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Epidemiological Pattern of Extended Spectrum Beta Lactamase Resistant *Klebsiella pneumoniae* (ESBL) isolated from Mastitic Milk in Baghdad

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ABSTRACT

Epidemiology is the study of the distribution and determinants of health-related states or events (denominators), including diseases and subclinical cases. Various methods can be used to carry out epidemiological investigations: surveillance and descriptive studies can be used to study distribution; analytical studies are used to study determinants. It is the cornerstone of public health, and shapes policy decisions and evidence-based practice by identifying risk factors for disease and targets for preventive healthcare. Opportunistic psychrotrophic, biofilm-producing and multidrug-resistant foodborne Carbapenemases producing or Extended Spectrum Beta Lactamase Resistant *Klebsiella pneumoniae* (*ESBL*/*KPC*) strains are pro-growing threat worldwide. This epidemiological surveillance focused on isolation and Identification of *K. pneumoniae* especially *ESBL* producers from Cows mastitic milk in Baghdad. Study design including collection and processing of forty five samples of mastitic milk from clinically infected Cows from regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya (fifteen samples from each region) during November (2016) to March (2017), in which they collected and processed according to modified dairy microbiological methodology in veterinary public health laboratory by McConkey agar. Gram stain, catalase, oxidase and negative staining technique with nigrosine and safranin for capsule detection. Electronic RapID™ ONE (4 hours) biochemical panel micro-tubes strep identification system compendium with reference colors chart and online confirmation microcodes data base software. Gold standard double staining technique, Microtiter Plate Assay for biofilm formation with methylene blue and safranin dyes. Antibiotics Susceptibility Pattern by Kirby-Bauer technique or disk diffusion method was proceed according to instructions of clinical laboratory standards institute (*CLSI*) or national committee for clinical laboratory standards (*NCCLS*) by using a Muller-Hinton agar and McFarland opacity tubes for checking resistance profile of isolates. Double diffusion inhibition technique or Oxoid Cefpodoxime Combination Kit determined *ESBL* resistance activity. Data were analyzed for significant differences by statistical package for social sciences software (*IBM SPSS*) in which a Chi-square was used. The results verify detection of twenty two strains (48.88%) of phenotypically indole negative *K. pneumoniae* out of forty five samples: twelve strains (26.66%) from Abu-Ghraib: eight strains (17.77%) were *ESBL* producers; five strains (11.11%) from Al-Fudhaliyah: two strain (4.44%) was *ESBL* producers and five strains (11.11%) from Al-Sadrya: two strain (4.44%) was *ESBL* producers. In conclusion, data verified isolation and identification of *K. pneumoniae* in Baghdad, in which *ESBL* producers were detected, thus I recommend confirmation of isolates by multiplex-PCR, monitoring of subclinical infected & carrier Cows, milk handlers, environment, utensils, equipment, milk cans, vectors, etc. through application of sanitation practices and hazard analysis critical control points (*HACCP*) strategies to overcome or reduce these public health problems.

Keywords: *Klebsiella pneumoniae*, *ESBL*, Mastitis.

1. INTRODUCTION

The epidemiological pattern and genetic makeup of *K. pneumoniae* has been associated with different types of infections as emergence of multidrug resistant strains [1-7]. Multidrug resistant strains cause serious nosocomial and community acquired infections that are hard to eradicate by using antibiotics. Moreover,

extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of *Klebsiella* and the development of multidrug resistant strains that produce extended spectrum beta-lactamases (*ESBL*) [8]. Epidemic strains of cephalosporin resistant *K. pneumoniae* (*CepharK*) have been associated with increased nosocomial morbidity and mortality [9]. Ten to thirty % of *K. pneumoniae* strains were plasmid mediated *ESBLs* of the *TEM* or *SHV* or *C-TXM* lineages [10], this may indicate chromosomal and extrachromosomal *ESBLs* genotypes. Emergence of Multidrug resistant strains is associated with four resistant strategies that diminish the effects of antibiotics: enzymatic modification and inactivation of antibiotics, restriction of drug targets access, alteration of or complete diminish of the drug target and phenotypic resistance profile [11].

