *Klebsiella* species was screened for MBL production by determining their susceptibility to imipenem and this was confirmed using imipenem-EDTA combined disc test for phenotypic detection of MBLs. *Klebsiella* species isolated showed highest resistance to augmentin (97.9%) and least resistance to nitrofurantoin (18.8%). Out of the 48 *Klebsiella* species isolated from poultry feaces, 7 (14.6%) produced MBL. The production of MBLs by *Klebsiella* species in the community portends serious public health concern since these organisms are resistant to the carbapenems. Efficient detection and sustainable intervention protocol to control the emergence and spread of antibiotic resistant bacteria in the community are necessary to contain this dilemma; and there is need for poultry farmers to guide against the legal implications of raising birds with MBLs producing bacteria.

Keywords: *Klebsiella* species, Poultry feaces, Metallo-β-lactamases, Antibiogram, carbapenem.

INTRODUCTION

According to Mills *et al*., 2019, and Salehi *et al*., 2019, *Klebsiella* spp are present and available across the nature. They can be found in the atmosphere, surface water, soil, waste, even in plants and the mucosal surfaces of animals such as mammals, which they live. These are opportunistic pathogens that colonize such mucosal surfaces without causing pathology; however, *Klebsiella* can transmit from mucosae to some other tissues which may cause infections e.g. bronchopneumonia, urinary tract infections (UTIs) and bloodstream infections (Bengoechea *et al*., 2019). Infections that could be as a result of *Klebsiella* species may increase morbidity as well as dealth rate (Tian *et al*., 2016). Several earlier report since 1981indicated that *Klebsiella* strains were resistant to various generations of cephalosporins, most especially the third generation, since 1981, these bacteria have become resistant to antibiotics (Ripabelli *et al*., 2018).

The current sudden large increase in the emergence as well as the spread of microbial resistance among in the community has caused negative effects on some of the prominent modern discoveries of medicine. Antibiotics has saved large number of people from diseases and death jus as a result of infections, nevertheless, the discovery of modern medicine may likely be threatened because of microbial resistance that has crippled to hospitals and non-hospital environments. The present of carbapenem-resistant Gram-negative bacteria in the community as well as hospital environments constitute a serious development in the field of infectious and diseases management and control (Chika *et al*., 2014). This is a possible danger that portends with major public health implications because they jeopardize the clinical significance of potent antibiotics that treats chronic infections.

Metallo-β-lactamases (MBLs) are enzymes that belong to class B beta-lactamase of Ambler, they hydrolyze and confer on bacteria the exceptional ability to resist the antimicrobial action of the carbapenems for example imipenem and meropenem (Chika *et al*., 2016). As cofactors for enzyme activity they need divalent cations of zinc, therefore the activity of these enzymes are mostly inhibited in-vitro by chelating agents such as (EDTA). MBLs have active hydrolyzing property that is against both the carbapenems and other β-lactam antibiotics (Zurfluh *et al*., 2013). The rise in antibiotic resistance among

bacteria, especially *Klebsiella* species by the generation of β-lactamases has caused the use of carbapenem antibiotics to increase. However, strains of this organism are generally resistant to the carbapenems due to the fact that they produce MBLs.

However, Carbapenem-resistant *K. pneumoniae* exhibited resistance to a level of critically relevant antimicrobial categories which results to its therapeutic effects (Magiorakos *et al*., 2012). *K. pneumoniae* employs carbapenem resistance with the aid of enzymatic hydrolysis via carbapenemases enzymes, overexpression of the efflux pump system and loss of porin expression (Durante-Mangoni *et al*., 2019). The manace of carbapenems resistance among these bacteria are remarkably uncommon in most countries. However, many developing countries where access to quality healthcare is still poor are yet to report the said situation. The adoption of antibiotics in the commercial and substantial rearing and production of livestock and poultry birds as well as in other veterinary activities has contributed immensely to the emergence and spread of drug resistant bacteria in the community.

Public Health Concern: The emergence of Metallo-beta-lactamase producing Klebsiella species in poultry feces raises alarms about public health. These bacteria tends to communicate antibiotic resistance to other animals and humans, and this could make the infections difficult to treat. As a result, this may have implications on food safety and human health (Hasan *et al*., 2014).

