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Research Paper

MOLECULAR CHARACTERIZATION OF ANTIMICROBIALRESISTANT ESCHERICHIA COLI ISOLATED FROM CLINICALLY DISEASED EGYPTIAN COWS AND CALVES

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Antibiotic resistance in *E.coli* has been reported over the last years leading to therapeutic problems worldwide. The aim of this study was to characterize the antimicrobial resistance of *E.coli* isolated from clinically diseased Egyptian cows and calves phenotypically and genotypically. Among 232 samples collected from mastitic milk samples, vaginal swabs from cows and nasal swabs from pneumonic calves, a total of 38 resistant *E.coli* strains were isolate. Recovered isolates were tested for antimicrobial susceptibility by Kirby-bauer's disc diffusion method against penicillin, cefuroxime, cefepime, ampicillin, amikacin, kanamycin, gentamicin, ciprofloxacin, nalidixic acid, doxycycline, trimethoprim-sulphamethazole and chloramphenicol. Resistant isolates were molecularly characterized for presence of integron and other antibiotic resistance genes located outside the integron structure. The integron was detected in 5(13.1%). The B-lactamase encoding genes (bla_{-TEM} and bla_{-SHV}); aminoglycosides resistance gene, *aphA1*; quinolones resistance gene, *qnrA*; sulfonamides resistance genes (*sulI* and *sulII*) and trimethoprim resistance gene *dhfrIa*, were also identified in 7(18.4%), 4(80%), 0(0%), 8(40%) and 5(50%) of recovered isolates, respectively.

Keywords: Antimicrobial resistance, Escherichia coli, Integron, Resistance genes, Multidrug resistance

INTRODUCTION

Enterobacteriaceae are environmentally adapted to be present in water, soil, human, insects and animals (Brenner *et al.*, 2005). *Escherichia coli*, an important member of the family *Enterobacteriacae*, are Gram negative rods, catalase positive, oxidase-negative, facultative anaerobes (Forbes *et al.*, 2007), non motile or motile by peritrichous flagella (Orskov, 1984; and Scheutz and Strockbine, 2005). *E.coli* is one of the main micro flora inhabit the intestinal tract of humans, birds and animals (Kaper *et al.*, 2004)

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causing no pathogenicity. Some *E.coli* strains are pathogenic that were reported to cause serious diseases worldwide such as diarrhea, urinary tracts infections and neonatal meningitis in human (Gray, 1995; Nataro and Kaper, 1998; Kaper *et al.*, 2004; Gyles, 2007; Hobman *et al.*, 2007; and Schmidt, 2010) In animals, these bacterial species represents the main bacterial cause of bovine mastitis (Wray *et al.*, 1997) and diarrhea which is the most common cause of mortality and morbidity in young calves. In birds, colibacillosis with other manifestations such as peritonitis, septicemia, pericarditis, perihepatitis, airsacculitis (extra intestinal lesions) are the most prevalent sequelae of infection with this pathogen.

Antimicrobial resistance in bacteria can be either acquired or intrinsic (Woodford and Ellington, 2007). The intrinsic resistance is through decrease outer membrane permeability (Slama, 2008), efflux pump (Kerr and Snelling, 2009) and inactivating enzymes to antibiotic (Mauldin *et al.*, 2010). Yet, acquired resistance is more significant due to the possibility of spreading of resistant genes from resistant to sensitive bacteria, These genes can be transferred through integron system, plasmids (Bennett, 2008) and transposons (Lambert, 2002; and Poole, 2004). Integrons are genetic elements that allow efficient capture and expression of exogenous genes by gene cassettes that can be mobilized to other integrons (Canal *et al.*, 2016). All integrons are composed of three major cores that allow the mobility of genes to other integrons or to secondary sites in the bacterial genome (Boucher *et al.*, 2007; and Cambray *et al.*, 2010). Integron consists of a gene encoding for a site specific recombinase (*Intl*), belong to the integrase family (Messier *et al.*, 2001), recombination site (*attl*) at which gene cassettes may be inserted and that is recognized by the integrase (Partridge *et al.*, 2000) and a promotor that directs transcription of genes encoded cassettes (Lévesque *et al.*, 1994; and Collis *et al.*, 1995).

There are two types of integron. The first type was mobile integron that commonly encoded antibiotic resistance genes and had a few gene cassettes. The second type was super integron that were located on chromosomes and have hundreds of gene cassettes (Mazel *et al.*, 1998; Rowe-Magnus *et al.*, 1999; Vaisvila *et al.*, 2001; and Mazel, 2006).

The aim of this study was to characterize the antimicrobial resistance of antibiotic-resistant *E.coli* isolated from clinically diseased Egyptian cows and calves phenotypically and genotypically.

