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Molecular and Epidemiological Characterization of Extended-Spectrum- β-Lactamase among Pathogenic *Enterobacteriaceae* Isolates from Riyadh Hospitals, Saudi Arabia

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ABSTRACT

Enterobacteriaceae belong to the normal enteric flora in humans and may cause infections. Enterobacterial strains are the most common cause of urinary tract infections but also cause pneumonia, sepsis, infections of wounds and of the intestine. Since the introduction of antibiotic agents, the amount and prevalence of β-lactam resistant enterobacteria has become an increasing problem. Many enter bacteria are opportunistic pathogens that easily acquire resistance mechanisms and genes, which make the situation menacing. These bacteria have acquired resistance and can hydrolyse extended spectrum by producing enzymes called extended-spectrum β-lactamases (ESBLs). Laboratory detection of ESBL-producing bacteria remains a challenge for the microbiology laboratory and is important both to avoid clinical failure due to inappropriate antimicrobial therapy and to prevent nosocomial outbreaks. This study investigates the genetic basis of extended-spectrum β-lactamases in *Enterobacteriaceae* isolates. DNA Microarray technology has recently been developed for the typing of bacterial isolates. The Future of DNA Microarray is very new significant tool for Gene discovery, Disease diagnosis by classify the types of cancer on the basis of the patterns of gene activity in the tumor cells, *pharmacogenomics* study of correlations between therapeutic responses to drugs and the genetic profiles of the patients and Toxicogenomics in which microarray technology allows to research the impact of toxins on cells. Some toxins can change the genetic profiles of cells, which can be passed on to cell progeny.

INTRODUCTION

Enterobacteriaceae is a heterogeneous family consisting of more than 30 genera and 150 species and subspecies [7]. They belong to the normal intestinal flora of most animals, including humans, and may cause intestinal and extra-intestinal infections. Medically important species of *Enterobacteriaceae* are *Escherichia coli* and *Klebsiella pneumoniae*. Pathogenic *E. coli* are characterised by shared O (lipopolysaccharide) and H (flagellar) antigens that define serotypes or serogroups. *E. coli* can be subdivided further into four main phylogenetic groups; A, B1, D and B2. Commensal strains of *E. coli* belong mainly to the A and B1 phylogenetic groups [30]. Pathogenic phylogenetic lineages involved in extra-intestinal infections are mainly derived from the B2 group and its sub-groups.

K. pneumoniae typically causes opportunistic infections of the urinary tract, in wounds and soft tissue and in the bloodstream [21, 31]. Infections caused by *K. pneumoniae* are often observed in hospital settings and are associated with high mortality rates in infants and the elderly [8]. Resistance to β-lactams can be inherent or acquired. Inherent resistance in a Gram-negative bacterium is due to an outer membrane that establishes a permeability barrier against the antibiotic. For example, Gram-negative bacteria are intrinsically resistant to penicillin G by virtue of their double membrane structure which prevents the antibiotic from accessing the cell wall target. Intrinsic resistance is not considered an important clinical problem since antimicrobial agents are or were not intended for use against intrinsically resistant bacteria. It is the acquired resistance that is of clinical importance.

Acquired resistance to β-lactams operates through different mechanisms: production of β-lactamases, changes in the outer membrane permeability or alterations to the PBPs [35]. β-lactamases are a group of enzymes that are most likely originally targeted for synthesis of the cell wall but have also evolved to degrade and inactivate β-lactam antibiotics. These flexible enzymes have been detected in both Gram-positive and Gram-negative bacteria, but these enzymes are especially important in Gram-negative bacteria as they are the most common cause of β-lactam resistance in this group of bacteria [1,15]. The outer membrane of Gram-negative bacteria plays an important role in serving as a diffusion barrier to extracellular solutes and interacts with the bacterial environment. Before a β-lactam reaches its PBP targets which are on

the outer surface of the cell membrane, it must diffuse across the outer membrane of the cell, by using the pores that are formed by porins, and then cross the periplasm. The porins, which represent one family of outer membrane proteins (Omps), form channels to permit diffusion of small hydrophilic solutes through the outer membrane. The porins are divided into two classes: specific and non-specific. In *E.coli,* OmpC and OmpF represent the non-specific porins that permit the general diffusion of small polar molecules. A loss of either of these porins has been related to antibiotic resistance (27). In *Klebsiella* sp. OmpK36 and OmpK35 are the homologues of OmpC and OmpF(26,3). Usually, *K. pneumoniae* strains express OmpK35 and OmpK36 while the ESBLproducing strains commonly express only one of these, normally OmpK36, or no porin at all [(17,8)].

The resistance is often due to impermeability or the presence of a β-lactamase alone. However, these factors work together - for any given external β-lactam concentrations, the β-lactam concentration in the periplasm maintains a steady-state level. Reduced permeability through porin loss reduces the steady-state periplasmic drug concentrations and thereby reduces PBP inactivation. Decreased permeability may act synergistically with the expression of β- lactamases or active efflux to confer higher levels of resistance [(5, 14)]. In some cases, β-lactamases that have only low activity. *In vitro* can confer resistance in a suitable impermeable host strain (14).

Extended spectrum β -lactamase (ESBL)-producing organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists. ESBLs are enzymes capable of hydrolyzing penicillins, broad-spectrum cephalosporins and monobactams, and generally derived from TEM and SHV-type enzymes. ESBLs are often located on plasmids that are transferable from strain to strain and between bacterial species.

Although the prevalence of ESBLs is not known, it is clearly increasing, and in many parts of the world 10–40% of strains of *Escherichia coli* and *Klebsiella pneumoniae* express ESBLs.

ESBL-producing *Enterobacteriaceae* have been responsible for numerous outbreaks of infection throughout the world and pose challenging infection control issues. Antibacterial choice is often complicated by multi-resistance. Many ESBL producing organisms also express AmpC β -lactamases and may be co-transferred with plasmids mediating aminoglycoside resistance. In

addition, there is an increasing association between ESBL production and fluoroquinolone resistance (16).

Extended spectrum cephalosporins (third generation cephalosporins such as ceftriaxone and cefotaxime) gained widespread clinical use in the early 1980s and were developed by the increasing prevalence of ampicillin-hydrolysing β -lactamases (TEM-1, TEM-2 and SHV-1) in *Enterobacteriaceae*. In addition, the clinical significance of ESBLs is discussed and recommendations for therapy are offered.

MATERIALS AND METHODS

A total of hundred sixty-three clinical isolates of *Enterobacteriaceae* were collected from patients admitted at the Clinical Microbiology Laboratory of the Riyadh Military Hospital (RMH), Riyadh, Saudi Arabia. The tested isolates of *Enterobacteriaceae* were isolated by laboratory team during the period of 2011- 2013. All the isolates were collected from different sources mostly from Ward, UR, MB, BI, PITENT SEX and AGE. The specimens processed were: 81 of *Escherichia coli* (n=59,15 and 17), 63 of *Klebsiella pneumoniae* (n=16,29 and 18),8 of *Enterobacter cloacae* (n=6 ,2 and 0), 3 of *Enterobacter aerogenes* from (n=3,0 and 0), 7 of *Proteus mirabilis* (n=4 ,2 and 1),1 of *Salmonella enteriditis* (n=1 ,0 and 0) from UR, MB and BI respectively. All isolates were preserved with 5% glycerol in a polypropylene tube labeled, then freeze-preserved at -70°C. Samples were cultured on blood agar and Muller Hinton Agar (Oxide). Identification of organisms was done by the standard laboratory techniques. Bacterial culture of *Klebsiella pneumoniae* and *E. coli* isolates were performed on blood agar *(OXOID, Basingstoke Hampshire, England)* add (*Hors blood TCS Biosciences Ltd Botolph Claydon Buckingham MK 182LRUK*), MacConkey agar *(OXOID, Basingstoke Hampshire, England)* and Mueller –Hinton agar (*BD, backton and company spark, MD211524 SA 38800 Le pont de claix, Frace*) with incubation for 20-24 h at 35-37°C. Frozen bacterial suspension was streaked on blood agar plate and incubated overnight at 37°C.

The bacterial identifications were performed by routine laboratory methods in the microbiology laboratory of RMH using MicroScan WalkAway 96 Plus system (Siemens Healthcare Diagnostic Inc, Germany).

Minimal inhibitory concentration (MIC) for 28 antibiotics was determined by MicroScan Walk-Away 96, which having panels for gram-negative bacteria (Neg Panel Type 30) according to the manufacturer's instructions. The antibiotics tested were Amikacin (Ak); Amoxicillin/K Clavulanic (Aug); Ampicillin (Am); Cefazolin (Cfz); Cefepime (Cpe); Cefotaxime (Cft); Cefoxitin (Cfx); Ceftazidime (Caz); Cefuroxime (Crm); Ciprofloxacin (Cp); Colistin (CI); Ertapenem (Etp); Fosfomycin (Fos); Gentamicin (Gm); Imipenem (Imp); Levofloxacin (Lvx); Meropenem (Mer); Mezlocillin (Mz); Moxifloxacin (Mxf); Nitrofurantoin (Fd); Norfloxacin (Nxn); Piperacillin/Tazobactam (P/T); Piperacillin (Pi); Tetracycline (Ti); Tigecycline (Tgc); Tobramicin (To); Trimethoprim/Sulfamethoxazole (T/S) and Trimethoprim (T) .

All isolates were screened for resistance genes by PCR, with specific Primers (Table 1). A Multiplex Real-Time polymerase chain reaction (MRPCR) was used on thirty clinical isolates of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* for simultaneous amplification number and diversity of genes potentially complicate genetic approaches to the rapid detection of transmissible extended-spectrum β -lactamase genes.

Genotyping by Pulsed Field Gel Electrophoresis used for molecular subtyping of extended-Spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae (Escherichia coli* and *Klebsiella pneumoniae)* using Pulsed field gel electrophoresis CHEF-DR III.

The Check- **Points molecular Microarray platform** (Check-MDR CT103QIAGEN®, QIAamp®, QIAcube® (QIAGEN Group, QIAGEN Inc Valencia, CA 91355) used for detection and identification of the TEM, SHV and CTX-M genes encoding Extended-Spectrum Beta-Lactamases in Highly Resistant *Enterobacteriaceae*.

Table 1: The Primers used for Detection of the Extended-Spectrum β-Lactamase by Multiplex Real-Time PCR

Primers	Sequence
CTX-M Gp1.F	**GGAATCTGACGCTGGGTAAA**
CTX-M Gp1.R	**GGTTGAGGCTGGGTGAAGTA**
CTX-MGp1.Pr 6-FAM-	**ACTATGGCACCACCAACGAT-BHQ-1**
CTX-M Gp9.F	**GGTGATGAACGCTTTCCAAT**

CTX-M Gp9.R	**TCAATTTGTTCATGGCGGTA**
CTX-MGp9.Pr HEX-	**CAGAGTGAAACGCAAAAGCA-BHQ-1**
SHV-5/12.F	**AGCTGCTGCAGTGGATGGT**
SHV-5/12.R	**CAATGCGCTCTGCTTTGTTA**
SHV-5/12.Pr Tx Red	**ACCGGAGCTAGCAAGCGG-BHQ-2**
VEB.F	**CAAATGCACAAGGATTGGAA**

RESULTS

A total of hundred sixty-three clinical isolates of *Enterobacteriaceae.* Table 2 showed the distribution percentage for all 163 isolates in different source: MB special for swabs / BI special for TB lab/ UR special for urine. *Escherichia coli* followed by *Klebsiella pneumonia, Enterobacter cloacae, Enterobacter aerogenes, Proteus mirabilis* and the latest were in *Salmonella enteritidis* 66%, 18, 7, 3.4.4.5 and 1.1 in UR and 31.2%, 60, 4.16., 4.16 and negative for *Enterobacter aerogenes* and *Salmonella enteritidis* in MB. 27%, 69, 49 for *Escherichia coli, Klebsiella pneumonia* and negative for *Enterobacter cloacae, Enterobacter aerogenes* and *Salmonella enteritidis* in for BI. The total highest percentage was showed *in Escherichia coli* (50%), 39% for *Klebsiella pneumonia.*

Table 2: Distribution percentage for all 163 isolates in different source: MB special for swabs / BI special for TB lab/ UR special for urine

	source							
Species	**UR**	Percent of isolates %	**MB**	Percent of isolates %	**BI**	Percent of isolates %	**Total**	Percent of all isolates %
Escherichia coli	59	66%	15	31.2%	7	27%	81	50%
Klebsiela pneumoniae	16	18%	29	60.4%	18	69%	63	39%
Enterobacter cloacae	6	7%	2	4.16%	-	-	8	5%
Enterobacter	3	3.4%	-	-	-	-	3	1%

aerogenes								
Proteus mirabilis	4	4.5%	2	4.16%	1	4%	7	4%
Salmonella enteritidis	1	1.1%	-	-	-	-	1	1%
Total /Average	**89**	**100%**	**48**	**100%**	**26**	**100%**	**163**	**100%**

The age of the all isolates from different clinical departments for the male and female. High percentage isolation from females (88%) than males (76%) (Table 2, Fig 1). It was noted that females reported with higher isolation than males.