Carbapenems (imipenem, meropenem and ertapenem) are recommended first-line therapy for severe infections caused by multidrug resistant *ESBL* producers [1, 12 & 13]. The emergence of carbapenem-resistant strains is a sophisticated problem, since consequently the antimicrobial treatment options are very restricted. Resistance to carbapenems may involve several combined mechanisms: modifications to outer membrane permeability and up-regulation of efflux systems associated with hyper production of *AmpC β* lactamases (Cephalosporinases, *ESBL*) or production of specific carbapenem-hydrolyzing β-lactamases (Carbapenemases). Carbapenemases present in *Enterobacteriaceae* can be either metallo-β-lactamases (*IMP*, *VIM*), expanded-spectrum Oxacillinases (*OXA-48*), or Ambler class A clavulanic-acid inhibited β-lactamases (*NMCA*, *IMI*, *SME*, *GES* & *KPC*) [14-16].

K. pneumoniae Carbapenemases (*KPCs*) are plasmid encoded carbapenems-hydrolyzing enzymes, which have the potential to spread widely through gene transfer. Routine antibiotic susceptibility testing failed to identify *KPC* producers. Combination therapy might be preferable to control *KPC* infections in immediate future [6]. The *KPCs* have become endemic in many countries but there is no optimal treatment recommendation available for bacteria expressing *KPCs*. Hurdle technology in food processing and preservation with phage therapy as a part of *HACCP* program were a new modern strategy to overcome these superbugs. Carbapenems are broad-spectrum antibiotics structurally very similar to penicillin but contain a Sulphur group at C1 position. Several Gram-negative pathogens have become resistant to these by acquiring any one or more of the following mechanisms: structural alterations in drug targets like penicillin binding proteins (*PBPs*), porin loss and upregulation of efflux pumps and expression of Carbapenemases [17]. All Carbapenemases are β-lactamases but not *vice versa*. These periplasmic enzymes hydrolyze beta lactam antibiotics either by alteration in the target site of the antibiotic that reduces its binding capacity or modification of the antibiotic so that it is no longer recognized by the target. Carbapenemases are divided into two major types: metallo β-lactamases (Class B) containing zinc at the active site (*NDM-1*) whereas serine β-lactamases (Classes A, C and D) containing serine at the active site. The *K. pneumonia*e Carbapenemases constitute the class A [17-19].

Initially biofilm producing *KPC* strains were restricted to sporadic outbreaks, but the situation became difficult because of global frequency and distribution in both developed and developing countries [20-25]. One of the most popular and traditional dairy food in Iraq is milk due to social nature. As we know, that raw milk might harboring many foodborne pathogens especially those causing food poisoning or intoxication in man, due to its water content or water activity and handlers under poor sanitary environment or from unhygienic animals from poor unclean raw milk or been actively infected or passively carriers with multidrug resistant pathogens especially from subclinical mastitic Cows in Iraq, as well as its containers could harboring the persistent and resident infectious biofilm foci of these pathogens. All these scenarios and others about real nature of milk in Iraqi environment with the low information about quality and quantity of raw milk, as well as environmental hygienic conditions of milk producing animals and their milk production and processing cycles under these circumstances linked with the case of subclinical and clinical mastitis in dairy farms in Iraq, and obscure information about prevalence and epidemiology of *K. pneumonia*e especially *ESBL* producers aiming to investigation of the occurrence, distribution and frequency of this pathogen in mastitic Cow's milk from regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya.

2. MATERIALS AND METHODS

2.1 Collection & Processing of Samples

Study design including collection and processing of forty five samples of mastitic milk from clinically infected Cows from regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya (fifteen samples from each region) during November (2016) to March (2017), in which they collected and processed according to modified dairy microbiological methodology and bacteriological analytic manual in veterinary public health laboratory (**26-31**). Samples were collected from clinically mastitic Cows via sterile containers carefully after cleaning of infected udder quarters with clean water and towel for each case, then get ridding first strips of infected milk from them to minimize contamination and processed adequately until transportation to work lab by ice box, then refrigerated at 4 ©C as critical control point in isolation and identification procedure of psychrotrophic *K. pneumonia*e.