MATERIALS AND METHODS

Sampling, Isolation, and Identification Procedures

A total of 232 samples were collected from cows and calves including 155 milk samples from mastitic cows, 4 vaginal swabs from metritic cows and 73 nasal swabs from pneumonic calves. All samples were obtained from different private farms in Damietta Governorate, Egypt, during the year of 2016. Milk samples were collected in sterile containers and swabs were collected by sterile cotton swabs in sterile nutrient broth, all samples transferred to our laboratory under refrigeration in coolers and analyzed within 6 hours of sampling at most. All isolates were inoculated on MacConkeys (Oxoid LTD, England) agar medium at 37 °C for 24 hr. Dark pink colony was picked up from each plate and sub cultured on Eosin Methylene Blue (EMB) (Oxoid LTD., England) agar plate. Blue black colony with green metallic sheen was taken for further analysis. A total of 40 suspected isolates of *E.coli* were

selected and further confirmed using the panel of biochemical tests suggested by referenced authors (Edwards and Ewing, 1986). Once identified, the isolates were preserved at -70 °C in brain heart infusion broth containing 20% glycerol (vol/vol) for further studie.

Antimicrobial Susceptibility Testing

Antimicrobial resistance phenotypes determined By Kirby-bauer's disc diffusion method according to the standards and interpretive criteria a described by CLSI (Clinical and Laboratory Standards Institute, 2008). The following antibiotics were used (Oxoid, Basingstoke, UK) penicillin G (P, 10 U), ampicillin (AMP, 10 µg), cefuroxime (CXM, 30 µg), cefepime (FEP, 30 µg), amikacin (AK, 30 µg), kanamycin (KAN, 30 µg), gentamicin (GN, 10 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 30 µg), doxycycline (DO, 30 µg), trimethoprim-sulphamethazole (STX, 25 µg) and chloramphenicol (C, 30 µg).

DNA Extraction and Determination of Antimicrobial Resistance Genes

For bacterial DNA extraction, bacterial colonies were washed with 150 µl sterile deionized water. Tubes were vortexed and the suspension was boiled 15 min and followed by 10000 g for 5 min. The supernatant was then used as DNA template. Six PCR protocols were applied to detect specific genes according to the resistance phenotype, as follows: *TEM* and *SHV* B-lactamase genes for isolates exhibiting penicillin, ampicillin, cefuroxime, cefepime resistance (Belaaouaj *et al.*, 1994; and Pitout *et al.*, 1998), *aphA1* for amikacin, kanamycin, gentamicin resistance (Maynard *et al.*, 2003), *qnrA* for ciprofloxacin, nalidixic acid resistance (Robicsek *et al.*, 2006), *sulI* and *sulII* for sulfonamides resistance (Mazel *et al.*, 2000; and Maynard *et al.*, 2003) and *dhfrIa* for trimethoprim resistance (Toro *et al.*, 2005). Primer sequences used in this assay are listed in (Table 1).

Detection of Integrons

The presence of integrons among *E. coli* isolates were assayed using primers hep35 and hep36 that amplify conserved regions of integron-encoded integrase genes intl1, intl2, and intl3 (White *et al.*, 2000) using PCR protocols published (Spindler *et al.*, 2012).

RESULTS AND DISCUSSION

Among 232 samples collected from clinically diseased cows and calves (mastitic milk, vaginal and nasal swabs), a total of 40 *E.coli* (13 from milk samples, 22 from nasal samples and 4 from vaginal samples) isolates were recovered. These isolates were examined for antimicrobial susceptibility testing. The number of isolates showing resistance to one antimicrobial agent were 25(65.8%) and Multiple Drug Resistance (MDR) was observed among 13(34.2%) isolates. The most commonly observed isolates showing resistance to antimicrobial agent were: ampicillin (97.4%), penicillin (84.2%), cefuroxime (50%), trimethoprim-sulphamethazole (31.5%), kanamycin (13.1%), ciprofloxacin (7.9%), nalidixic acid (7.9%), cefepime (2.6%), gentamicin (2.6 %), amikacin (2.6%).

The differences in resistance between different antimicrobial agents within the same class were listed in (Table 2). In this study a total of 38 (95%) *E.coli* isolates showed antimicrobial resistance mainly against penicillin, cefuroxime, ampicillin, kanamycin and trimethoprim-sulphamethazole. Of interest, 95% of isolates were multidrug-resistant isolates observed in the that was higher than the proportion of multidrug-resistant isolates in previously published studies where the