Table 3: Distribution percentage for all 163 *Enterobacteriaceae* isolates with sex group

Species	**Number in Males**	**Percent of isolates %**	**Number in Females**	**percentes of isolates %**	**percentes of all isolates %**
Escherichia coli	34	20.7%	47	53.4%	49%
Klebsiella pneumoniae	32	19.5%	32	36.4%	39%
Enterobacter cloacae	3	1.8%	5	5.7%	5%
Enterobacter aerogenes	1	0.6%	2	2.3%	2%
Proteus mirabilis	6	3.6%	1	1.1%	4%
Salmonella enteritidis	-	-	1	1.1%	1%
Total / Average	**76**	**100%**	**88**	**100%**	**100%**

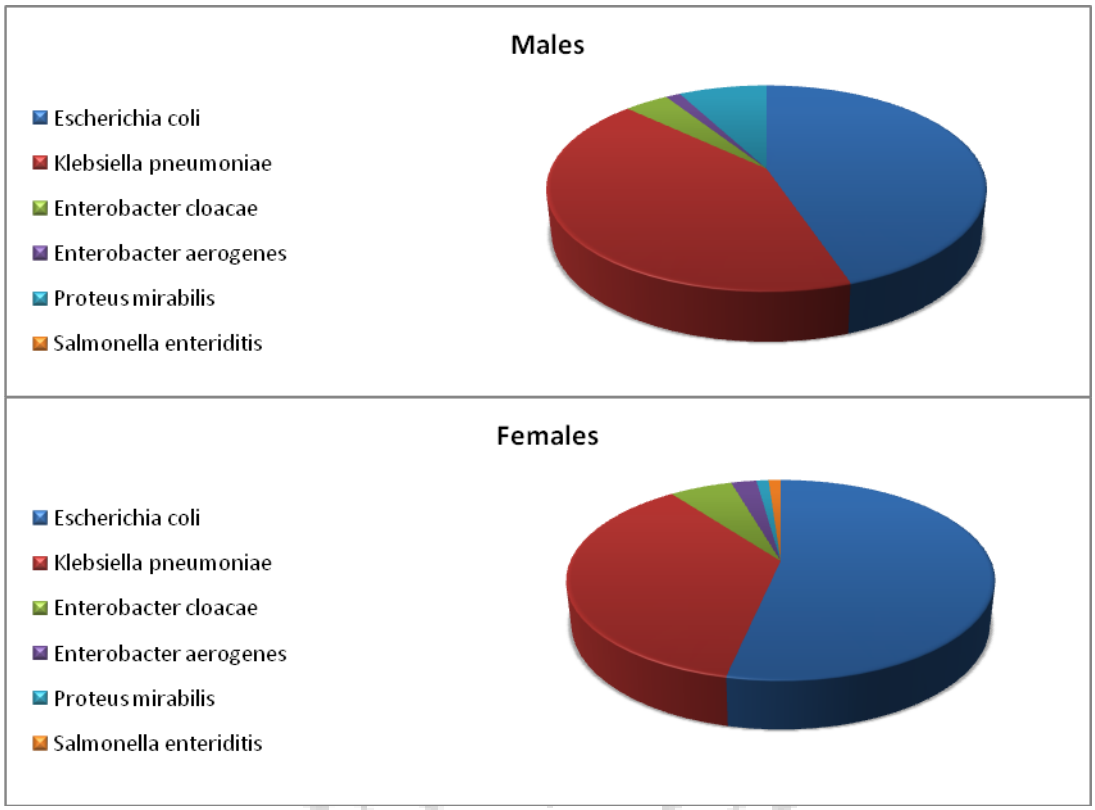


Fig 1. The percentage of the hundred sixty-three isolates between sex group females and males.

Antibiotic susceptibilities:

Bacterial isolates were identified by antibiotic susceptibilities were determined for all isolates with commercial dehydrated panels provided by Dade Behring MicroScan (Sacramento, Calif). According to the manufacturer's recommendations, and interpreted according to NCCLS criteria (National Committee for Clinical Laboratory Standards (26) production. Antibiotics and Susceptibility Teste were applied to hundred sixty-three isolates of *Enterobacteriaceae* isolates were exposed to Twenty-eight different antibiotics included the groups of Beta-lactams as (Penicillins) and Cephalosporins (Cephems), Monobactams and Carbapenems. *Escherichia coli* showed sensitivity to antibiotics with 53% for 72 isolates and resistant were 9 with 34%. *Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter aerogenes, Proteus mirabilis* and *Salmonella enteritidis* were 49, 8, 2, 5, 1 with 36, 5, 1, 4,1% for sensitivity and 12, 1, 2, 2 with 46, 4, 8, 8% for *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter aerogenes, Proteus mirabilis* only *(*Fig 2 and Fig 3).

The panels were selected on the basis of the concentrations and types of β-lactam drugs in the panel from among a number of panels available to the routine clinical laboratory [26,37]. The antibiotics aztreonam, cefazolin, cefoperazone, cefotaxime, ceftazidime were selected as they are potentially useful for the detection and differentiation of the β- lactamases present in GNB strains.

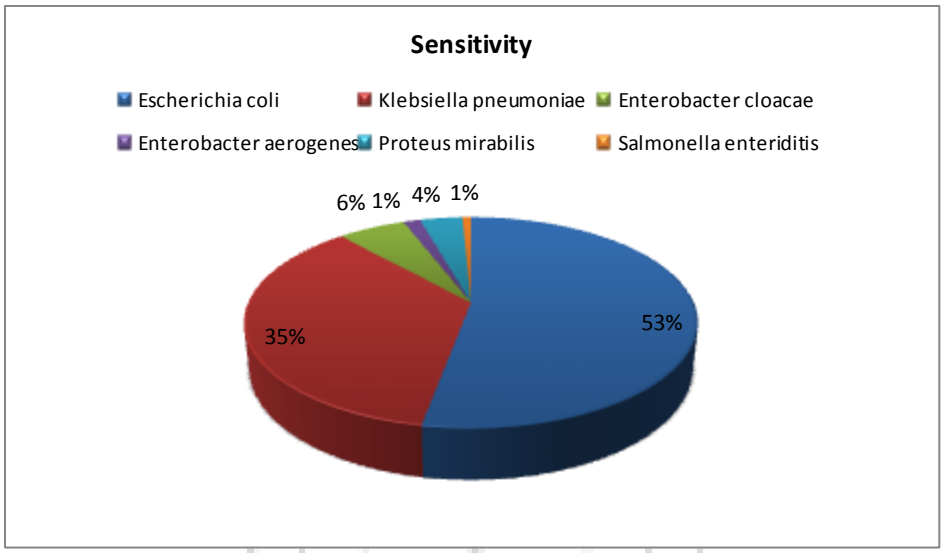


Fig 2: The percentage of bacteria isolates that exhibited sensitivity to antimicrobial agents.

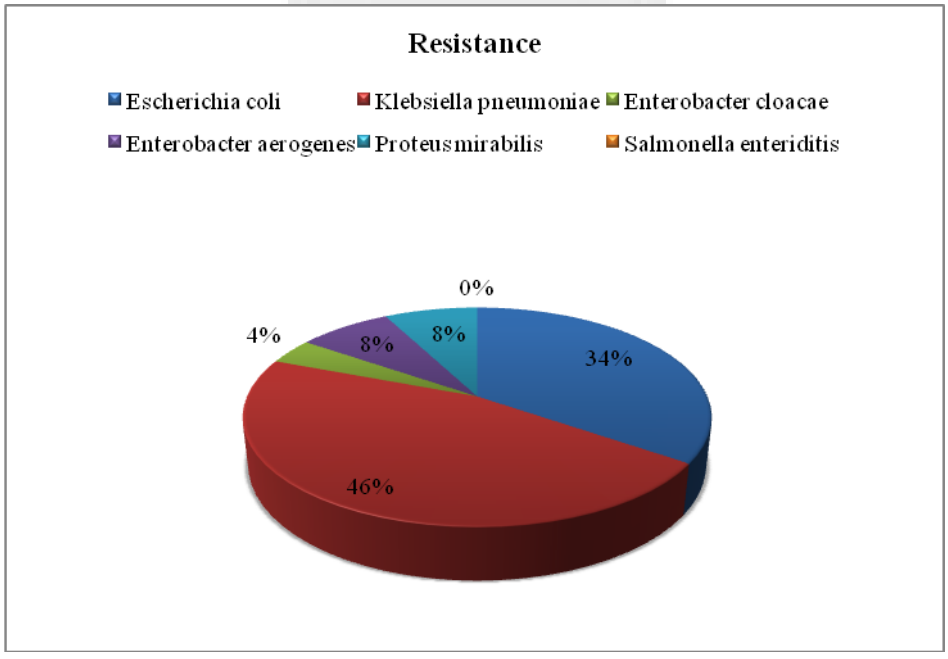


Fig 3. The percentage of bacteria isolates that exhibited resistance antimicrobial agents

Molecular epidemiological Identification:

Detection of Extended- Spectrum β-Lactamase by Multiplex Real-Time PCR polymerase chain reaction

(M RTPCR):

Thirty isolates of *K. pneumonia* and *E coli* were analyzed targeting the different genes using different primers (Table 1). The number and diversity of genes potentially complicate genetic approaches to the rapid detection of transmissible extended- spectrum β--lactamase genes. Multiplexed real-time PCR assay applied based on targets identified genes and used this to detect relevant genes in thirty consecutive clinical isolates of extended-spectrum β -lactamase (ESBL)-producing *K. pneumonia and E. coli* of *Enterobacteriaceae.*

Molecular detection by Real time polymerase chain reaction was done to detect the target genes by using Rotor-Gene Multiplex PCR) by dyes commonly used in multiplex, real-time PCR on Rotor-Gene 6000 cyclers at excitation maximum (365±20 to 680±5nm) and emission maximum (460±15 to 712nm) (Table 4). The results were read on (530±5/ 680±5nm) fluorescence. The isolates of *K. pneumonia* 1,3,6,8,9,10,12,13,14 and 15 were positive for CTX- M Gp1, the isolates 2,10,11,12,14 were positive for SHV-5/12 while all isolates for CTX-M Gp9 and VEB gene were negative. The isolates for *E. coli* were positive for CTX-M Gp1 gene in isolates 1,3,4,7,8,10,11,13,14 and 15 for. Isolates 2 and 12 were positive for CTX-M Gp9 and all *E. coli* isolates were negative for SHV-5/12 and VEB genes. Fig. 2 showed the analysis of fifteen isolates (1-15) of *K. pneumonia* genes by Multiplex Real-Time PCR polymerase chain reaction (M TPCR) A: 160 Green Channel: 470±10, B: Yellow Channel: 530±5, C: Red Channel: 625±10 and D: Crimson Channel: 680±5 fluorescence wavelength (nm). In Fig. 3 the analysis of fifteen isolates (16-30) of *E.coli* genes by Multiplex Real-Time PCR polymerase chain reaction (M TPCR) represented as separate channels as A: Green Channel: 470±10, B: Yellow Channel: 530±5, C: Red Channel: 625±10 and D: Crimson Channel: 680±5 fluorescence wavelength (nm).

Table 4: Detection of *K. pneumonia* and *E. coli* genes by Multiplex Real-Time PCR polymerase chain reaction (M RTPCR)

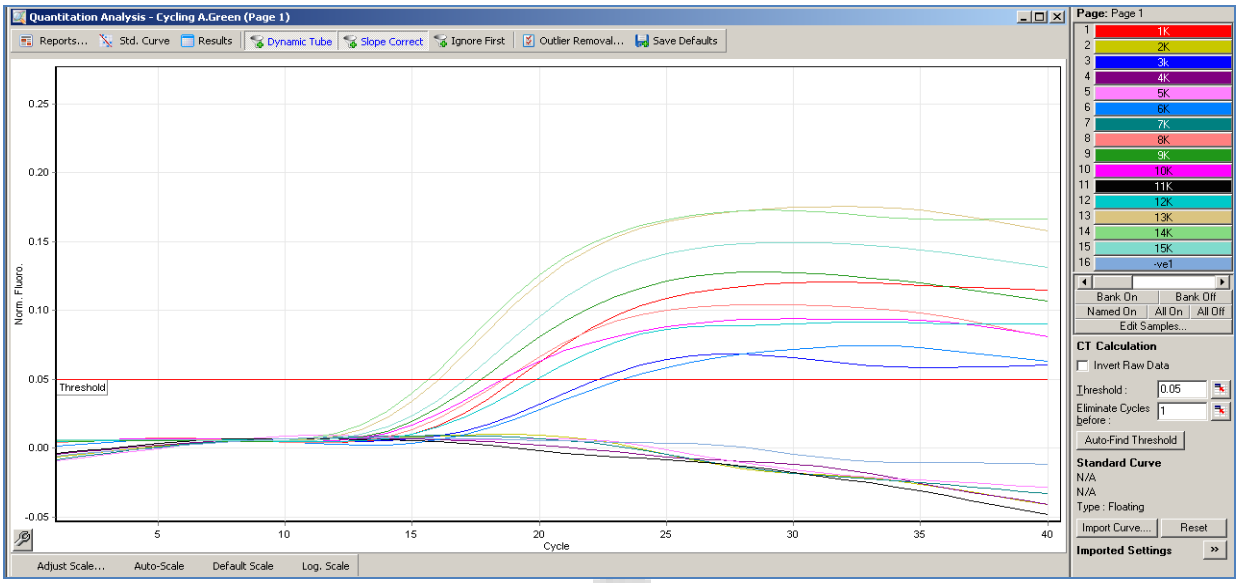
				Detection Cycle (Threshold)**			
No.	**Color**	**Bacterial Code***	**No. of Sample**	**CTX-M Gp1 (Green)**	**CTX-M Gp9 (Yellow)**	**SHV-5/12 (Crimson)**	**VEB (Red)**
1		1K	9936	19.09	---	---	---
2		2K	21585	---	---	15.26	---
3		3k	21440	22.32	---	---	---
4		4K	34648	---	---	---	---
5		5K	10375	---	---	---	---
6		6K	10309	23.30	---	---	---
7		7K	21430	---	---	---	---
8		8K	10314	18.62	---	---	---
9		9K	35355	17.70	---	---	---
10		10K	10611	18.55	---	17.83	---
11		11K	11024	---	---	15.33	---
12		12K	10501	19.92	---	19.33	---
13		13K	22629	16.05	---	---	---
14		14K	36247	15.65	---	18.26	---
15		15K	23352	17.09	---	---	---
16		1E	40152	18.78	---	---	---
17		2E	33056	---	15.73	---	---
18		3E	35176	20.88	---	---	---
19		4E	34859	17.33	---	---	---
20		5E	21659	---	---	---	---