2.2 Isolation & Identification Procedure

All samples were processed according to modified food microbiological techniques in which they mixed toughly by vortex and refrigerated at 4 ©C for 48 hours for resuscitation of psychrotrophic *K. pneumonia*e, then

remixed, diluted and inoculated in buffered double strengthen power tryptone soya yeast extract broth (TSBYE) (one part sample (10 ml) to nine part diluent (90 ml) and incubated at 37 ©C for 24 hours to encourage reviving of *K. pneumonia*e with capsule production and biofilm formation, then cultured in McConkey agar in duplicates for each sample and incubated at 37 ©C for 48 hours [26-31]. Pure large mucoid and capsulated glistening pink colonies with viscous threads were picked up and recultured in TSBYE for increasing seed of denominator, then cultured in double strengthen power tryptone soya yeast extract agar (TSAYE) for further identification. Gram stain, catalase, oxidase and negative staining technique with nigrosine and safranin for capsule detection were proceeds. Electronic RapID™ ONE (4 hours) biochemical panel micro-tubes strep identification system compendium with reference colors chart and online confirmation microcodes data base software was used for identification procedure according to company leaflet instructions [32].

2.3 Biofilm Formation Assay

Double staining technique, Microtiter Plate Assay with methylene blue and safranin dyes was used for detection of biofilm formation and secretion. Quantitavely and Qualitative detection of slime producer strains was determined by culturing the isolates on modified buffered double strengthen power TSBYE using adherence assay on large U-shape 24 well tissue culture plates as described previously by Christensen *et al*. and others [33-35]. An overnight culture grown in TSBYE at 37 ©C was transferred and diluted in microtiter plate as 0.1ml to 0.5ml freshly prepared TSBYE inoculated for each well. Each isolate was tested in duplicate. Wells with sterile TSBYE alone was served as controls. The plates were incubated for 24 hours at 37°C. Furthermore, the culture was removed and plates were washed three times with phosphate-buffered saline to remove non-adherent cells and dried in an inverted position. Adherent biofilm was fixed with 2% sodium acetate and was stained with of 10% methylene blue and safranin for 5 min. Then, unbound stain was removed and the wells were washed three times with PBS. Plates were settled 2-3 hours for dryness then stained layers and dots of biofilm in bottom and around internal rims of wells were photographed, measured and scored according to the degree of formation, type of stain and type of isolate.

2.4 Antibiotics Susceptibility Pattern (Resistance Profile)

A Kirby-Bauer technique or disk diffusion method was dependent according to instructions of clinical laboratory standards institute (*CLSI*) or national committee for clinical laboratory standards (*NCCLS*) by using a Muller-Hinton agar and McFarland opacity tubes [36-38]. A test procedure was done by selecting well-isolated (4-5) colonies of *K. pneumonia*e from freshly inoculated overnight TSAYE, touched tops of these colonies by a loop then, transferred to freshly prepared (4-5) ml TSBYE tubes and incubated for 2 hr. at 37 ©C in order to reach a standard 0.5 opacity of McFarland tubes or approximately 10^4-10^5 cfu/ml standard inoculum broth. Preparation of freshly agar plate's cultures of Muller-Hinton then dried in incubator before testing procedure. A sterile cotton swab was dipped into the adjusted suspension and rotated several times and pressed firmly on the inside wall of the tube above the fluid level for removing excess inoculum from the swab. Streaking the surfaces of Muller-Hinton agars (4-5) times with the rim by a swabs then left inoculated agars for (10-15) minutes to absorb the inoculum before applying selected antimicrobial disks by pressing down via rotated multiple automatic applicator to ensure complete contact with the agar surface and distributed evenly. The plates were inverted and placed in an incubator at 37 ©C for (18-24) hrs. Then reading the plates and interpretation the results [39 & 40]. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone was related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium.