Table 1: Primers Used for PCR

		Nucleotide sequences (5′ to 3′)	Target	Reference
Integron/ resistance genes	Integron hep35 hep36	TGCGGGTYAARGATBTKGATTT CARCACATGCGTRTARAT	Conserved regions of integron-encoded integrase genes*intI1*, *intI2*, and *intI3*	(White e al., 2000)
	B-lactams *TEM*-F *TEM*-R *SHV*-F *SHV*-R	ATTCTTGAAGACGAAAGGGC ACGCTCAGTGGAACGAAAAC CACTCAAGGATGTATTGTG TTAGCGTTGCCAGTGCTCG	bla_{TEM} bla_{SHV}	(Belaaouaj et al., 1994) (Pitout et al., 1998)
	Aminoglycosides *aphA1*-F *aphA1*-R	ATGGGCTCGCGATAATGTC CTCACCGAGGCAGTTCCAT	*apk(3')- Ia (aphA1)*	(Maynard et al., 2003)
	Quinolones *qnrA*-F *qnrA*-R	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	*qnrA*	(Robicsek et al., 2006)
	Sulfonamides *sulI*-F *sulI*-R *sulII*-F *sulII*-R	TGGTGACGGTGTTCGGCATTC GCGAGGGTTTCCGAGAAGGTG CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	*sulI* *sulII*	(Mazel et al., 2000) (Maynard et al., 2003)
	Trimethoprim *dhfrIa*-F *dhfrIa*-R	GGAGTGCCAAAGGTGAACAGC GAGGCGAAGTCTTGGGTAAAAAC	*dhfrIa*	(Toro CS et al., 2005)

percentage is 90.7% in New York State, USA (Srinivasan *et al.*, 2007) and in other studies the proportion of multidrug-resistant isolates was ranged from 15% to 74% (Anderson, 1989; DANMAP, 2001; Lanz *et al.*, 2003; and Lehtolainen *et al.*, 2003). These results suggested that the intensive use of antimicrobials in farm animals might contributed, at least in part, to the higher antimicrobial resistance rate.

In this study the majority of *E.coli* isolates were resistant to ampicillin (97.4%) that was in line with previously published studies in which ampicillin resistance was reported in 98.4% (Srinivasan *et al.*, 2007). Similarly, ampicillin resistance was

Table 2: Results of Antimicrobial Sensitivity Tests and Characterization of Integron in E.coli Isolates

Antimicrobial agent (s) tested	Antimicrobial class	Number of resistant strains	Integron positive	Integron negative
AMP	β-Lactams	37 (97.4%)	5 (100%)	32 (96.9%)
P	β-Lactams	32 (84.2%)	5 (100%)	27 (81.8%)
CXM	β-Lactams	19 (50 %)	4 (80 %)	15 (45.5%)
FEP	β-Lactams	1 (2.6%)	0 (0 %)	1 (3 %)
STX	Potentiated sulfonamides	12 (31.5%)	4 (80 %)	8 (24.2%)
KAN	Aminoglycosides	5 (13.1%)	3 (60%)	1 (3%)
AK	Aminoglycosides	1 (2.6%)	0 (0%)	1 (3%)
GN	Aminoglycosides	1 (2.6%)	0 (0%)	1 (3%)
CIP	Quinolones	3 (7.9%)	1 (2.6%)	2 (6%)
NA	Quinolones	3 (7.9%)	2 (40%)	1 (3%)
DO	Tetracyclines	0 (0%)	0 (0%)	0 (0%)
C	Phenicol	0 (0%)	0 (0%)	0 (0%)
Total		38 (100%)	5 (100%)	33 (100%)

Note: AMP, ampicillin; P, Penicillin; CXM, Cefuroxime; FEP, Cefepime; STX, Trimethoprim-sulphamethazole; KAN, Kanamycin; AK, Amikacin; GN, Gentamicin; CIP, Ciprofloxacin; NA, Nalidixic acid; DO, Doxycycline; C, Chloramphenicol.

reported in 88% of *E.coli* isolated from cows with mastitis (Lehtolainen *et al.*, 2003) and in 92% of *E.coli* isolated from calves (Gunn *et al.*, 2003). On the other hand, in *E. coli* isolated from animals, the ampicillin resistance was low (21%) (Lanz *et al.*, 2003).

In this study integrons were detected in 13.1% of antimicrobial resistant *E.coli* isolates (Figure 1) (Table 2). In previously reported studies, integrons were carried by 27.6% of *E.coli* isolates from human and (13.4%) community-acquired *Enterobacteriacae* infection (Daikos *et al.*, 2007).

A total of six resistance genes were screened (Table 3). The B-lactams resistance was attributed to bla_{TEM}, bla_{SHV} and was screened among 38 B-lactams resistant isolates. Of these isolates, 5(13.2%) isolates were carrying bla_{TEM}, 2(5.3%) were carrying bla_{SHV}. In Gram negative bacteria resistance to B-lactams is due to B-lactamases enzyme. The most common Blactamases described in Gram negative bacteria is *TEM, SHV* (Bradford, 2001). In *E.coli*, *TEM* is responsible for 90% of ampicillin resistance (Livermore, 1995).