Hence, this study is aim at determining the antibiogram and the production of MBL by *Klebsiella* species isolated from Poultry droppings in Owo metropolis.

MATERIALS AND METHODS

Sample Collection

Between January and February 2022, freshly passed poultry droppings of apparently healthy chickens were aseptically collected into appropriately labeled sterile capped universal bottles from Rufus Giwa Polytechnic, Owo (RUGIPO) poultry farm using sterile spatula. The samples were preserved in ice packs and transferred to Microbiology unit laboratory, Department of Science Laboratory Technology, RUGIPO for immediate bacteriological analyses.

Ethical approval and informed consents

No ethical approval was required; however, during the collection of samples; verbal permission was taken from the farm owners and farm workers as well

Isolation of *Klebsiella* species

1g of the poultry feacal samples was weighed into 10ml of de-ionized water to make a stock solution, after which serial dilution was carried out. 1ml each of the serial diluents (10^{-2}, 10^{-4} and 10^{-6}) was transferred into properlly labeled sterile Petri dishes. Aseptically EMB and MAC Agar already cooled to around 45^0C was separately transferred into the aliquots of samples in the petri dishes and was swirled gently, it allowed to solidified and it was incubated at about 35-37^0C for 24-48 hours (Egea *et al*., 2012). Distinct colonies were sub-cultured on freshly prepared MAC; repeated streaking was done to obtain pure culture of *Klebsiella species* prior to biochemical tests. *Klebsiella species* produce pink to purple colonies on EMB agar and pink/red colonies on MAC agar. All the suspected *Klebsiella species* isolates were thereby identified using standard microbiological techniques (Cheesbrough, 2005).

Maintenance of bacteria stock culture

Each of the isolated *Klebsiella species* were kept as stock cultures for neccessary studies on nutrient agar (Oxoid, UK) slants in Bijou bottles. This was done by streaking the isolates on the agar slants, and incubating at 35-37^0C for 18-24 hrs. After incubation, the inoculated slants were stored in the refrigerator at room temperature and these stock cultures was used as source of *Klebsiella species* isolates for further bacteriological studies.

Morphological Characterization of Isolates

A 24-hour old pure culture of the isolates was characterized and the different morphologies were recorded

Gram Staining

This was carried out according to the method described by (Becerra *et al*, 2016)

Biochemical Characterization of the Isolates

The isolates were further identified through a panel of biochemical tests which were carried out following standard procedure. The tests carried out include motility, catalase, oxidase, citrate, indole, Methyl Red, Voges-Proskauer and carbohydrate utilization which include glucose, lactose and maltose.

Preparation and Standardizing Inoculum Suspension

The inoculum suspension was prepared by picking 2-3 colonies of a 24-hour culture with a sterile wire loop and was suspended in 5M normal saline, the suspension was mixed with a vortex mixer. The turbidity of the suspension was standardized to match the 0.5 McFarland's standard which corresponds to approximately 1.5 x 108 cfu/ml and this was done by comparing the test suspension with barium sulphate suspension by placing the tubes in front of a white paper with black lines.

Antimicrobial susceptibility test of the *Klebsiella* species isolated

Antibiotic susceptibility test of the *Klebsiella* species was carried out against nine antimicrobial agents using the standard Kirby-Bauer disc diffusion method (CLSI, 2018). The antibiotics used include the following classes of antibiotics: beta-lactam combination agent (amoxicillin/clavulanate 20/10µg), cephem (cefotaxime 30 µg, ceftazidime 30 µg, cefixime 5 µg), carbapenem (imipenem 10 µg), aminoglycosides (gentamicin 10 µg), fluoroquinolone (ciprofloxacin 5 µg, ofloxacin 5 µg), and nitrofurans (nitrofurantoin 300 µg).The antibiotic disks were placed on Mueller Hinton agar plates inoculated with the standardized inoculum and incubated at $35^0C \pm 2^0C$ for 18-24 hours. The zones of inhibition were measured in millimeter and interpreted based on CLSI guidelines (CLSI, 2018). Antibiotic growth inhibition zone diameter data were compared with standard reference values in order to classify the isolates as sensitive, intermediate resistance or resistant to a particular antibiotic (CLSI, 2018). Isolates showing resistance to at least two different classes of antibiotics were considered as multidrug resistant strains (Magiorakos *et al*., 2012).