2.5 *ESBL* Combination Disk Technique

Double diffusion inhibition technique or Oxoid Cefpodoxime Combination Kit determined *ESBL* resistance activity in food laboratory [32]. Combination discs were a blend of cephalosporin and clavulanic acid on a single disc, which are, used in conjugation with a plain cephalosporin for *in vitro* detection of *ESBLs* strains that do not produce inducible *AmpC* enzymes. The kit contains the following: Cefpodoxime/clavulanic acid (*CD01*) 10/1µg & Cefpodoxime (*CPD10*) 10µg. The isolates were tested by comparing the inhibition zones given by cefpodoxime (10-mg) and cefpodoxime-plus-clavulanate (10- plus 1-mg) disks. The presence of clavulanate enlarged the zones for all of *ESBL*-producing *klebsiellae* by ≥ 5 mm, whereas zones for cefpodoxime-susceptible isolates and cefpodoxime-resistant isolates with *AmpC* and *K1* β-lactamases were enlarged by ≤ 1 mm. Good discrimination was achieved with either the *NCCLS* (*CLSI*) or British Society for Antimicrobial Chemotherapy (*BSAC* Standardized Disc Sensitivity Testing Method) [41-44]. Combination discs should be used by qualified personnel trained to handle category 2 resistant pathogens, and be competent in basic microbiological techniques including antibiotic susceptibility testing. The discs need to be placed on sensitivity media (Muller-Hinton agars) with sufficient space between the discs to allow the formation of clearly defined zones of inhibition and combination of them. A freshly prepared standardized inoculum 0.5 McFarland from each isolate on TSBYE was used in test procedure (10^4-10^5 cfu/ml). Ceftazidime (*CAZ*) (30-mg) disks (Oxoid) were tested in parallel as a control [43]. Zone diameters were measured to the nearest millimeter. A difference of ≥ 5 mm between the zones of the *CD01* (10- plus 1-mg) and *CPD* (10-mg) disks was taken to indicate *ESBL* production, as advocated by the manufacturer. The zones of the *CAZ* (30-mg) and *CPD*

(10-mg) disks additionally were compared against the susceptibility criteria of the *NCCLS* and *BSAC* for predicting *ESBL* production [44].

2.6 Statistical Analysis

Chi-square (æ ²) analysis was used for checking significant differences among data, through statistical package for social sciences software [45] with the formula of

æ ² = *(O-E)²/E*, in which:

O = Observed positive isolates of *K. pneumonia*e from total samples.

E = Expected negative samples free from *K. pneumonia*e from the original total samples.

3. RESULTS AND DISCUSSION

3.1 Isolation & Identification Profile

The results verify detection of twenty two strains (48.88%) of phenotypically indole negative *K. pneumoniae* out of forty five samples: twelve strains (26.66%) from Abu-Ghraib: eight strains (17.77%) were *ESBL* producers; five strains (11.11%) from Al-Fudhaliyah: two strain (4.44%) was *ESBL* producers and five strains (11.11%) from Al-Sadrya: two strain (4.44%) was *ESBL* producers. Total *ESBL* producers were twelve (26.66%) phenotypic strains. Detection Profile and isolation percentages were illustrated in tables (1 & 2):

Table 1: Isolation of phenotypically indole negative *K. pneumoniae* from mastitic Cow's milk.

Region	Number of Samples	Isolation % (45)
Abu-Ghraib	15	12 (26.66%) A*
Al-Fudhaliyah	15	5 (11.11%) B
Al-Sadrya	15	5 (11.11%) B
Total	45	22 (48.88%)

*: Indicate highest isolation percentages from Abu-Ghraib.
A,B: Indicate significant differences (æ²) vertically at level (P≤0.05).

Table 2: Isolation of *ESBL/KPC* strains from mastitic Cow's milk.

Region	Number of Samples	Isolation % (45)
Abu-Ghraib	15	8 (17.77%) A*
Al-Fudhaliyah	15	2 (4.44%) B
Al-Sadrya	15	2 (4.44%) B
Total	45	12 (26.66%)

*: Indicate highest isolation percentages from Abu-Ghraib.
A,B: Indicate significant differences (æ²) vertically at level (P≤0.05).

Microbial quality and quantity of milk and dairy products depends on hygienic measurements during milk production and dairies manufacturing, thermal processing, storage temperature and duration, animals feeding, season, area, plant sanitation, quality of starter cultures, presence of phages, quality of rinsing water, etc. so that, the examination for the microbial load of specific microorganisms is, therefore, an integral part of any quality control or quality assurance plan and it may be applied to a number of areas: raw materials, intermediate samples, finished products, or environmental/equipment sites [46-48]. Large mucoid and glistening pink colonies with viscous threads on McConkey agar with *ERIC* panel aid in confirmation of phenotypically indole negative *K. pneumoniae* strains from mastitic milk samples from regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya. Isolation profile indicate presence of infectious foci of this pathogen in these areas due to poor sanitary conditions during and after milk production as well as climatic conditions and inadequate management practices for milk producing animals and milk cans as well as infective individuals and carriers with vectors and other interconnected factors and stressors encourage epidemiological distribution and frequency pattern of these emerging opportunists. Contamination and pollution can occurs at any stage of milk production, handling, transportation and storage [31, 32, 49 & 50].