In this study B-lactamase encoding genes were detected in 18.4% of B-lactams resistant *E.coli* isolates. Of these isolates (13.2%) isolates were carrying bla_{TEM}, (5.3%) were carrying bla_{SHV}

Table 3: Characterization of Antimicrobial Resistance Genes in E.coli Isolates

Antibiotic	Number of resistance strains	Tested resistance genes	Number (%) of positive isolates
AMP,P	37	*TEM* *SHV*	5 (13.5%) 2 (5.4%)
SXT	12	*sulI* *sulII* *dhfrIa*	1 (8.3%) 7 (58.3%) 5 (41.6%)
KAN	5	*aphA1*	4 (80%)
CIP	3	*qnrA*	0 (0%)

Note: AMP, ampicillin; P, Penicillin; STX, Trimethoprim-sulphamethazole; KAN, Kanamycin; CIP, Ciprofloxacin.

(Figures 2-4). Other reports recorded that bla_{TEM} are the most frequent gene associated with animal origin ampicillin resistant *E.coli* (Brinas *et al.*, 2002). In other study bla *TEM* was found in 21.8% of *E.coli* isolated from cattle in Germany (Guerra *et al.*, 2003).

The presence of *dhfrla, sulI and sulII* were screened among 12 Trimethoprim-sulphamethazole resistant isolates. Of these isolates 5(50%) were carrying *dhfrla*, 1(10%)

Figure 1: PCR for Detection of Integron, M Abbreviated to Marker (1 Kb), These Samples Showing Positive Result (491 bp)

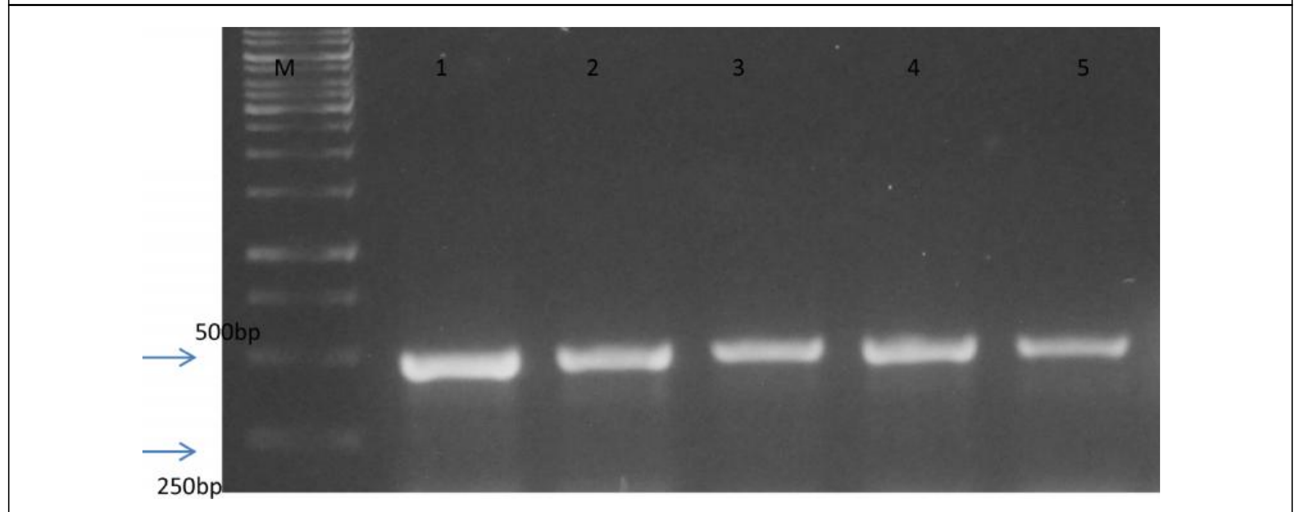


Figure 2: PCR for Detection of TEM, M Abbreviated to Marker (1 Kb), Samples Showing 6, 7, 8, 9 Negative Results, Samples 1, 2, 3, 4, 5 Showing Positive Results for (1150 bp)