No.	Color	Bacterial Code*	No. of Sample	Detection Cycle (Threshold)**			
				CTX-M Gp1 (Green)	CTX-M Gp9 (Yellow)	SHV-5/12 (Crimson)	VEB (Red)
21		6E	35466	---	---	---	---
22		7E	10533	15.80	---	---	---
23		8E	35566	16.91	---	---	---
24		9E	22165	---	---	---	---
25		10E	35571	17.59	---	---	---
26		11E	36483	17.36	---	---	---
27		12E	37723	---	20.03	---	---
28		13E	11390	16.43	---	---	---
29		14E	37545	17.45	---	---	---
30		15E	22939	19.69	---	---	---

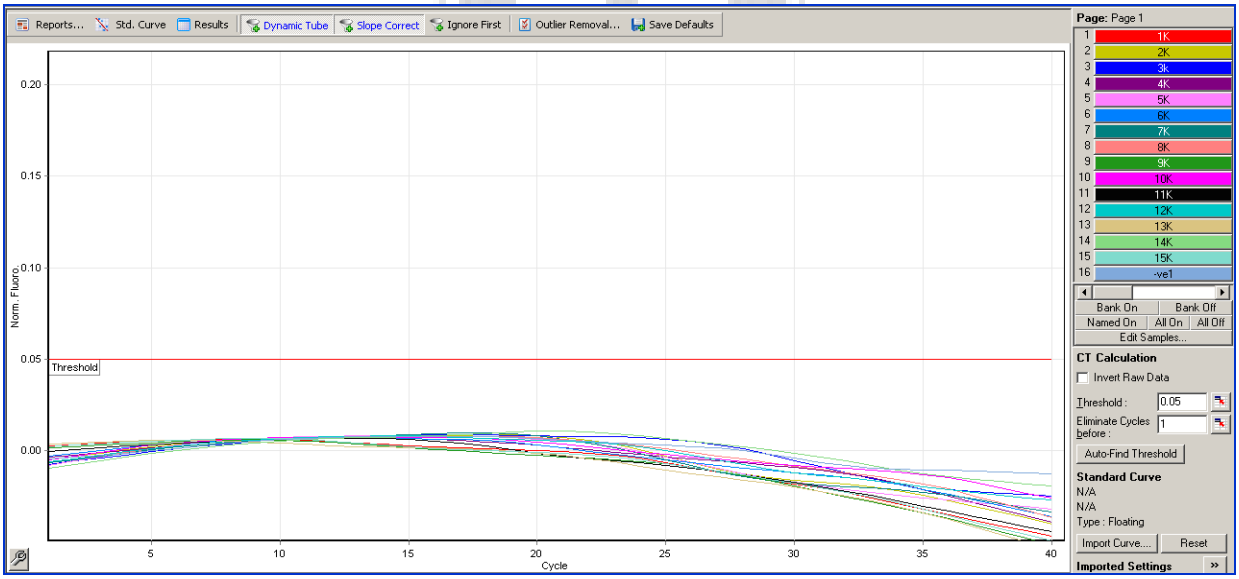
* K represents *K. pneumoniae* samples and *E* for *E coli.*

** If the sample has a number, it means it is a positive sample (has the gene). If it hasn't (---) it means the sample is negative (doesn't have the gene).

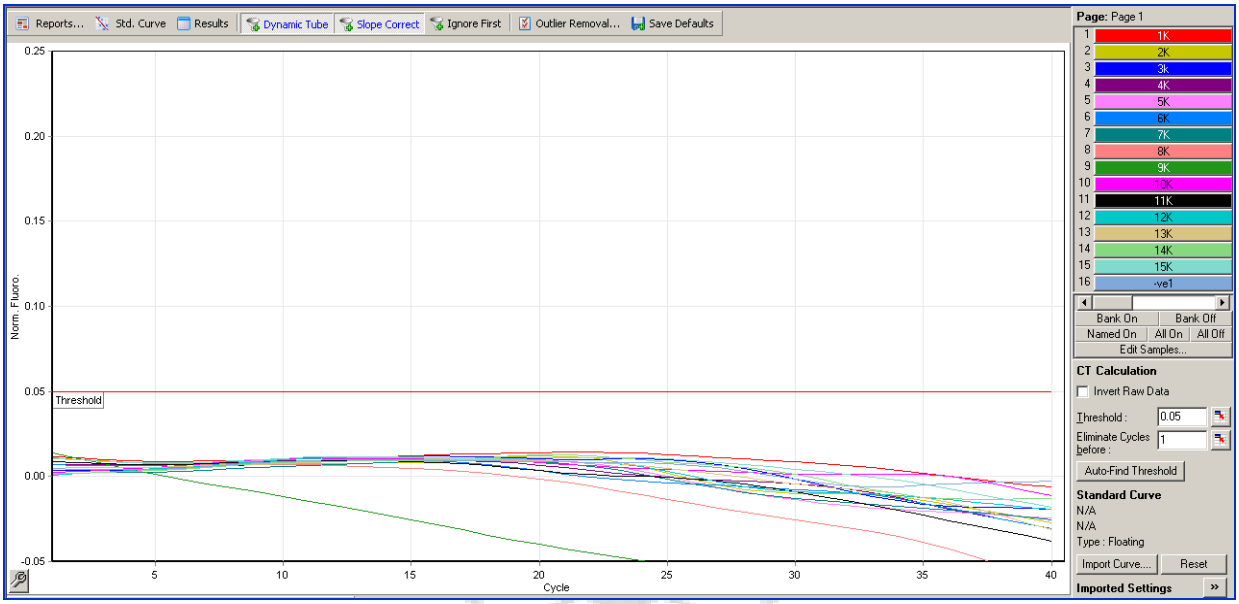
A: Green Channel:470±10



B: Yellow Channel:530±5



C: Red Channel: 625±10



D: Crimson Channel: 680±5

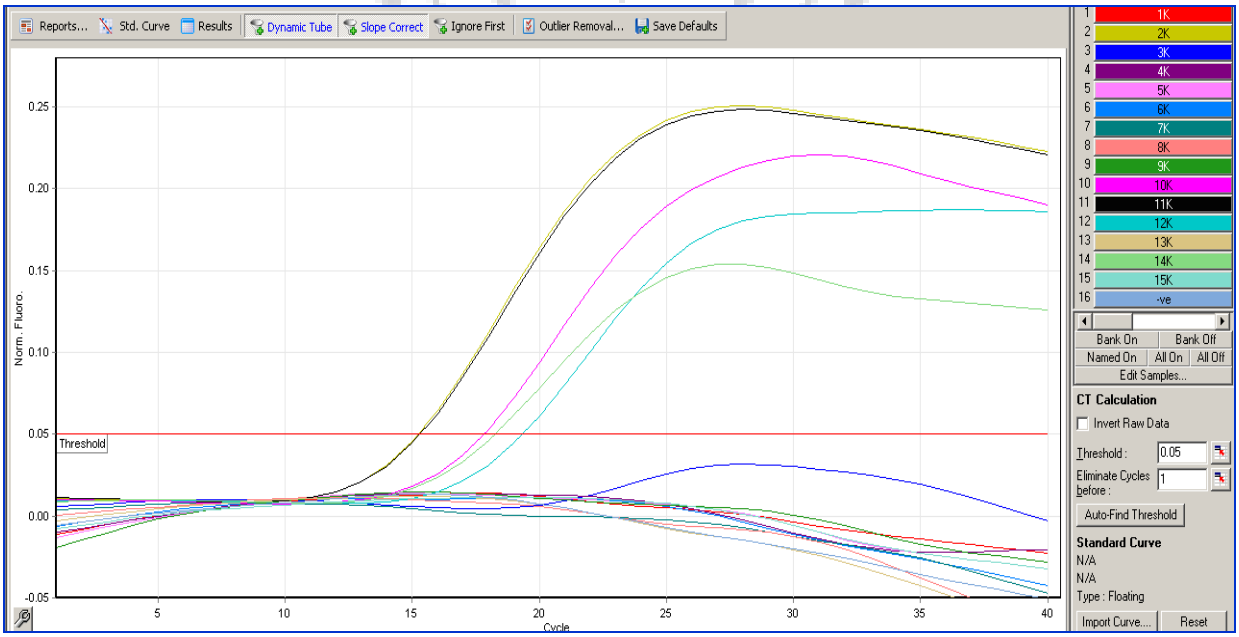
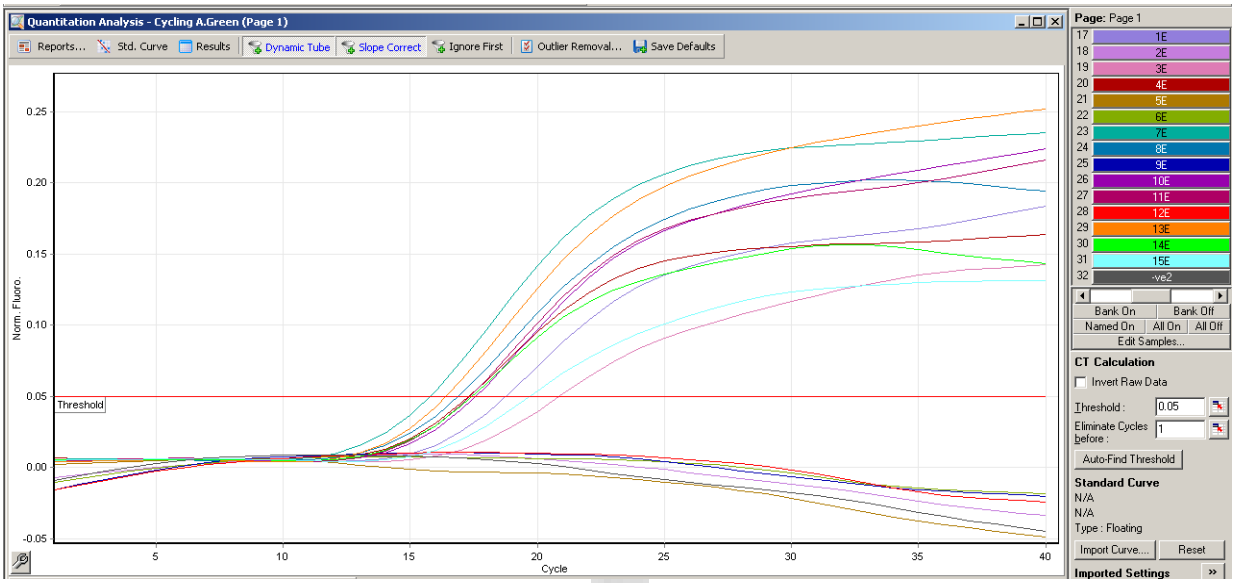
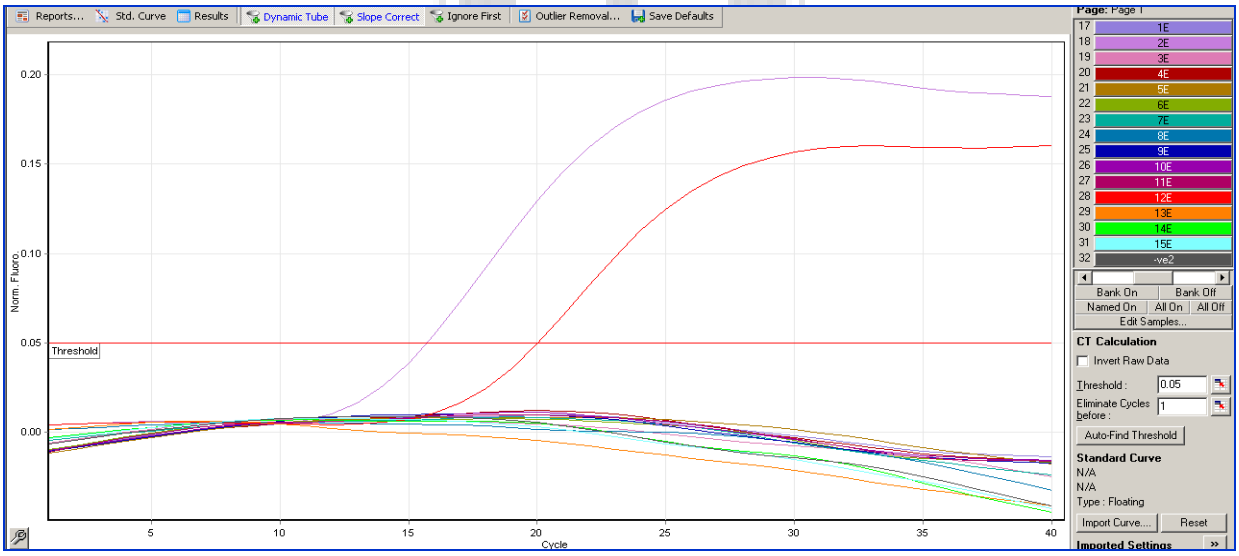


Fig 2: Analysis of fifteen isolates (1-15) of *K. pneumonia* genes by Multiplex Real-Time PCR polymerase chain reaction (M TPCR) A: Green Channel: 470±10 , B:Yellow Channel: 530±5 , C: Red Channel: 625±10 and D: Crimson Channel: 680±5 fluorescence wavelength (nm) .