Isolation profile and epidemiological pattern of mastitis causes may fluctuate according to different factors from hidden unrecognized subclinical to immunocompromised cases to careless adulteration during processing of mastitic milk especially during unsanitary cleaning of milk cans that contains resident adherent biofilm infectious foci of *K. pneumoniae* in the bottom of these containers. Absence of cyclic monitory Compton metabolic profile programs for milk producing animals and their udder or quarters hygiene shifting tests like California mastitis test, Whiteside test and Somatic cell count as a widely used marker for prediction of udder health and milk quality, aid in developing of these hidden unrecognized hazards. In the healthy mammary gland, the predominant cell types are macrophages followed by lymphocytes, polymorphonuclear cells and epithelial cells, they classified as wondering leukocytes especially macrophages & polymorphonuclear neutrophils, which

constitutes 95% of somatic cells and they are important in mastitis cases. Low number of milk samples in this project with high ratio of isolation was unacceptable in this time, but it's indicate the real face of contamination in Iraqi environment in spite of presence of high quality of clean milk in Iraq, but in low number and areas. Risk factors of these foodborne pathogens were evident through transportation from livestock to community and nosocomial relationship that facilitate distribution of biofilm producing and multidrug resistant pathogens in Iraqi environment, but actually in low number and cases, but remain dangerous.

3.2 Biofilm formation

Gold standard microtiter tissue culture assay with double staining technique by methylene blue and safranin dyes plus McConkey agar results (large viscous mucoid pink colonies) obviously indicate biofilm formation and secretion. Red and blue adherent layers and dots of biofilm around and in the bottom of microtiter plate tubes indicate presence of biofilm producing isolates as in Figure 1, in which hypermucoviscosity in these clinical isolates were correlated with high multiple antibiotic resistance index and developing of *ESBL* producers phenotypes [25].

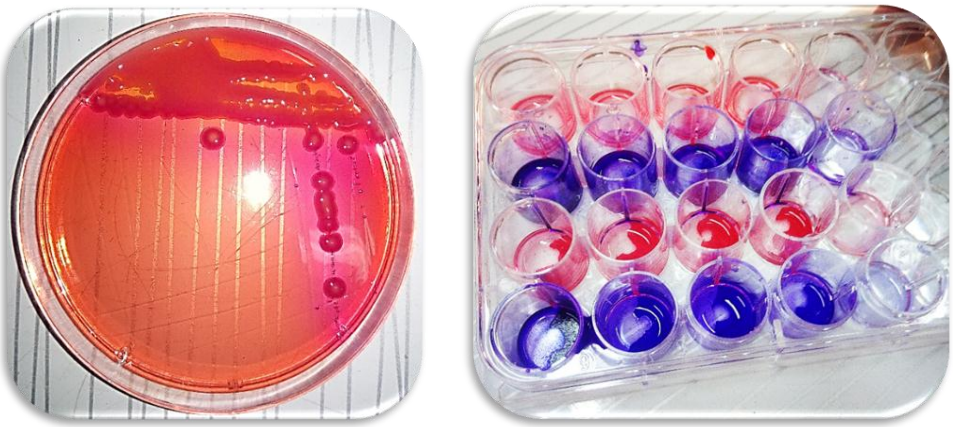

Figure 1: Colonies of Extended Spectrum Beta Lactamase (*ESBL*) Resistant *K. pneumoniae* (*KPC* strain) on McConkey agar after 48 hrs. at 37 ©C as large mucoid & pink colonies (lactose fermenter) with biofilm formation texture. Biofilm formation on modified microtiter plate assay with double staining technique (methylene blue & safranin). Noticed deposited mucous layers & dots on bottom & around microtiter tubes.