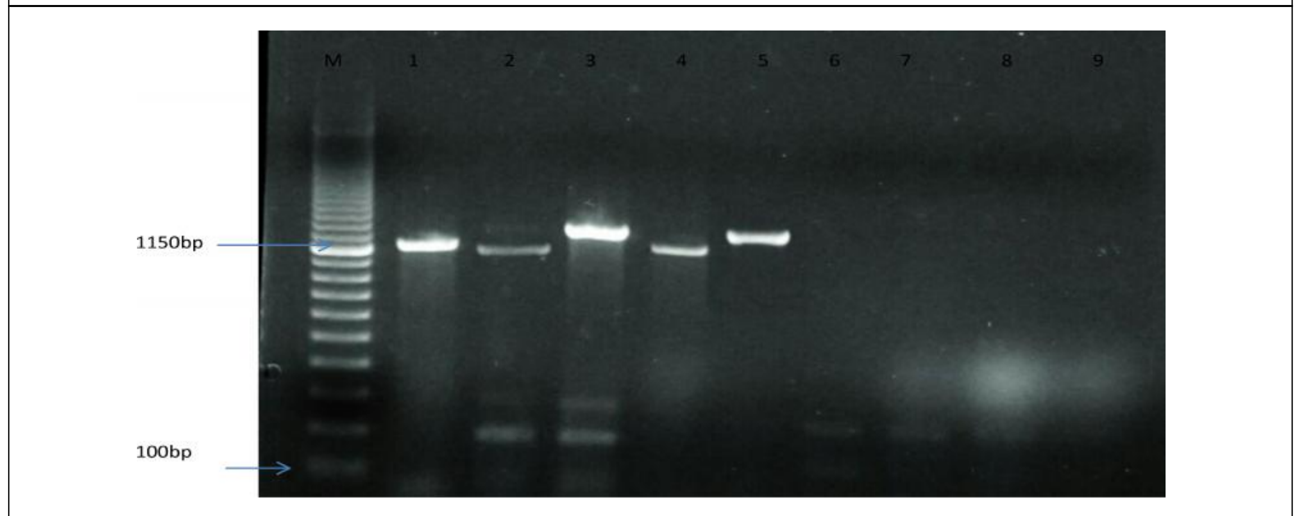


Figure 3: PCR for Detection of SHV, M Abbreviated to Marker (1 Kb), Samples 1, 2, 4, 5, 6 Showing