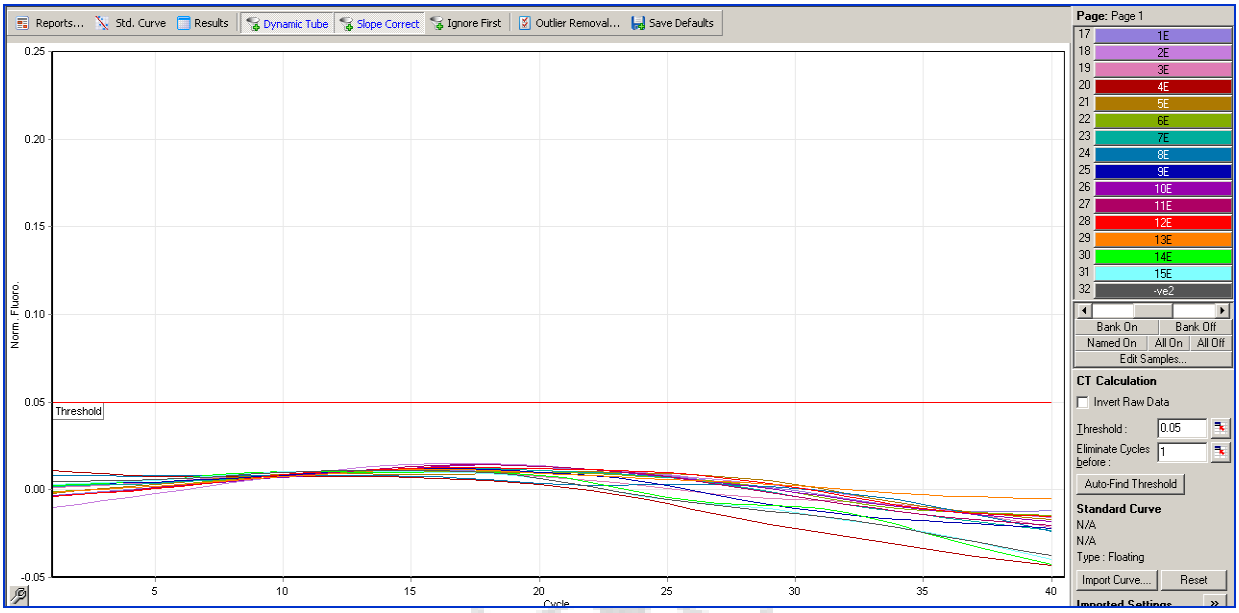
A: Green Channel:470±10



B:Yellow Channel:530±5



C: Red Channel: 625±10



D: Crimson Channel: 680±5

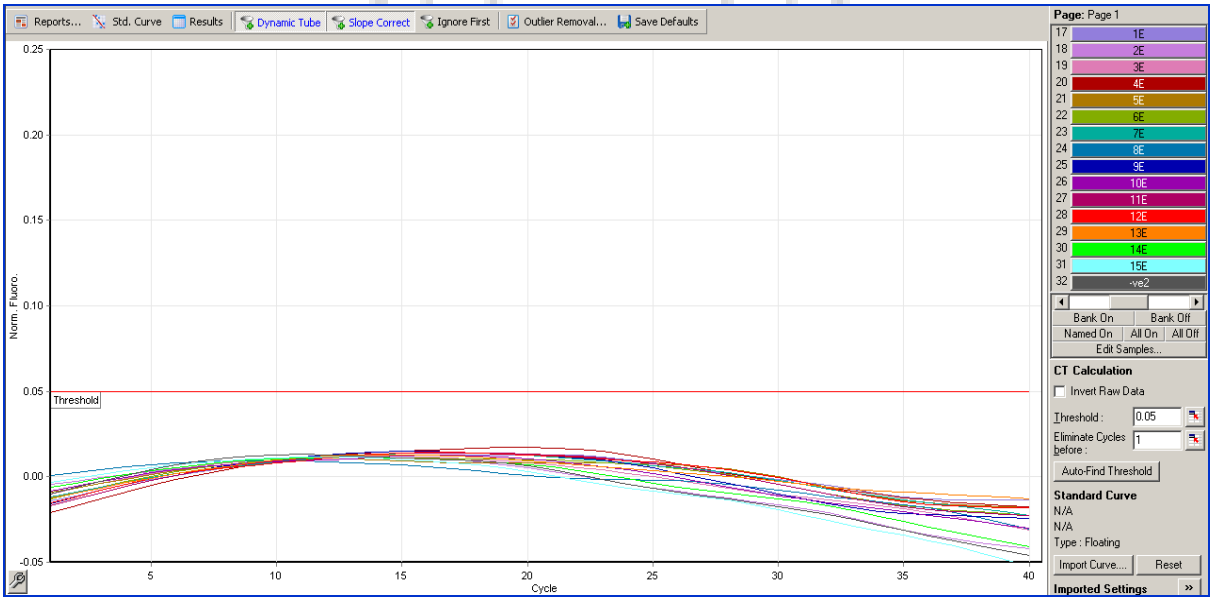


Fig 3: Analysis of fifteen *isolates* (16-30) of *E.coli* genes by Multiplex Real-Time PCR polymerase chain reaction (M TPCR) A: Green Channel: 470±10, B: Yellow Channel: 530±5, C: Red Channel: 625±10 and D: Crimson Channel: 680±5 fluorescence wavelength (nm).

Molecular subtyping of *Escherichia coli* typing by Pulsed field Gel electrophoresis (PFGE):

PFGL analysis of fifteen clinical isolates *E. coli* samples for ESBL production has been carried out by using CHEF- DR 111 systems from Bio- Rad according to Corona – Nikamura *et al*. Isolates were genotyped using PFGE. Genomic DNA from each fifteen isolates of *E. coli* were isolated in an agarose-embedded form and subjected to in-gel enzymic digestion using 10 U of *Spe* I digested DNA. The samples were examined by PFGE to establish a potential clonal relationship among them. Analysis showed that (Table 5, 6) isolates had identical band patterns on the gel, indicating a clonal relationship between these strains. Fig. 4 had fifteen band pattern that didn't resemble the others indicating no clonal relationship to these strains. Lan nine represents positive control. Fig 5 shows pulsotype were analyzed by both computer – assisted program (BioNumerics Software) and by manual or visual comparison of each banding patterns.

Table 5: Dendrogram similarity between isolates of *E. coli*

Dendrogram | Lane Similarity | Dendrogram Similarity | Cluster Groups

Lane	e_ coli sample:9	e_ coli samples:1	e_ coli samples:10	e_ coli samples:11	e_ coli samples:12	e_ coli samples:13	e_ coli samples:14	e_ coli samples:15	e_ coli samples:2	e_ coli samples:3	e_ coli samples:4	e_ coli samples:5	e_ coli samples:6	e_ coli samples:7	e_ coli samples:8	e_ coli samples:Control
e_ coli sample:9	100	40	60	60	54	62	48	48	48	48	40	48	54	54	74	70
e_ coli samples:1	40	100	40	40	40	40	40	40	40	40	50	40	40	40	40	40
e_ coli samples:10	60	40	100	82	54	60	48	48	48	48	40	48	54	54	60	60
e_ coli samples:11	60	40	82	100	54	60	48	48	48	48	40	48	54	54	60	60
e_ coli samples:12	54	40	54	54	100	54	48	48	48	48	40	48	63	76	54	54
e_ coli samples:13	62	40	60	60	54	100	48	48	48	48	40	48	54	54	62	62
e_ coli samples:14	48	40	48	48	48	48	100	73	60	53	40	53	48	48	48	48
e_ coli samples:15	48	40	48	48	48	48	73	100	60	53	40	53	48	48	48	48
e_ coli samples:2	48	40	48	48	48	48	60	60	100	53	40	53	48	48	48	48
e_ coli samples:3	48	40	48	48	48	48	53	53	53	100	40	78	48	48	48	48
e_ coli samples:4	40	50	40	40	40	40	40	40	40	40	100	40	40	40	40	40
e_ coli samples:5	48	40	48	48	48	48	53	53	53	78	40	100	48	48	48	48
e_ coli samples:6	54	40	54	54	63	54	48	48	48	48	40	48	100	63	54	54
e_ coli samples:7	54	40	54	54	76	54	48	48	48	48	40	48	63	100	54	54
e_ coli samples:8	74	40	60	60	54	62	48	48	48	48	40	48	54	54	100	70
e_ coli samples:Control	70	40	60	60	54	62	48	48	48	48	40	48	54	54	70	100

Table 6: Cluster groups are represented at distance 0.4 for fifteen isolates of *E. coli.*

Dendrogram | Lane Similarity | Dendrogram Similarity | Cluster Groups

Lane	Group
e_coli sample:9	3
e_coli samples:1	7
e_coli samples:2	5
e_coli samples:3	4
e_coli samples:4	6
e_coli samples:5	4
e_coli samples:6	1
e_coli samples:7	1
e_coli samples:8	3
e_coli samples:Control	3
e_coli samples:10	2
e_coli samples:11	2
e_coli samples:12	1
e_coli samples:13	3
e_coli samples:14	5
e_coli samples:15	5

Positive control Sample 10

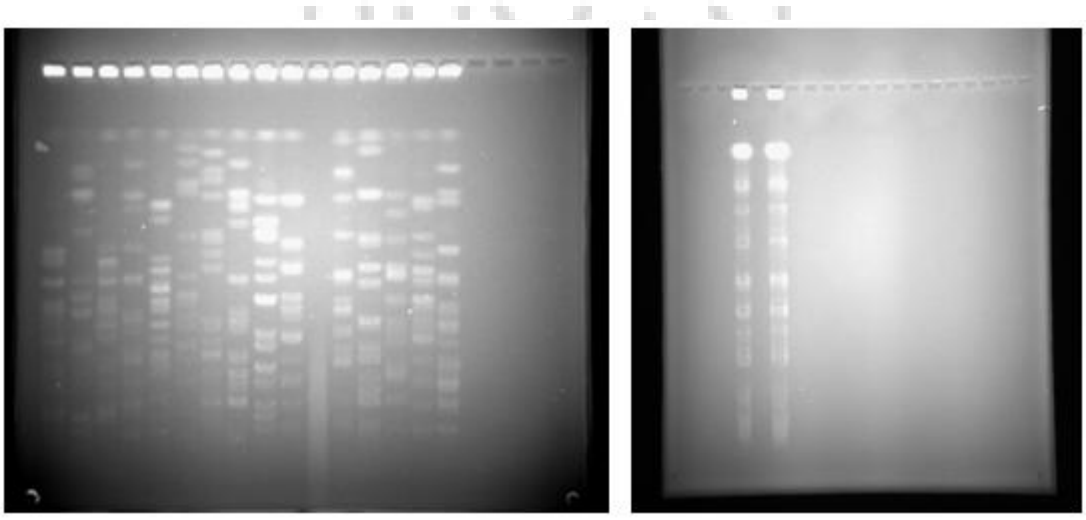


Fig 4.: Genotyping by Pulsed Field Gel Electrophoresisto *E. coli* isolates

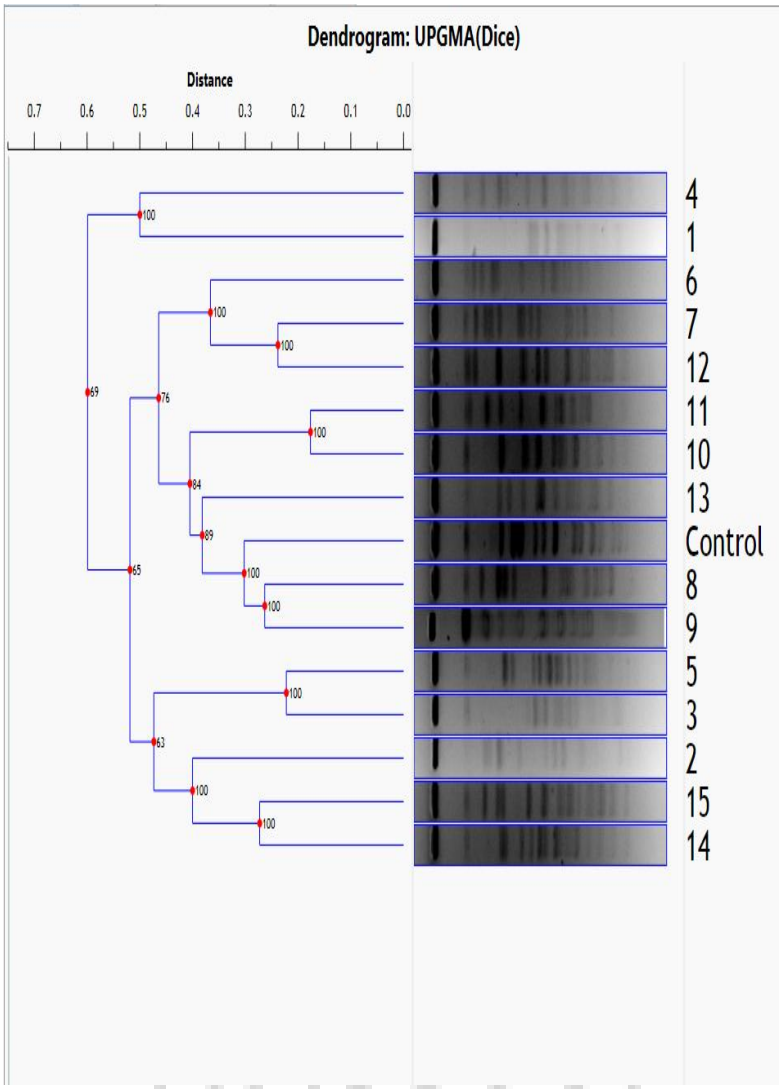


Fig 5: Denedogram similarity of fifteen isolates of *E. coli*

Microarray for Detection and Identification of extended spectrum β lactamase Resistant *Enterobacteriaceae*

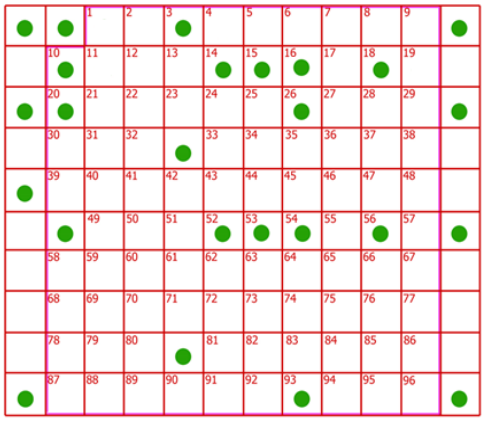
The method of DNA microarray consisted of several steps involving DNA extraction, amplification and labeling of target DNA with specific oligonucleotide probes immobilized on porous membrane support. Careful optimization of oligonucleotide probes and hybridization conditions ensured specific identification of all control isolates producing ESBLs and carbapenemases in a single array. The method developed has been applied successfully for the detection of ESBL and carbapenemase genes in a series of clinical isolates of *Enterobacteriaceae, Klebsiella pneumoniae* and *E. coli.* The Check-MDR CT103 microarray,

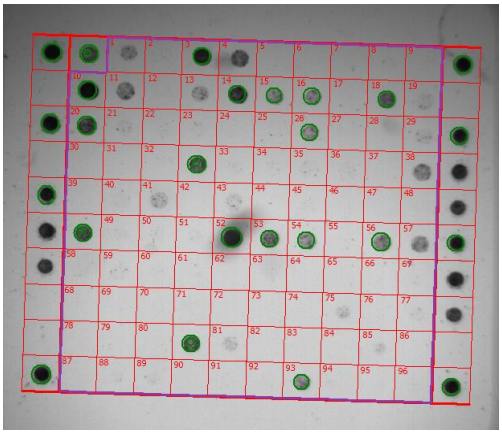
aimed at identifying bacteria producing extended-spectrum β –lactamase (ESBL) (SHV, TEM, and CTX-M) and carbapenemase (KPC, OXA-48, VIM, IMP, and NDM-1), was evaluated on a total of thirty Gram-negative isolates expressing various β -lactamases. The sensitivity and specificity were 100% for most tested genes, suggesting that this assay allows accurate identification of common ESBL and carbapenemase producers from bacterial cultures. The green colour represents the control in all isolates tested. In *Escherichia coli* isolates number one was positive for ESBL,CTX-M group (type 1, ND **) SHV SNP (WT + 238S genes). The isolates (2,6,1 5,16,24 and 25) were positive for ESB, CTX-M group (1, type-15 like *) and TEM SNP type (WT) gene. Isolate seven was positive for ESB, CTX-M group (1, type-15 like *) and fourteen was positive for ESB, CTX-M group type 9 and TEM SNP type (WT) gene (Fig 6).