Isolates' MBL Production Screening

All isolates that were resistant to imipenem by recording a zone of inhibition diameter less than 23 mm were suspected of producing the enzyme metallo-β-lactamase (Aibinu *et al*., 2007). They were further subjected to confirmation test phenotypically.

Phenotypic Confirmation of MBL- Positive Isolates

The turbidity of a culture of the isolated *Klebsiella* species was adjusted to 0.5 MacFarland standards, then inoculated aseptically on freshly prepared Muller-Hinton agar plates. Later, antibiotic disk containing 10 µg imipenem and infused with 1 µg of EDTA as well as another imipenem disc free of EDTA were placed aseptically on the freshly inoculated agars. The dishes were incubated for 24 h at 30ºC, then inhibition zones were taken and interpreted using the criteria set by CLSI (2018). According to Chika *et al*. (2016), a variation of 7 mm or more recorded in the zones of inhibition between the imipenem disc infused with EDTA and disc without EDTA confirmed the phenotypic production of MBL.

Results

Identification of the Isolates

Table 1 showed that all the isolates were Gram-negative rods. They were motile, catalase, indole, citrate, Voges-Proskauer, glucose, lactose and maltose positive but were methyl-red and indole negative. They are mucoid, lactose-fermenting (pinkish) colonies on MAC agar; and large, mucoid, lactase-fermenting, non-green metallic sheen colonies on EMB agar.

Antibiotic susceptibility patterns of the isolated *Klebsiella* species from poultry feaces

Table 2 showed the varying levels of susceptibilities and resistances exhibited by the *Klebsiella* species isolated from poultry feaces to the test antibiotics. The *Klebsiella* species isolates from the poultry feaces showed highest resistance to augmentin (97.9%) and least resistance of 18.8% to nitrofurantoin

Distribution of MBL-producing *Klebsiella* species isolated from poultry feaces

Table 3 showed that from the 9 poultry feacal samples aseptically collected, 48 *Klebsiella* species were isolated. Out of 48 *Klebsiella* species isolated, 12 (25.0%) were suspected to be MBL-producers, 7 (14.6%) were confirmed to be actual producers of MBL while 41 (85.4%) were non-producers of MBL.

Antibiotype of the MBL-Producing *Klebsiella* species

Table 4 showed that three (42.8%) of the seven isolates that produced MBL resisted six classes of antibiotics (NIT-IMP-CPR-GEN-AUG-CAZ), another two (28.6%) resisted five classes (IMP-CPR-CRX-AUG-GEN) and two (28.6%) were resistant to four (CAZ-CPR-AUG-GEN) different classes of antibiotics, hence they were considered multidrug resistant.

Table 1 :. Gram reaction and biochemical characterization of the isolates from the poultry feaces

S/N	Isolates	Shape	Gram Reaction	MOT	CAT	CIT	IND	MR	VP	GLU	LAC	MAL	Morphological Appearance on Culture Media	Probable Organism
1-48	All	Rod	-	+	+	+	-	-	+	+	+	+	Mucoid Lactose-fermenting (pinkish) colonies on MAC; and large mucoid lactose-fermenting, non-green metallic sheen colonies on EMB	*Klebsiella* species

KEY: S/N = Serial number, + = Positive, - = Negative, MOT = Motility, CAT = Catalase, OXI = Oxidase, CIT = Citrate, IND = Indole, MR = Methyl red, VP = Voges-proskauer, GLU = Glucose, LAC = Lactose, MAL = Maltose, MAC = MacConkey Agar, EMB = Eosin Methylene Blue Agar

Table 2: Antibiotic susceptibility patterns of all the *Klebsiella* species (n = 48) isolated from poultry feaces