Negative Results, Samples 3 Showing Positive Results (885 bp)

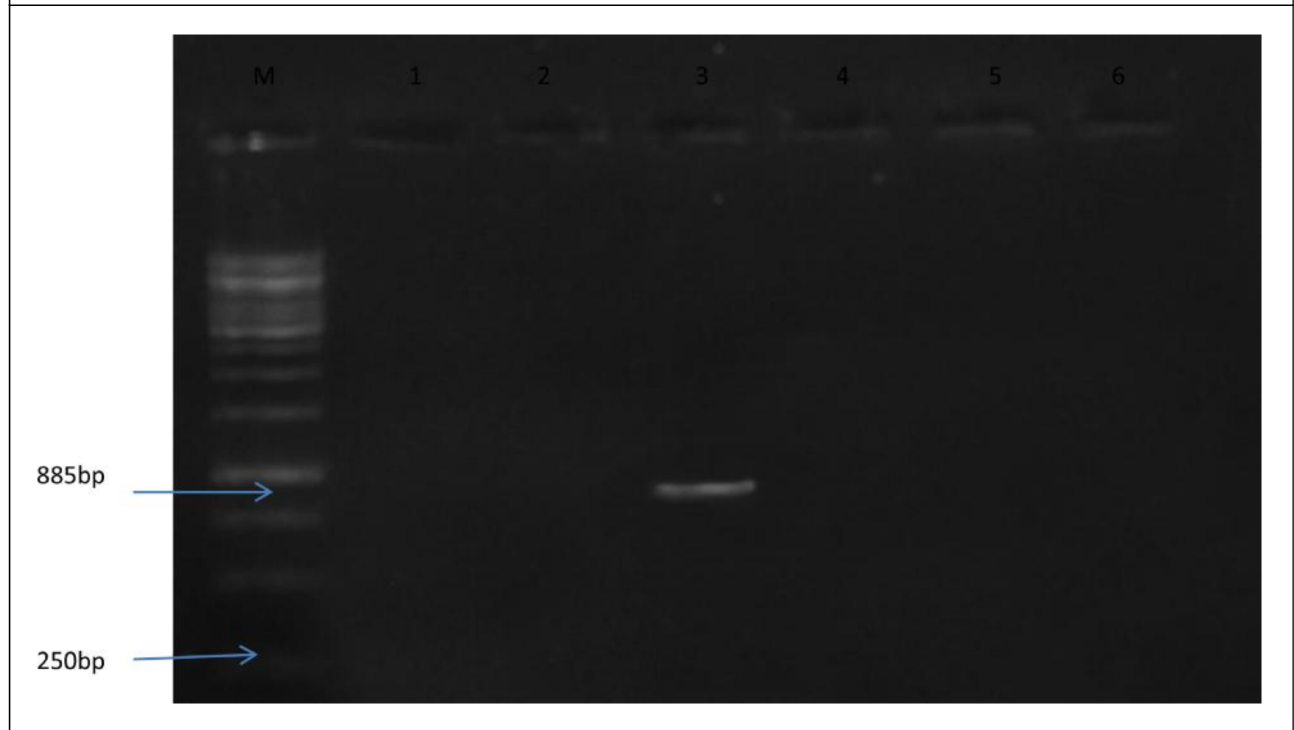
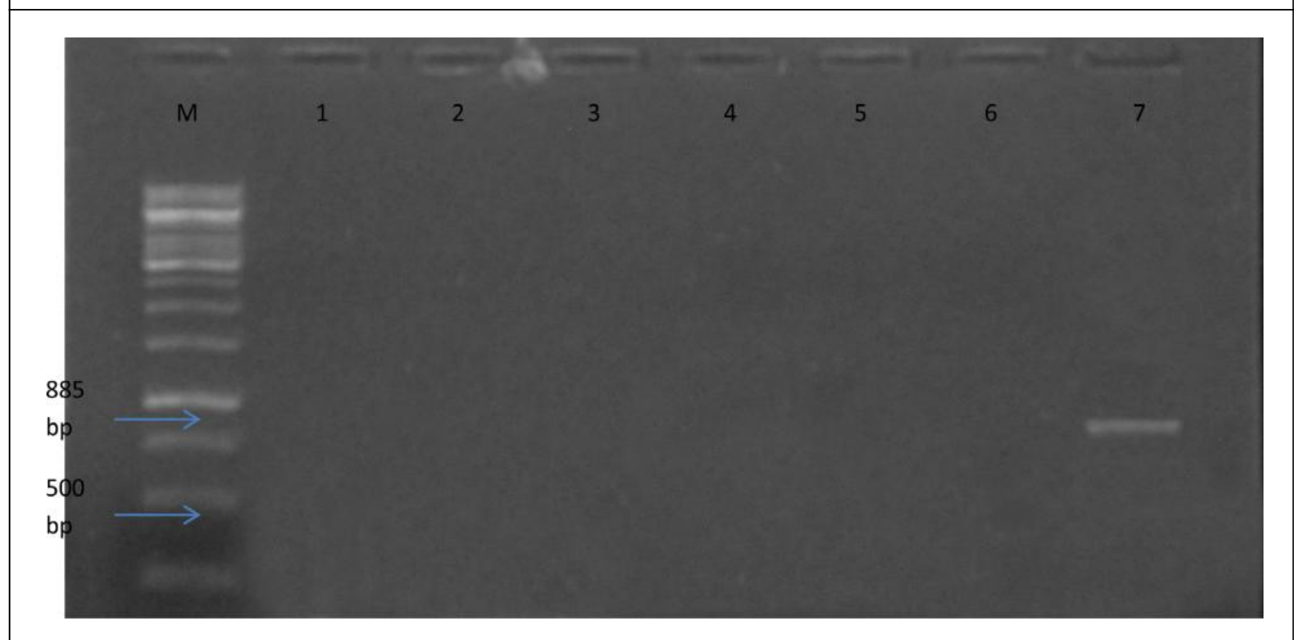