The isolates twelve, twenty one, twenty eight and twenty nine were positive for the (ESB, CTX-M group type 9), (ESB, AMPC, AMPC type (DHA), CTX-M group (1, type-15 like *) and TEM SNP type (WT) gene.

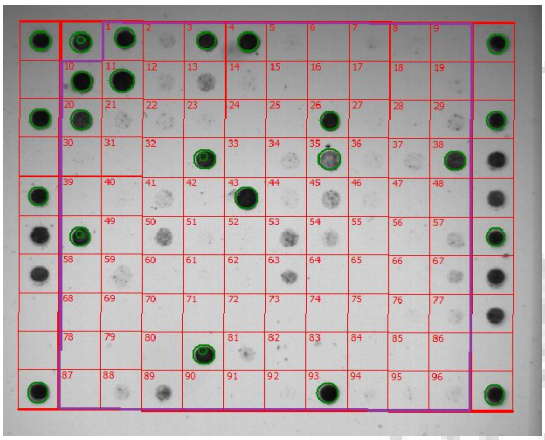
(ESB, CTX-M group (1, type-15 like *), SHV SNP type (WT)., TEM SNP type (WT) genes respectively. The detection of genes in *Klebsiella pneumonia* (Fig 7) were as follow: the isolates three and four were positive for ESBL, CTX-M group type (1, ND **) SHV SNP type (WT + 240K) TEM SNP type (WT), CARBA, ESBL , CARBA type (OXA-48), SHV SNP type (WT + 238S + 240K) genes respectively. Isolates (8, 9, 10,11,13,26, 27) were positive for ESBL, CTX-M group (1, type-15 like *), SHV SNP TYPE (WT) and TEM SNP type (WT). The isolates seventeen, eighteen and nineteen had the genes (AMPC, AMPC type (DHA), SHV SNP type (WT) and TEM SNP type (WT), (ESBL, CTX-M group (1, type-15 like *) and SHV SNP type (WT + 238S + 240K) . The isolates twenty two, twenty three and thirty were positive for (ESBL, SHV SNP type (WT + 238S), CARBA, ESBL, CARBA type (OXA-48) and SHV SNP type (WT + 238S + 240K), (TEM SNP type (WT) as well the isolate twenty (TEM SNP type (WT). The sensitivity of the microarray was 95%; and the specificity 100% using molecular characterization of ESBLs.