Biofilm producing and multidrug resistant *K. pneumoniae* isolates detection was another very important and dangerous risk evident in this study because of persistent source of contamination and pollution of environment, peoples, animals, foods, feeds, etc. from these infectious foci and active and passive carriers. Gold standard double staining technique, Microtiter Plate Assay for biofilm formation with sensitive methylene blue and safranin dyes assist in detection of biofilm producing isolates especially those owing resistance profile to Cefpodoxime/clavulanic acid as indicated in another studies [5, 7, 25, 51 & 52]. Production of biofilm or slim exopolysaccharides protect the isolates from harsh conditions during their life cycle and supply new virulent strains in environment. Genetic makeup and plasmids mediated of these strains may need more interpretation along with the restudy the relationship among environment, milk producing animals and handlers in distribution of these infectious foci in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya. Risk of biofilm multidrug resistant strains incriminate in difficult to treatment cases of pneumonia, urinary tract infections, etc.

All isolates were encapsulated and biofilm producers, but those isolated from Abu-Ghraib region show hypermucoviscosity especially *ESBL* producers, this may indicate they are genetically well equipped variants that hyper resistant to all selected antimicrobials with no zone of inhibition in accordance with other isolates as correlated in another studies about *K. pneumonia and P. aeruginosa* [5, 7, 25, 51 & 52].

3.3 Antibiotic Susceptibility Pattern

Resistance Profile of biofilm producing isolates were evident for selected antimicrobials and interpreted according to tables of *CLSI* [37 & 38] as indicated in table 3.

Table 3: Pattern of Resistance, Intermediate and Susceptible isolates of *K. pneumoniae* (total 22 isolates) from mastitic Cow's milk in Baghdad.

Antibiotics	Concentration	Resistance %	Intermediate %	Susceptible %
Ceftazidime (CAZ)				
Cefotaxime (CTX)	30 µg			
Aztreonam (AZ)		22 (100) [A]	None (0) [B]	None (0) [B]
Imipenem (IMI)	10 µg			
Meropenem (MEM)				

A,B: Indicate significant differences (æ [2]) among total isolates (22) for selected antibiotic horizontally at level (P≤0.05)

Antibiotic resistant pattern may be partially linked to the genetic capacity of isolate to the formation of capsular polysaccharides and viscous thread and mucoid in texture large pink biofilm colonies on McConkey agar as noticed in this study and linked to the study of Al-Shammary in Iraq [50], that show increased secretion of biofilm producing isolates from milk and soft cheese, may be due to genetic makeup of these active isolates. Study isolates were resistant to all antibiotics; this may indicate development of resistance pattern from biofilm producing mastitis strains. The ability to resist antibiotics may reflect the inelegant strategy and behavior of *K. pneumoniae* to overcome, modulating and buffering environmental and inoculation conditions according to their life cycle *in vivo* and *in vitro*. Risk of biofilm multidrug resistant strains incriminate in difficult to treatment cases of pneumonia, urinary tract infections, etc.

Chromosomal genetic information expressing carbapenemases carried on DNA, extrachromosomal segments and transposons. Chromosomally encoded carbapenem inactivating enzymes act as defense mechanism in these bacteria and have a role in regulation of cell wall synthesis especially capsule and biofilm formation [6]. The expression of these plasmid encoded enzymes gets upregulated by various factors like intrinsic hydrolytic activities shown by *KPC* variants, the genetic relatedness and control of carbapenemase production, and the presence or absence of other broad-spectrum β-lactamases and porin mutations [53]. It is necessary to establish how the bacterium is able to show resistance to all classes of antibiotics and the mutations that are responsible for resistance. Accumulation and transfer of *KPC* resistance determinants rapidly through horizontal gene transfer leads to an increase in mortality and morbidity. The *KPC* enzyme confers resistance to all β-lactam agents including penicillins, cephalosporins, monobactams, and carbapenems [53].

3.4 *ESBL* Producer Variants

Total *ESBL* producers were twelve (26.66%) phenotypic strains out of forty five samples, in which they resident from all regions especially in Abu-Ghraib cases. Those variants noticed during calculating and subtracting the differences in millimeter between diameters of Cefpodoxime/clavulanic acid (*CD01*) 10/1µg (must be the larger zone) & Cefpodoxime (*CPD10*) 10µg (must be the smaller zone) zone of inhibition, so that any result larger than 5 millimeter indicate phenotypically *ESBL* production as in Figure 2.