S/N	Antibiotics (µg)	No. (%) susceptibility	No. (%) resistance
1	AUG (30)	1 (2.1)	47 (97.9)
2	OFL (5)	8 (16.7)	40 (83.3)
3	CAZ (30)	9 (18.8)	39 (81.3)
4	CRX (30)	3 (6.3)	45 (93.8)
5	GEN (10)	33 (68.8)	15 (31.3)
6	CXM (5)	2 (4.2)	46 (95.8)
7	NIT (300)	39 (81.3)	9 (18.8)
8	CPR (5)	7 (14.6)	41 (85.4)
9	IMP (10)	36 (75.0)	12 (25.0)

KEY: S/N = Serial number, No. = Number, AUG = Augmentin, OFL = Ofloxacin, CAZ = Ceftazidime, CRX = Cefuroxime, GEN = Gentamisin, CXM = Cefixime, NIT = Nitrofurantoin, CPR = Ciprofloxacin, IMP = Imipenem

Table 3: Distribution of MBL-producing *Klebsiella* species isolated from poultry feaces

Bacteria	Sample	No. of samples	No. of Isolates screened	No of isolates suspected to produce MBL N (%)	MBL positive N (%)	MBL negative N (%)
***Klebsiella* species**	Poultry feaces	9	48	12 (25.0)	7 (14.6)	41 (85.4)

KEY: No. = Number, N = number of isolates, % = percentage, MBL = Metalllo-Beta-Lactamase

Table 4: Antibiotype of MBL-producing Klebsiella species isolated from Poultry feaces

Antibiotype	Classes of Antibiotics	Number (%) MBL+ve Klebsiella species
NIT-IMP-CPR-GEN-AUG-CAZ	6	3 (42.8)
IMP-CPR-CRX-AUG-GEN	5	2 (28.6)
CAZ-CPR-OFL- GEN-AUG	5	0 (0.0)
NIT-CPR-GEN-CXM-AUG	5	0 (0.0)
CRX-CPR-OFL-GEN-AUG	5	0 (0.0)
CXM-IMP-GEN-OFL	4	0 (0.0)
NIT-IMP-CRX-AUG	4	0 (0.0)
CAZ-CPR-AUG-GEN	4	2 (28.6)
CXM-CPR-OFX-AUG	4	0 (0.0)
CAZ-OFX-AUG	3	0 (0.0)
CXM-GEN-AUG	3	0 (0.0)
CAZ-AUG	2	0 (0.0)
CPR-AUG	2	0 (0.0)
TOTAL		

KEY: AUG = Augmentin, OFL = Ofloxacin, CAZ = Ceftazidime, CRX = Cefuroxime, GEN = Gentamisin, CXM = Cefixime, NIT = Nitrofurantoin, CPR = Ciprofloxacin, IMP = Imipenem

DISCUSSION

This study intended to determing the antibiogram and the generation of MBL by *Escherichia coli* isolates recovered from poultry feaces in Owo metropolis, Ondo State, Nigeria. *Klebsiella* species is a member of the Enterobacteriaceae family and they are commonly found in the gastrointestinal tract of warm blooded animals, from where they contaminate the environment through feacal contamination.

Antibiotic resistance that occurs in non-hospital environment is a public health threat that has risked the efficacy of some available antimicrobial agents and this phenomenon is partially due to the abuse of antibiotics for both human and non-human. In poultry, antibiotics are usually adopted for the treatment and control of bacterial related diseases. When these antibiotics are introduced to the birds over long period of time, usually at low levels, some species of bacteria become resistant (Kilonzo-Nthenge *et al*., 2008). More scientific researches show that these resistant bacteria, such as pathogens, can be transmitted to humans through foods (Van Looveren *et al*., 2001).