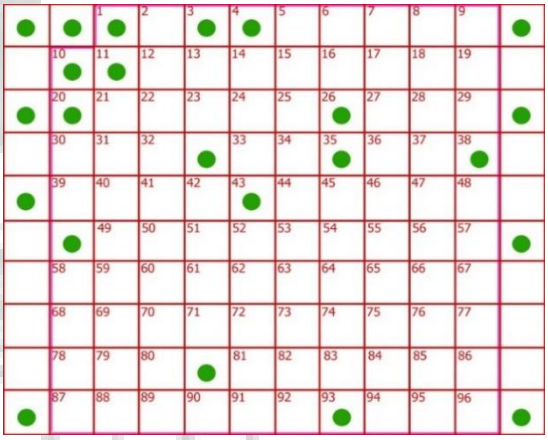
(21659) *E. coli*



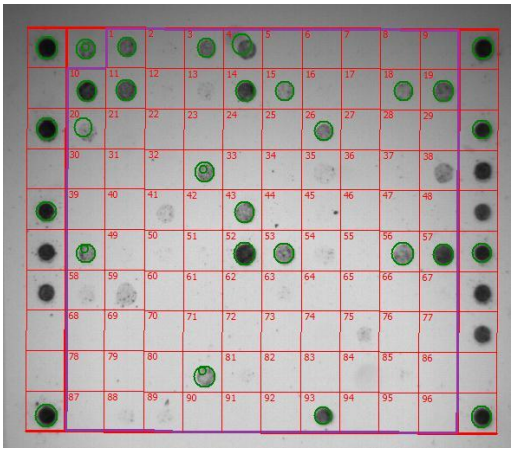


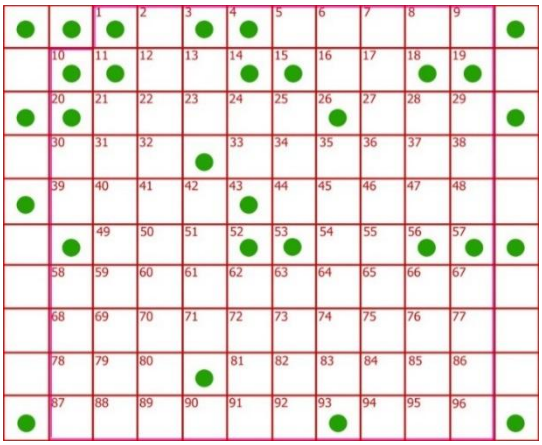
(36483) *E. coli*



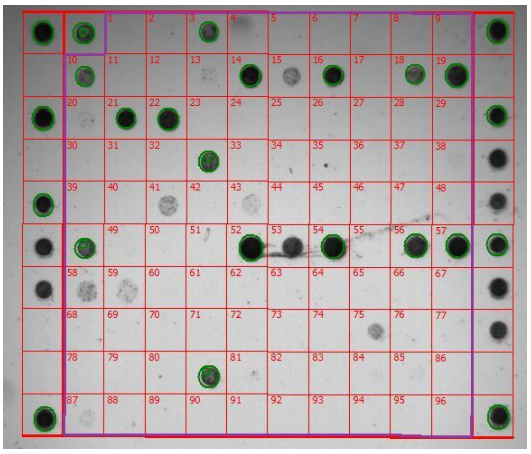


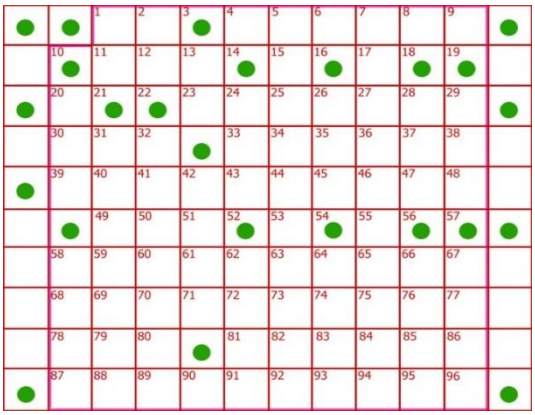
(22939) *E. coli*



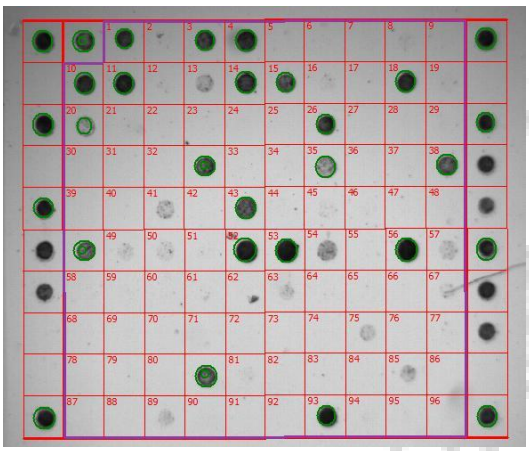


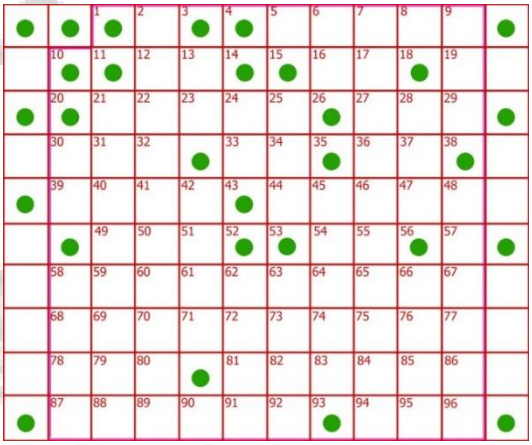
(10533) *E. coli*



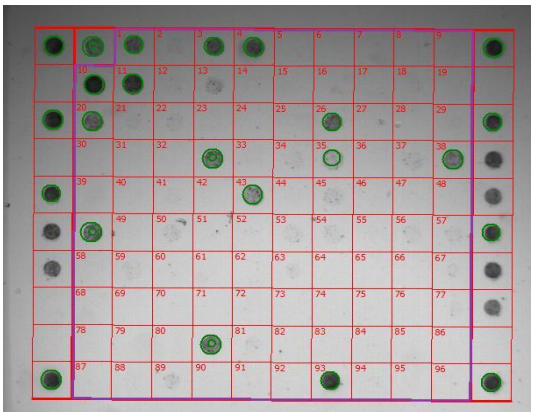


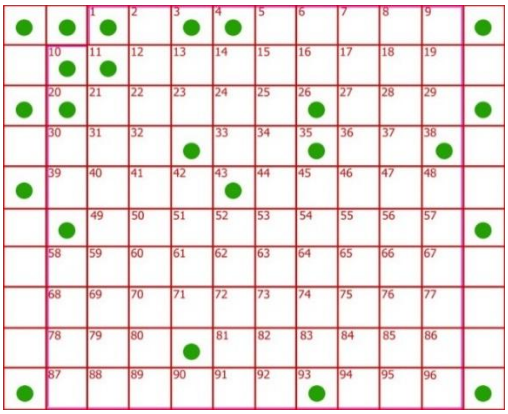
(37446) *E. coli*



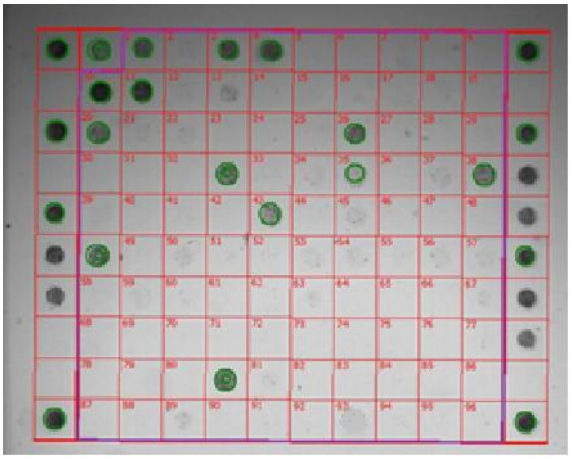


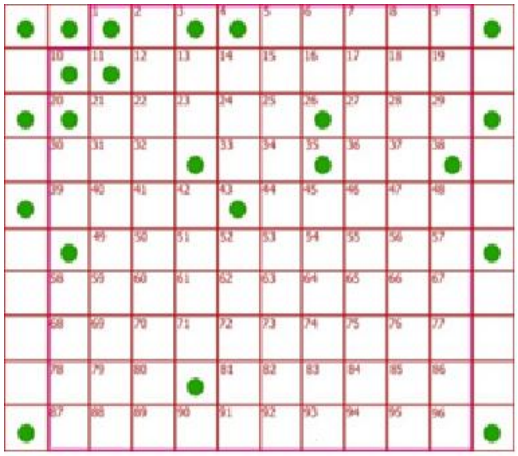
(37723) *E. coli*



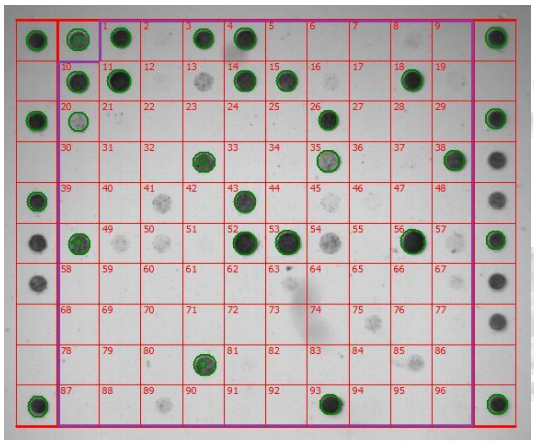


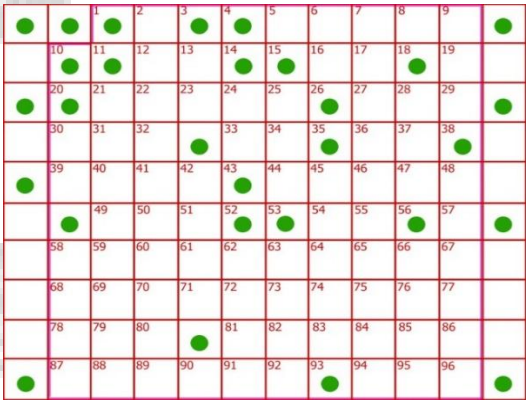
(35571) *E. coli*



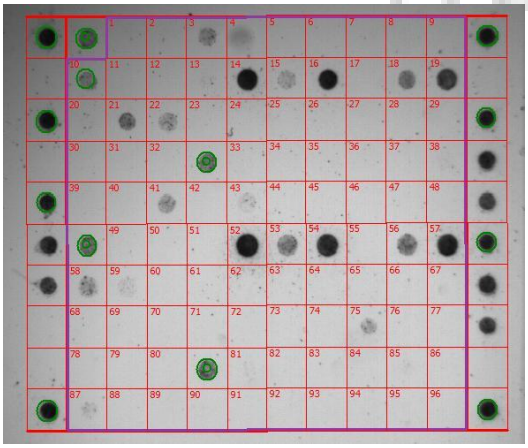


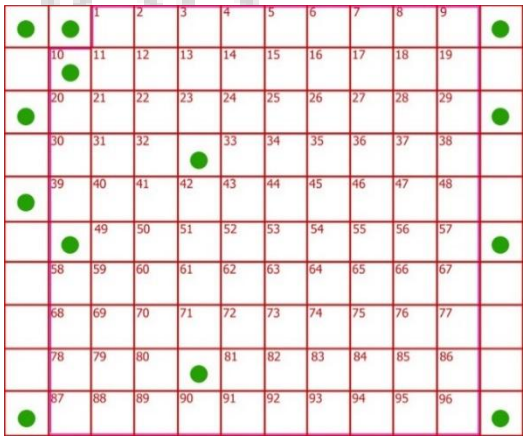
(35566) *E. coli*



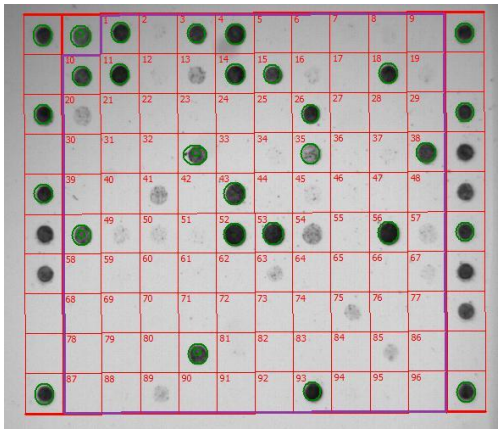


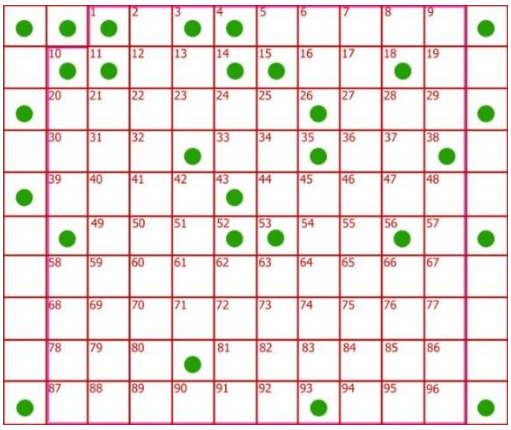
(03344) *E. coli*



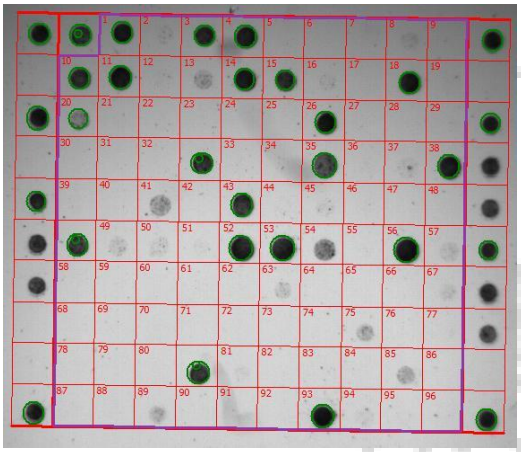


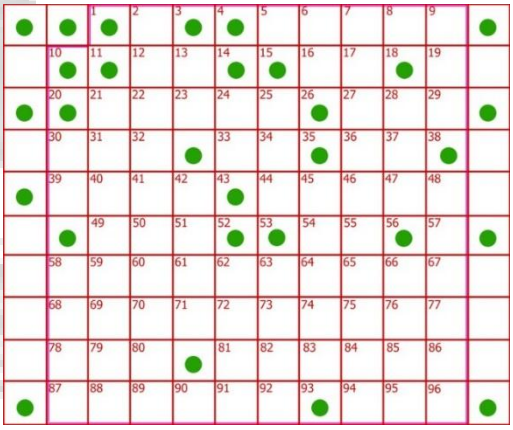
(35176) *E. coli*



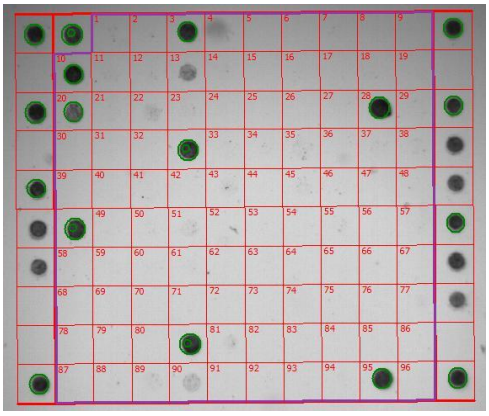


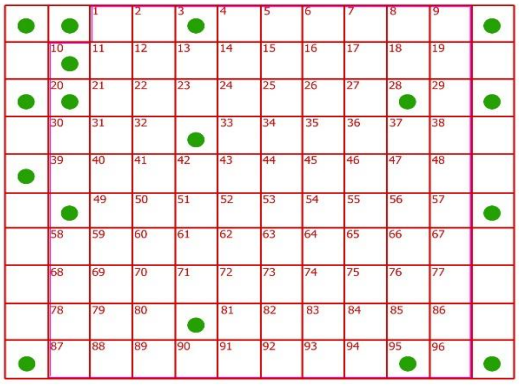
(38697) *E. coli*





(20568) *E. coli*





(37724) *E. coli*

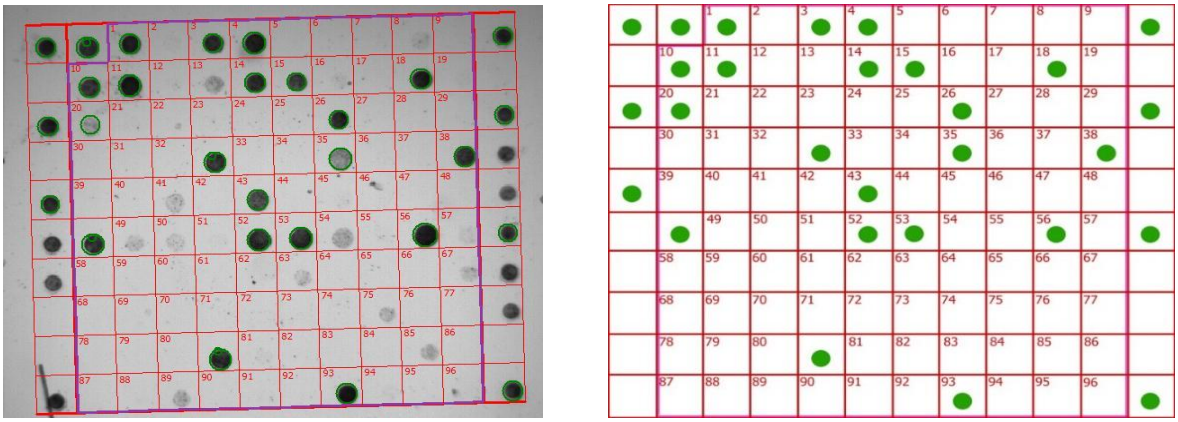
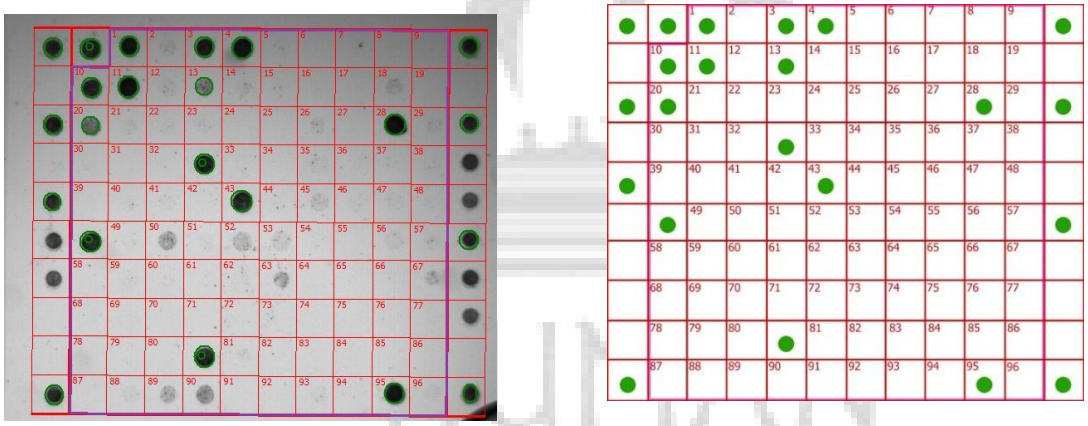
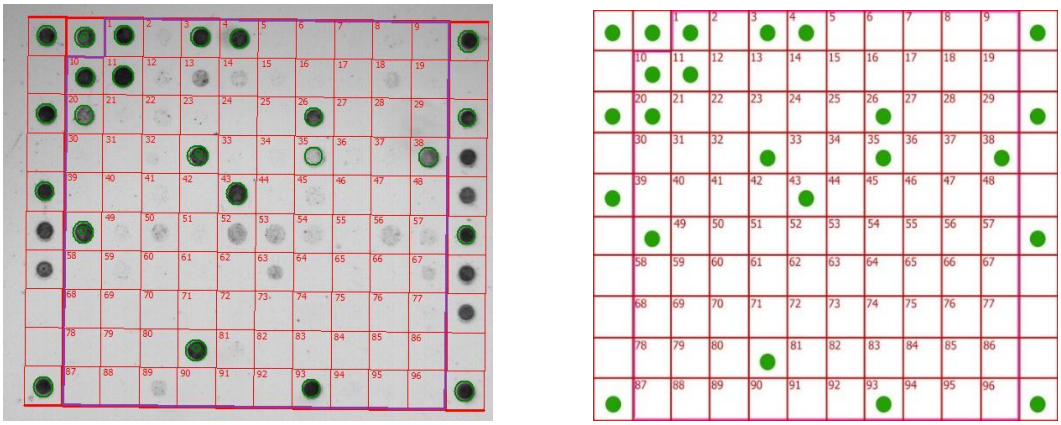


Figure 6: Image of DNA microarray pictures obtained with the Check-MDR CT103 microarray identifying fifteen *E. coli* isolates producing extended-spectrum β–lactamase (ESBL) (SHV, TEM, and CTX-M)