Figure 4: PCR for Detection of SHV, M Abbreviated to Marker (1 Kb), Samples 1, 2, 3, 4, 5, 6 Showing Negative Results, Sample 7 Showing Positive Result (885 bp)



carrying *sulI* and 7(70%) carrying *sulII* (Figures 5-7). In this study sulfamethoxazole encoding genes were detected in 40% of sulfamethoxazole resistant *E.coli* isolates. Of these isolates, a total of 8(10%) isolates were carrying *sulI,* (70%) were carrying *sulII*. Therefore, *sulII* was found in higher

Figure 5: PCR for Detection of sulI, M Abbreviated to Marker (1 Kb), Samples 1, 3, 4, 5, 6 Showing Negative Results, Sample 2 Showing Positive Result (789 bp)

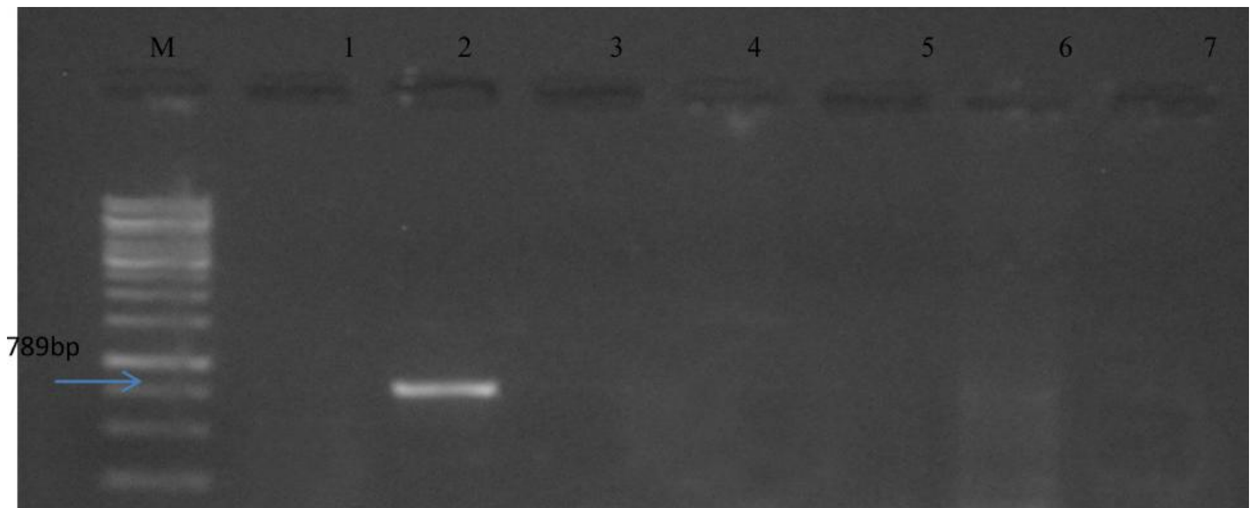
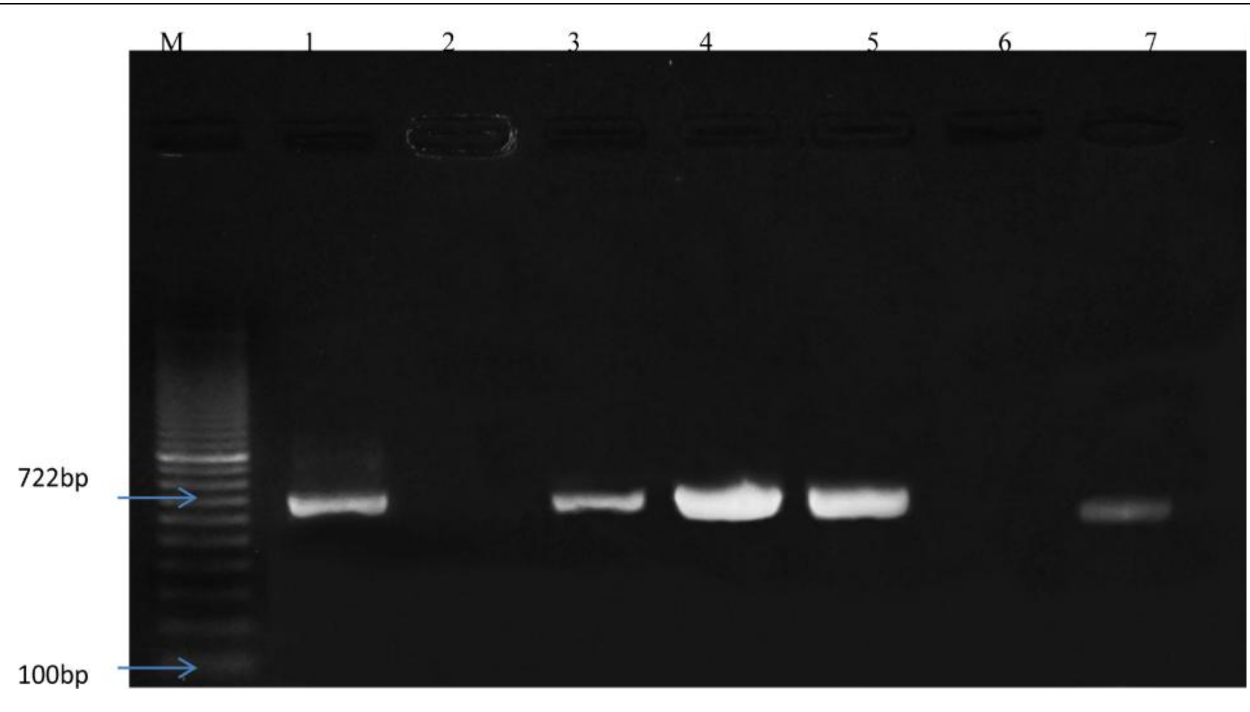


Figure 6: PCR for Detection of sulII, M Abbreviated to Marker (1 Kb), Samples 2, 6 Showing Negative Results, Sample 1, 3, 4, 5, 7 Showing Positive Result (722 bp)



frequency than *sulI* in sulfamethoxazole resistant *E.coli*. In contrast higher prevalence rate of *sulI* (29.6%) compared to *sulII* (25%) was observed among *E.coli* from mastitic cow (Srinivasan *et al*., 2007). In this study a low proportion of one isolate (1%) of these resistant isolates possessed both *sulI* and *sulII* that was in line with previous studies reported that one isolate possessed both *sulI* and *sulII* (Srinivasan *et al*., 2007; and Navajas-Benito *et al*., 2017).