Klebsiella species is one of the most important Gram negative bacteria that results at several health problems due to its high resistance to some antibiotics. This organism is responsible for a large amount of both community-acquired and nosocomial infections in the globe. Most strains of *Klebsiella* are naturally multidrug resistant due production of enzymes e.g metallo beta-lactamase with the unique ability to hydrolyze and cause some broad-spectrum antibiotics like the carbapenems inefficacious (Chika *et al*., 2014; Walsh *et al*., 2005). The production of metallo beta-lactamases (MBLs) in Gram negative bacteria most especially the *Enterobacteriaceae* family such as *Escherichia coli* and *Klebsiella* species are responsible for the multidrug resistant nature of these organisms. Metallo beta-lactamases (MBLs) are majorly plasmid-borne, and mediate bacterial resistance to the carbapenems such as imipenem– that are clinically administered to treat multidrug resistance infections and those caused by extended spectrum beta-lactamase (ESBL)-producing bacteria (Chika *et al*., 2014; Walsh *et al*., 2005). A total of 9 consecutive, non-duplicated Poultry feacal samples were used for this study. The result of the Gram reaction and characterization of the isolates in this study is according to the characteristics of *Klebsiella* species as previously reported by other researhers (Cheesbrough, 2005). The results of the bacteriological investigation showed that a total of 48 *Klebsiella* species isolates were gotten and isolated from the aseptically collected 9 poultry feacal samples. This result is not the same with the report of Chika *et al.* (2016) who isolated 33 *Klebsiella* species from 50 cloacal swab samples of poultry birds. This difference may arise as a result of sampling techniques used.

Antimicrobial resistance is no doubt an affair of concern in human and veterinaries. Poultry farms are good platforms for the evolution and spread of antibiotic resistant bacteria (Luo *et al*., 2020). These antibiotic-resistant bacteria is likely to reach the humans by direct contact, and also by food products from animals (Van den Bogaard and Stobberingh, 2000). Hence, the reduction and elimination of antibiotics for other purposes that are not veterinary therapitic or management and treatment of infections in animals is essential. This can be achieved by improving on methods of animal husbandry, eliminating diseases in animals, adequate use of existing and development of new vaccines (van den Bogaard and Stobberingh, 2000).

The isolated *Klebsiella* species showed different levels of resistance to the tested antibiotics used in this study. They showed highest resistance to augmentin (97.9%) and least resistance to nitrofurantoin (18.8%) which is Beta-Lactam Combination Agent and nitrofuran respectively. The resistance rate of the isolated *Klebsiella* species to imipenem in this study (25.0 %) is low compared to the result reported in previous studies by (Chika *et al*., 2016). Bacterial resistance to the carbapenems especially imipenem and meropenem is an indication that metallo beta-lactamase (MBL) enzymes will be produced by the organism and this was previously reported (Chika *et al*., 2014 and Pitout *et al*., 2007). The resistance rate of the isolates to gentamicin (31.3%) in this study is comparatively similar but a little bit higher than the result (30.0%) reported by Chika *et al.* (2016). Also, 85.4% and 83.3% of the

isolates in this research showed resistance to ciprofloxacin and ofloxacin respectively both of which are fluoroquinolone unlike the report of Chika *et al***.** (2016) in which resistance to ciprofloxacin and ofloxacin were 80.0% and 70.0% respectively, values which were higher than this present study. Furthermore, the *Klebsiella* species showed high resistance to the cephalosporins used in this study including cefuroxime (93.8%), cefixime (95.8%), and ceftazidime (81.3%). The 81.3% resistance of the isolates to ceftazidime is higher than the 80.0% reported by Chika *et al***.** (2016). In this study, *Klebsiella* species had been reported to show varying rates of resistance and susceptibility to the cephalosporins, flouroquinolones and the aminoglycosides elsewhere (Fashae *et al*., 2004). The disparity in resistance in this study compared with other studies may be related to the antimicrobial agent frequently used for treatment in diverse geographical areas.