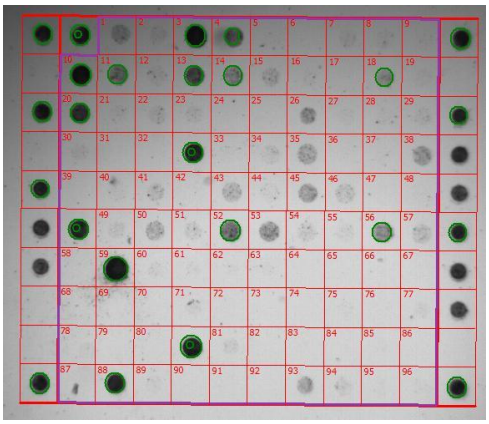
(21440) *K. pneumoniae*

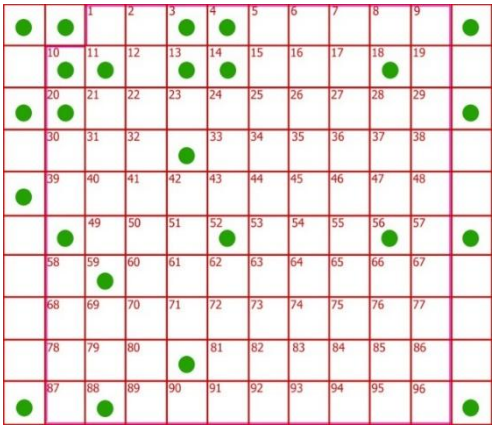


(11024) *K. pneumoniae*

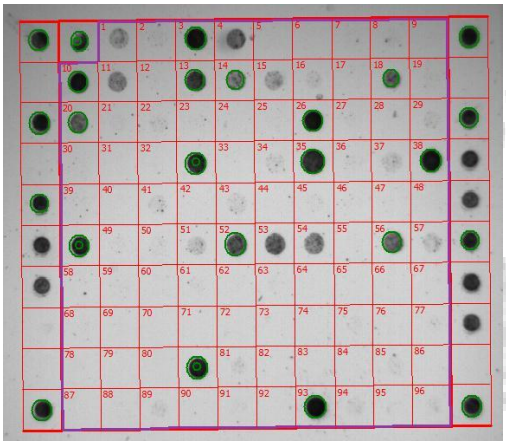


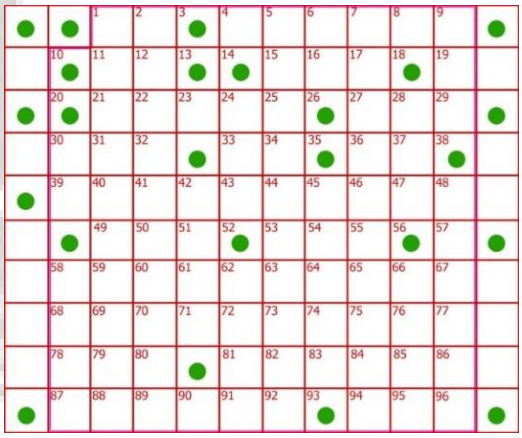
(10309) *K. pneumoniae*



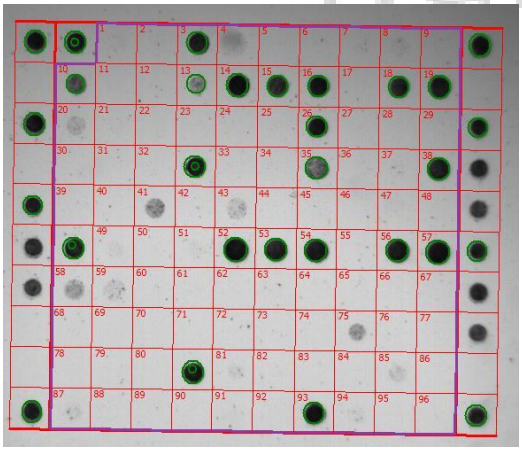


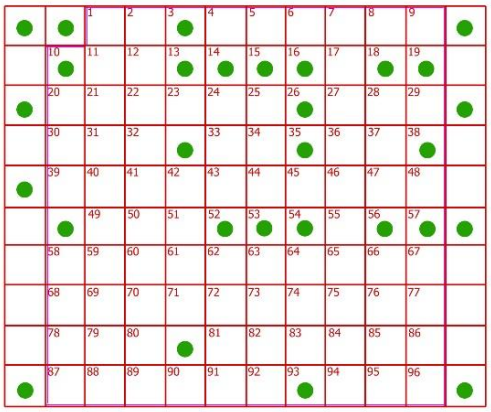
(21585) *K. pneumoniae*



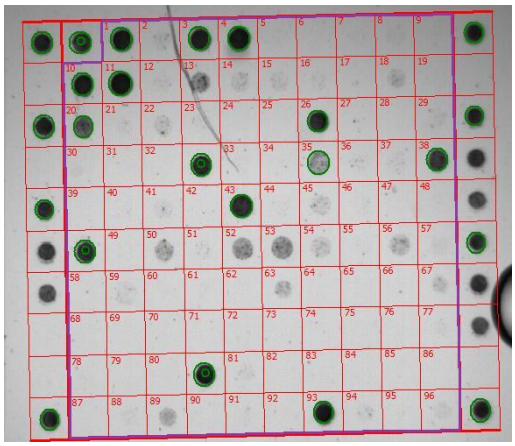


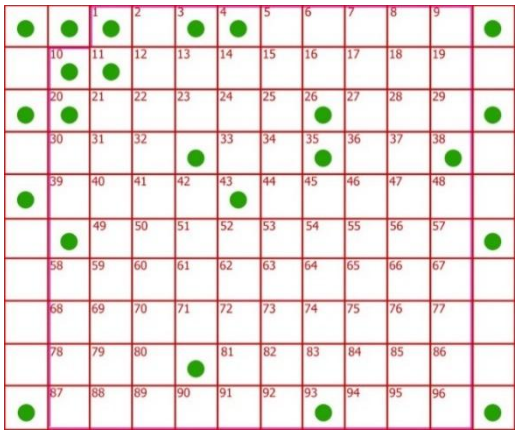
(9936) *K. pneumonia*



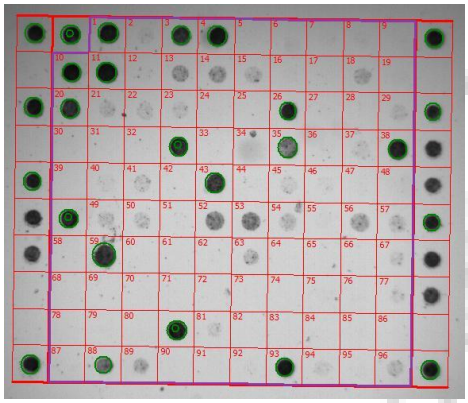


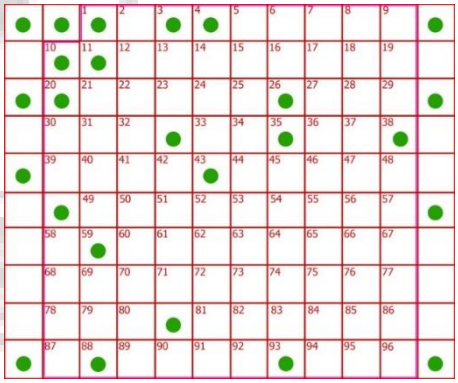
(23096) *K. pneumoniae*



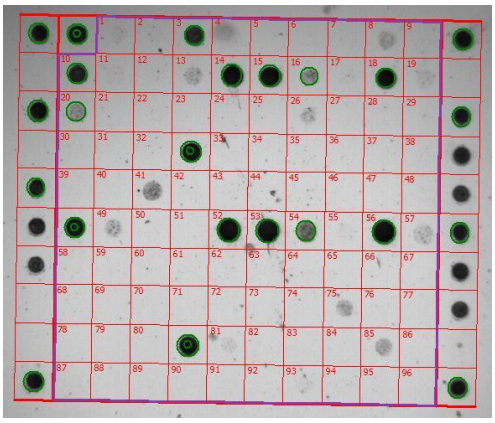


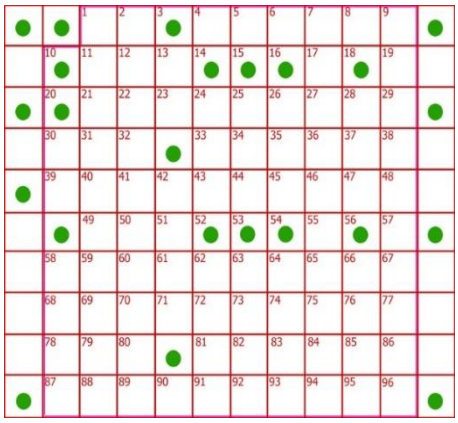
(23352) *K. pneumoniae*



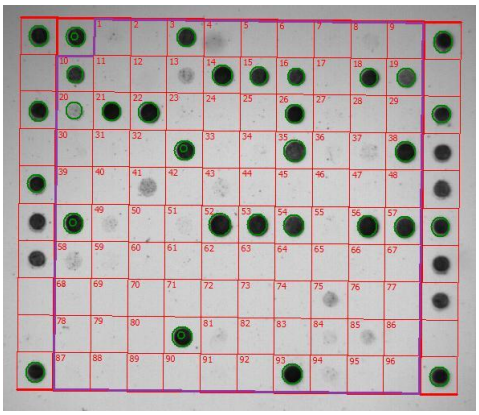


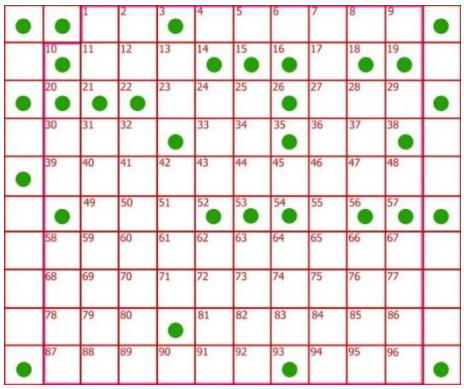
(21430) *K. pneumoniae*



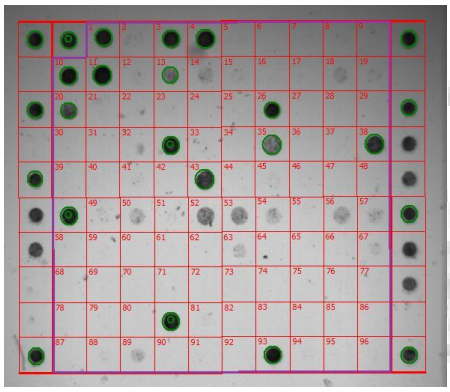


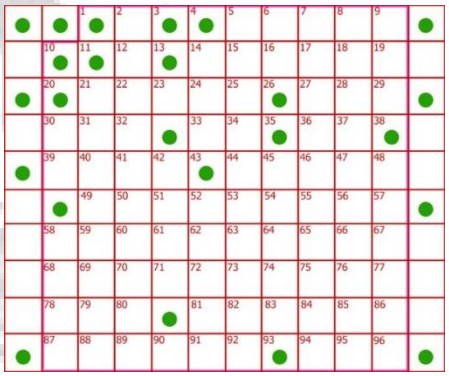
(35355) *K. pneumoniae*



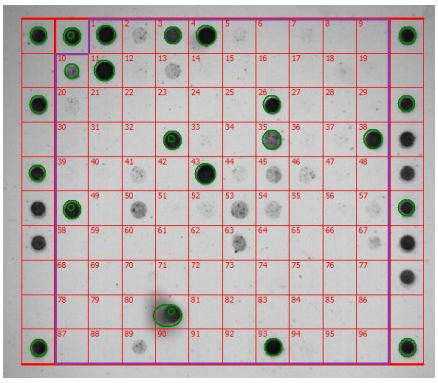


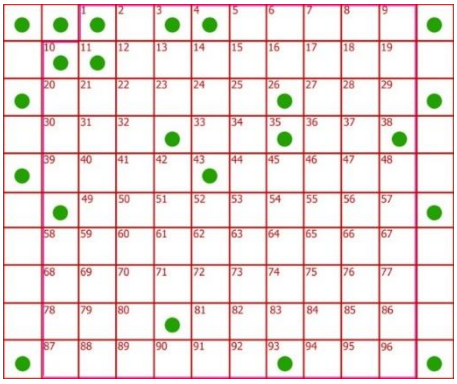
(10611) *K. pneumoniae*



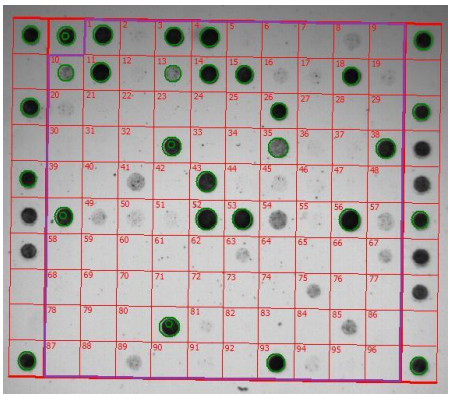


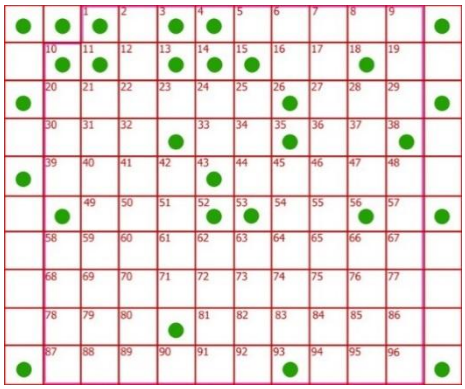
(37494) *K. pneumoniae*



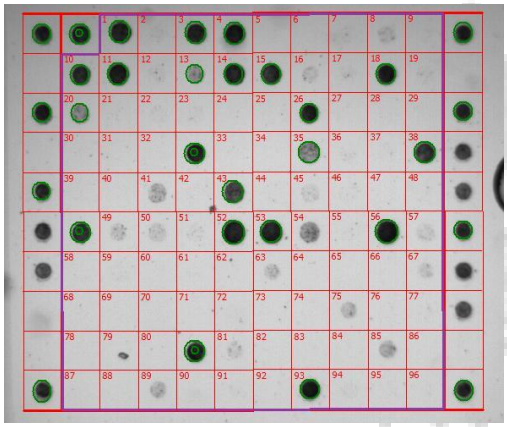


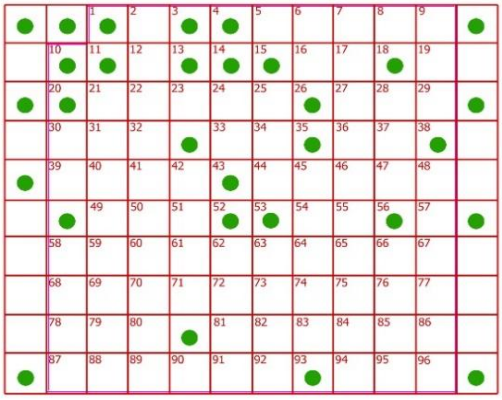
(34648) *K. pneumoniae*



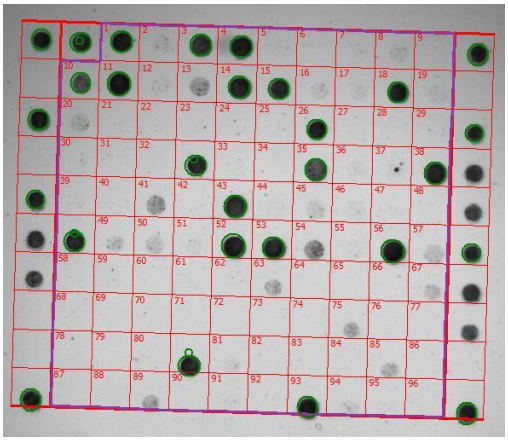


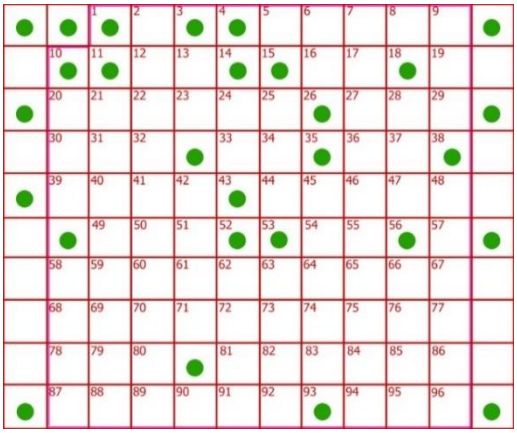
(36247) *K. pneumoniae*



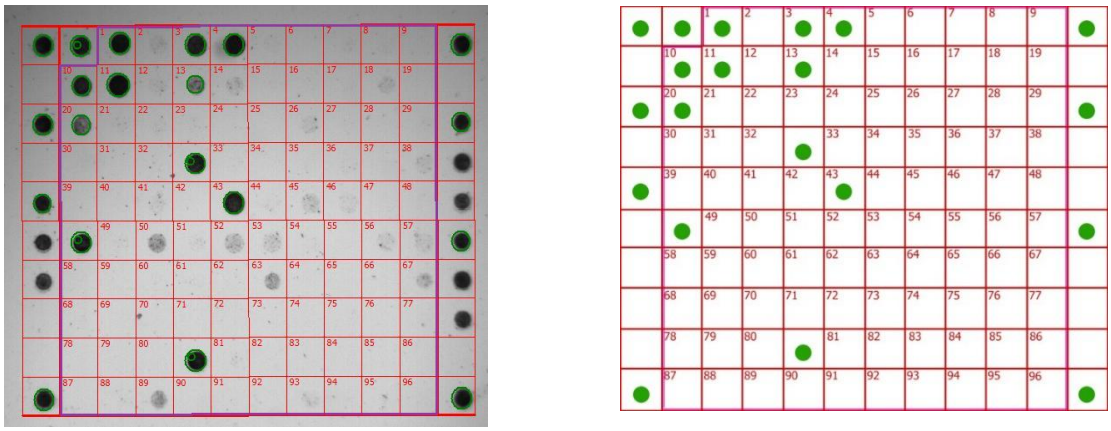


(23736) *K. pneumoniae*





(23772) *K. pneumoniae*



(37603) *K. pneumoniae*

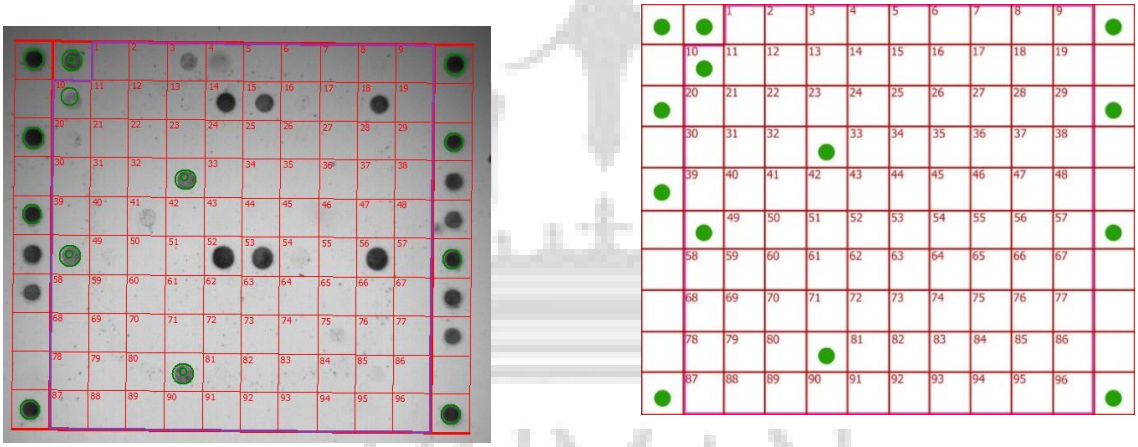


Figure 7: Image of DNA microarray pictures obtained with the Check-MDR CT103 microarray identifying fifteen *K. pneumoniae* isolates producing extended-spectrum β–lactamase (ESBL) (SHV, TEM, and CTX-M)

DISCUSSION

This study was done on hundred sixty three isolates from different sources concluded as the highest number was *E. coli* followed by *K. Pneumonia, Enterobacter cloacae, Proteus mirabilis, Enterobacter aerogenes and Salmonella enteritidis.* ESBls resistance was detected in 81 (50%) of isolates of *E. coli* and 63 (39%) in those with *K. pneumonia*e. 5%, 1%, 4% and 1% for *Enterobacter cloacae, Enterobacter aerogenes, Proteus mirabilis and Salmonella enteritidis*

respectively collected from all sources were used in this study. The present study revealed that there is a high frequency isolates of ESBls producing strains of *E. coli a*nd *K. pneupmiae* in both community and hospital. This has a significant implication for patients, management. Further drug resistance surveillance in other hospitals and molecular characteristics of ESBls isolates is necessary (6). Reported study *Escherichia coli* predominated followed by *Klebsiella* species, *Enterobacter* species with least common organisms are *Proteus* species (6).

The high percentage isolation from female (88%) than males (76%). It was noted that females reported with higher injury rate than males. The distribution pattern of ESBL producing *E. cloacae* varies in different studies suggesting that these infections are not gender specific and the ratio of female patients attending the hospital during the study period might be higher than the male patients. ESBL producing organisms were prevalent among all age groups and there was no statistically significant difference among various age groups. This increasing prevalence of ESBL producing organisms as shown by this data in all groups draw the attention toward the overall increase in ESBL producing organisms load.

The distribution percentage for all 163 isolates in different source: MB special for swabs / BI special for TB lab/ UR special for urine. In our study, *Escherichia coli* followed by *Klebsiella pneumonia, Enterobacter cloacae, Enterobacter aerogenes, Proteus mirabilis* and the latest were in *Salmonella enteritidis* 66,18,7,3.4.,4.5 and 1.1% in UR and 31.2,60.4,4.16, 4.16% and negative for *Enterobacter aerogenes and Salmonella enteritidis in* in MB. Twenty seven, 69,4% for *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis* and negative for *Enterobacter cloacae, Enterobacter aerogenes and Salmonella enteritidis in* for BI. The total highest percentage were showed in *Escherichia coli* (50%), (39%) for *Klebsiella pneumonia*.

The study was done to determine the frequency of extended spectrum beta-lactamases (ESBLs) producing *Escherichia coli* and *Klebsiella pneumonia.* The MicroScan walkaway 96 plus which having panels for gram negative bacteria (Neg/BP/Combo NM35 panels), is designed for the identification of gram-negative bacilli. We evaluated its ability to accurately identify *Enterobacteriaceae* that is routinely encountered in a clinical laboratory. This study approached 89% accuracy for the identification of gram-negative organisms encountered in the hospital laboratory. In our study, *Escherichia coli* showed sensitivity to antibiotics with 53% for 73

isolates and resistant were 9 with 34%. *Klebsiella pneumoniae, Enterobacter cloacae, Enterobacteraerogenes, Proteus mirabilis* and *Salmonella enteritidis* were 49,8,2,5,1 with 36,5,1,4,1% for sensitivity and 12,1,2,2 with 46,4,8,8% for *Klebsiella pneumonia, Enterobacter cloacae, Enterobacter aerogenes, Proteus mirabilis* only. Ceftazidime (ESBL) was positively ESBL produced by *Escherichia coli, Klebsiella pneumonia, Enterobacter cloacae, Enterobacter aerogenes* and *Proteus mirabilis* 74, 53, 6, 2, 7 (52.1, 37.3, 4.1, 5%). Cefotaxime (ESBL) was positively ESBL produced by *Escherichia coli, Klebsiella pneumonia, Enterobacter cloacae , Enterobacter aerogenes , Proteus mirabilis* and *Salmonella enteritidis* 76, 58, 8, 3, 7, 1 (50, 38, 5, 2, 4, 1%). This is probably because the ESBL is located on a plasmid that can be transferred from one organism to another rather easily and can incorporate (22).