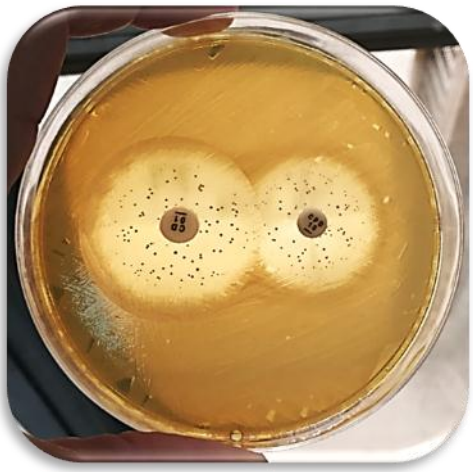

Figure 2: *ESBL* producer variants isolated from Cows mastitic mill from Abu-Ghraib region in Iraq, and detected in Cefpodoxime combination disk on Muller-Hinton agar.

Double inhibition technique may reveal phenotypically the presence of extra resistance enzymatic series of *ESBL* producers especially in isolates from Abu-Ghraib region. Cefpodoxime/clavulanate combination discs (*CD01*) were sensitive and specific for detecting *ESBL* producing strains [13, 41-43]. This behavioral extra resistance strategy may be linked to biofilm secretion and capsule formation as noticed from study of Al-Shammary [51]. This resistance strategy confirmed by selected antibiotic susceptibility pattern for selected antimicrobial agents as revealed in table **3**. The predicted risk from phenotypic species identification, dissemination and emergence of *KPC* producers as a serious threat to public health because it drastically limits the treatment options. It not only confers resistance to carbapenems, but also to almost all other β-lactams as noticed in table **3**. Identifying *KPCs* is still a challenge for many clinical microbiology laboratories. In antimicrobial susceptibility tests higher *MICs* of the antibiotic used could be due to increased levels of expression of these enzymes and low penetration of drug into periplasmic space [54].

Carbapenemases were slowly emerging and present a new detection challenge, partially because carbapenem resistance does not always accompany enzyme production, as well as *KPC* lineage may phenotypically different from *NDM-1*. *ESBLs* are of increasing concern as mutants of classical *TEM* and *SHV* plasmid-mediated types but the *CTX-M* types, which are derived from the chromosomal β-lactamases of *Kluyvera spp*. [54]. They are prevalent in *K. pneumoniae* as nosocomial hospital strains, but I noticed them in mastitic milk as an indicator of mastitis in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya. Oxoid Cefpodoxime Combination Kit [32] might aid in phenotypic detection of *ESBL* mediated *KPC* strains. Two detection strategies are in common use: using ceftazidime or cefpodoxime as an indicator drug, and considering *klebsiellae* with reduced susceptibility to these drugs to be resistant to all oxyimino-aminothiazolyl cephalosporins [44] or screening for synergy between extended-spectrum cephalosporins and clavulanic acid. Synergy can be detected by double-disk tests, although the optimum separation of the disks is strain variable. Alternatively, commercial systems, such as E tests and Vitek [55 & 56], can be used. One obvious strategy is to compare the inhibition zones of cephalosporin disks with and without clavulanate added. This method has been used by several researchers and comparison of the zones given by cefotaxime (30 μg) and ceftazidime (30 μg) disks with or without clavulanate (10 μg) added is now advocated by the *NCCLS* [44]. Such disks are available from several suppliers (Becton Dickinson, MAST, and Oxoid) [57].

4. CONCLUSION

Data verified infection of dairy Cows in Baghdad with biofilm producing and multidrug resistant strains of *K. pneumoniae* with noticed *ESBL* producers phenotypes, thus I recommend genotype confirmation of isolates by multiplex-PCR, monitoring of subclinical infected & carrier Cows, milk handlers, environment, utensils, equipment, milk cans, vectors, etc. through application of sanitation practices to overcome or reduce these public health problems., as well as application of up to date diagnostic tools for reducing dangerous foci of *K. pneumoniae* and their transmission as soon as possible with treating of diseased cases carefully, monitoring active and passive carriers, and designing future hazard analysis critical control points strategies to controlling the epidemiological distribution and frequency pattern of *K. pneumoniae* in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya. One of the future promised food processing strategies is the application of active lytic phages therapy through what we known as Clustered regularly interspaced short palindromic repeats (*CRISPR*) and their associated genes (*CAS*) program in conjugation with bacteriocins like *Klebocin* purified from local isolates of *K. pneumoniae* as a hurdle technology.

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