The undue use of antibiotics for growth promotion and prevention of diseases in farm animals has impressed selective pressures that induce more resistance among bacteria in the community. Out of the 48 isolates of *Klebsiella* species phenotypically screened for the production of metallo-β-lactamase (MBL), only 7 (14.2%) isolates were positive for the production of this enzyme. The other 41 (85.4%) isolates of *Klebsiella* species did not express MBL phenotypically by the method used in this study. The production of MBL is this study is similar to a previous study conducted by Yusuf *et al*. (2012) and Chika *et al***.** (2016) in which MBL was detected in *Klebsiella* species from hospital origin and local poultry farm. However, the prevalence of MBL-producing *Klebsiella* species in this study (14.2%) is lesser than the 18.2% and 41.7% respectively reported by Chika *et al***.** (2016) and (2017) who isolated *Klebsiella* species from poultry birds. This result is similar to the work of Chakraborty *et al*., 2010; Franklin *et al*., 2006 who reported similar prevalence of metallo-beta-lactamase-producing Gram negative bacteria from clinical isolates. This shows the impact of antibiotic abuse in the community. The present study has demonstrated that *Klebsiella* species from poultry feaces produces MBLs. This organism is endowed with the ability to be resistant to the carbapenems. Therefore, the detection of multidrug resistant bacteria in community in this region should be given a boost in order to assuage the emergence and spread of MBL-producing bacteria through the food chain.

The observation from this study that showed the MBL-producing *Klebsiella* species exhibiting multiple drug resistance (MDR) to a combination of six (NIT-IMP-CPR-GEN-AUG-CAZ), five (IMP-CPR-CRX-AUG-GEN) and four (CAZ-CPR-AUG-GEN) different classes of antibiotics respectively is similar and comparable to the report of a study carried out by Fielding *et al*. (2012) on *Klebsiella* species isolated from Free-range chicken samples collected from street vendors from the informal settlement of Langa, in the Western Cape Province of South Africa where approximately 40% of the isolates were multidrug resistance which may have been due to their expression of some enzymes that inactivate these drugs. In addition, the present result showing all the MBL-producing isolates as MDR is in agreement with previous researchers` report (Elmonir *et al*., 2021; Hayati *et al*., 2019) where all the isolates were MDR. Furthermore, the result of this present study is comparable to the report of Nirwati *et al*. (2019) and Atterby *et al*. (2019) who reported that 54.49% and 96% of the isolates were MDR resprctively.

Regulations and Guidelines: Depending on the jurisdiction, there may already be existing regulations and guidelines in place to address the issue of antibiotic resistance in food-producing animals. These regulations aim to minimize the risk of transmitting antibiotic-resistant bacteria to humans (World Organisation for Animal Health, 2015).

Risk Assessment and Mitigation: To address the legal implications, there may be a need for risk assessment studies, evaluating the potential impact on public health and the environment. Based on the findings, strategies for risk mitigation, such as improved farming practices or targeted use of antibiotics, may be recommended and enforced (Food and Agriculture Organization of the United Nations, 2018).

CONCLUSION AND RECOMMENDATION

CONCLUSION

This study reported the occurrence of *Klebsiella* species with multidrug resistance from poultry feaces. The *Klebsiella* species also expressed MBL phenotypically, and this allows them to be resistant to the carbapenems. The abuse\abuse of antibiotics in the rearing and production of poultry birds contributes a greatly to the emergence and spread of antibiotic resistant bacteria in the community through selective pressure. This antibiotic residues in poultry products and other food producing animals can cause drug resistant bacteria to take its cause, and be transmitted to humans via food.

Enforcement and Compliance: The legal implications involve enforcement and ensuring compliance with existing regulations. Authorities responsible for food safety and public health may monitor poultry farms, testing for the presence of antibiotic-resistant bacteria and taking appropriate actions if violations occur. (Van Boeckel *et al*., 2015).

RECOMMENDATION

The reduction and eventual elimination of antibiotics for purposes other than veterinary therapy or treatment of infections in animals becomes essential. This can be achieved by improving on the nmethods of animal husbandry, elimination of diseases in animals, use of existing and the development of new vaccines. Generally, these interventions will help in reducing the development and occurences of resistant bacterial infections, thus prolonging or restoring the potency of existing antibiotics. Importantly, a programme that will aim at prevention of antibiotic resistance includeing an active system of surveillance for resistance, an active and effective infection control programme to minimize secondary spread of resistance, and the sensible use of antimicrobials in animal production systems.

Penalties and Liability: If poultry farms are found to be in violation of regulations and knowingly fail to address the issue of Metallo-beta-lactamase producing Klebsiella species, legal penalties such as fines or suspensions may be imposed. Liability may also be considered if evidence suggests harm caused to human health or environment due to negligence (Ghanbarpour *et al*., 2016).

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