Genetic material coding for resistance to other antimicrobial classes (12). Inherent resistance in a Gram-negative bacterium is due to an outer membrane that establishes a permeability barrier against the antibiotic. For example, Gram-negative bacteria are intrinsically resistant to penicillin G by virtue of their double membrane structure which prevents the antibiotic from accessing the cell wall target (35). The molecular analysis proved the phenotypic study for detecting and characterize the extended Spectrum β-lactamase (ESBLs) producing *Enterobacteriaceae* isolates. Conducted molecular detection by PCR to study the resistance genes of ESBL-producing *Enterobacteriaceae* such as CTX-M, SHV, VEB and evaluate the diagnostic methods used in clinical laboratories. Applying a microarray based genotypic test of *Enterobacteriaceae* isolates for detection and identification of multi genes encoding Extended-Spectrum Beta- Lactamases as TEM, SHV and CTX-M group genes. The molecular and epidemic analysis will help to investigate the prevalent gene among the *Enterobacteriaceae* clinical isolates.

Multiplex PCR amplification assay for the detection of the blaTEM, blaSHV and blaCTX-M genes in clinical isolates of *E. coli* and *K. pneumoniae* was used in this study because this assay has been shown to have the advantage of rapidly screening large numbers of clinical isolates in addition to the fact that the isolated DNA was suitable for further molecular epidemiological studies if required (20). Molecular detection by Real time polymerase chain reaction was done to detect the target genes by using Rotor-Gene Multiplex PCR), by dyes commonly used in multiplex, real-time PCR on Rotor-Gene 6000 cyclers at excitation maximum (365±20 to 680±5nm) and emission maximum (460±15 to 712nm)**.** In our study, the results were read on

(530±5/ 680±5nm) fluorescence. The isolates of *K. pneumonia* 1,3,6,8,9,10,12,13,14 and 15 were positive for CTX- M Gp1, the isolates 2,10,11,12,14 were positive (23) for SHV-5/12 while the all isolates for CTX-M Gp9 and VEB gene were negative. The isolates for *E. coli* were positive for CTX-M Gp1 gene in isolates 1,3,4,7,8,10,11,13,14 and 15 for isolates 2 and 12 were positive for CTX-M Gp9 and all *E. coli* isolates were negative for SHV-5/12 and VEB genes. Conducted a study Mohsen et al.,2009, (19) the majority of the *bla*CTX-M belonged to CTX-M group 1 (89%), as also reported in Switzerland (31) France (13) and Austria (14). Five (3.5%) isolates were positive for *bla*CTX-M genes from the CTX-M group 9, and 1 (0.7%) isolate was positive for CTX-M group 25/26. The remaining 79 (56.4%) isolates were negative for *bla*CTX-M genes. These data could indicate the ESBL phenotype is due to production of ESBLs other than CTX-Ms. However, the negative PCR results in this report do not negate the possibility that modified *bla*CTX-M were present in these isolates. Due to the increased complexity of β-lactam resistance in gram-negative organisms, the key to effective surveillance is the use of both phenotypic and genotypic analyses in concert (4). This high ESBL frequency may have been caused by the excessive use of broad-spectrum antibiotics in our hospital and to a higher level in our community setting, together with a lack of attention to laboratory screening of ESBL production by clinical isolates. On the other hand, the high rate of ESBL production could possibly be due to the spread of 1 single clone and/or plasmid within our hospital setting. Owing to a number of limitations, we could not exclude this possibility by determining plasmid profiles and pulsed-field gel electrophoresis patterns of the isolates (178). The method could be useful locally for investigating epidemic outbreaks and would be suitable for use in regional or national reference facilities. The PFGE typing of ESBL-producing isolates showed various DNA banding profiles (28). In our study had fifteen band pattern that didn't resemble the others indicating no clonal relationship to these strains. Lan nine represent positive control (22). DNA Microarray (Check-MDR CT102) for Rapid Detection of TEM, SHV, and CTX-M Extended-Spectrum β-Lactamases. This technology has the potential to detect an almost unlimited number of genes within one reaction mixture (21). The production of β -lactamases is the predominant cause of resistance to β -lactam antibiotics in Gram-negative bacteria. Extended-spectrum β -lactamases (ESBLs) are the most widespread and clinically significant enzymes which are active against practically all penicillins and cephalosporins. So far as many clinical isolates produce more than one b-lactamase and due to the high diversity of these enzymes, their determination at the

molecular level ensures adequate information for the identification of antimicrobial resistance. In the present research, we have developed the technology of oligonucleotide microarray for the identification of genes encoding ESBLs and carbapenemases. This novel commercially available DNA microarray system may offer an attractive option for rapid and accurate detection of CTX-M, TEM and SHV, ESBL genes in *Enterobacteriaceae* in the clinical laboratory. The worldwide prevalence of extended-spectrum beta-lactamase β- producing members of *Enterobacteriaceae* (ESBL-E) is increasing rapidly (2). Controlling ESBL-E is difficult, as the resistance genes are located on plasmids and may be transferred between species and even different genera of the *Enterobacteriaceae* family (27). The rapid laboratory detection of this resistance trait is important to guide antimicrobial therapy and to take appropriate infection control measures. The performance of a microarray for the detection of extended-spectrum beta-lactamases was determined on a collection of thirty highly resistant members of the family *Enterobacteriaceae* collected from patients at the Clinical Microbiology Laboratory of the Riyadh Military Hospital 2011-2013 in The Riyadh, KSA. The microarray had a significantly higher specificity than the phenotypic assays (24). It also detects characterizes the resistance genes, providing epidemiological insight (11). The study, a high frequency of ESBL producing organisms was found especially among *Escherichia coli* and *Klebsiella pneumonia*. ESBL positivity in isolates also uses of antimicrobials in this set up. As confirms the large dissemination of the resistance genes of ESBL-producing *Enterobacteriaceae* such as CTX-M, SHV, TEM, and the results of antibiotic susceptibility revealed high rates of resistance to the cephalosporin, penicillin, and monobactam. These results should draw (25) the attention of the presence to the serious consequences of increasing antimicrobial resistance.

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