Figure 7: PCR for Detection of sulII, aphA1, M Abbreviated to Marker (1 Kb), Samples 3, 7 Showing Negative Results, Samples 1, 2 Showing Positive Results for sulII (722 bp), Samples 4, 5, 6 Showing Positive Results for aphA1 (600 bp)

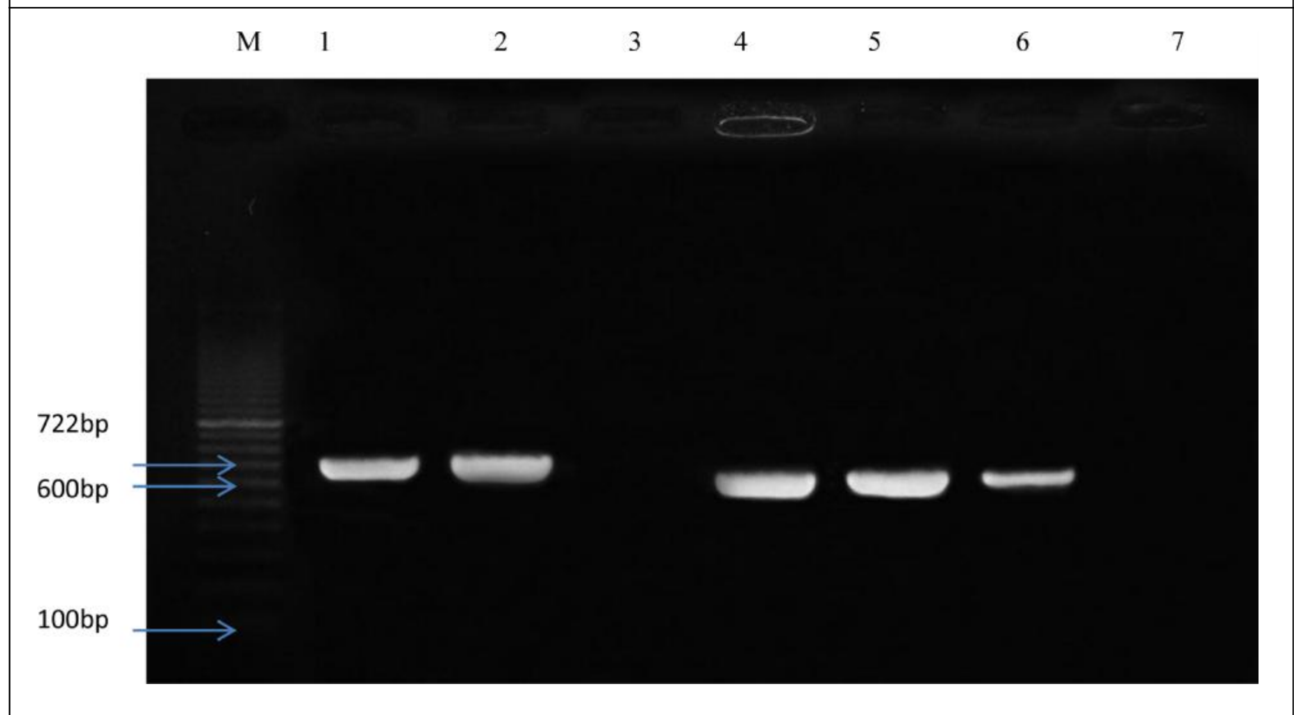
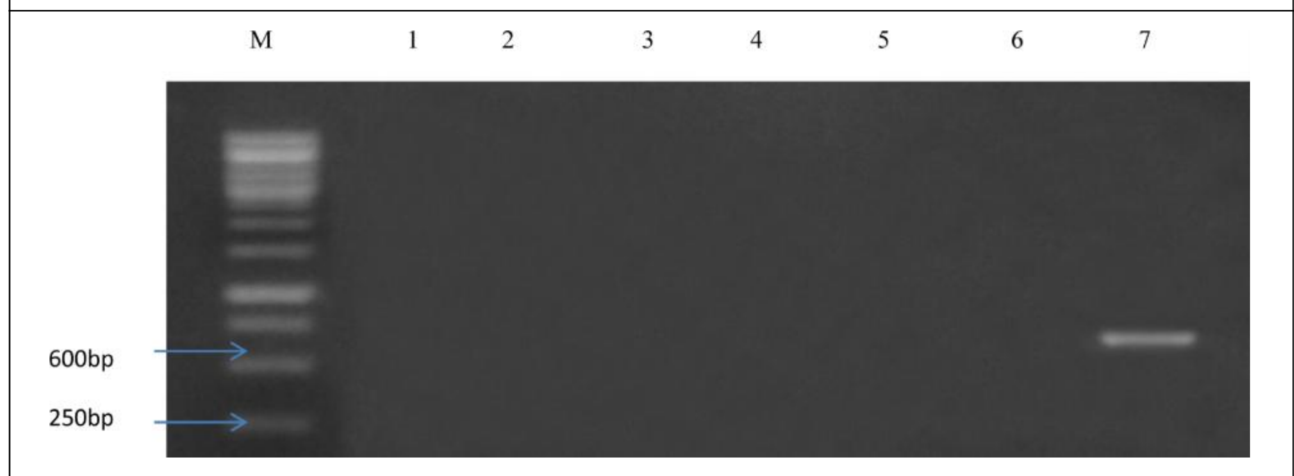


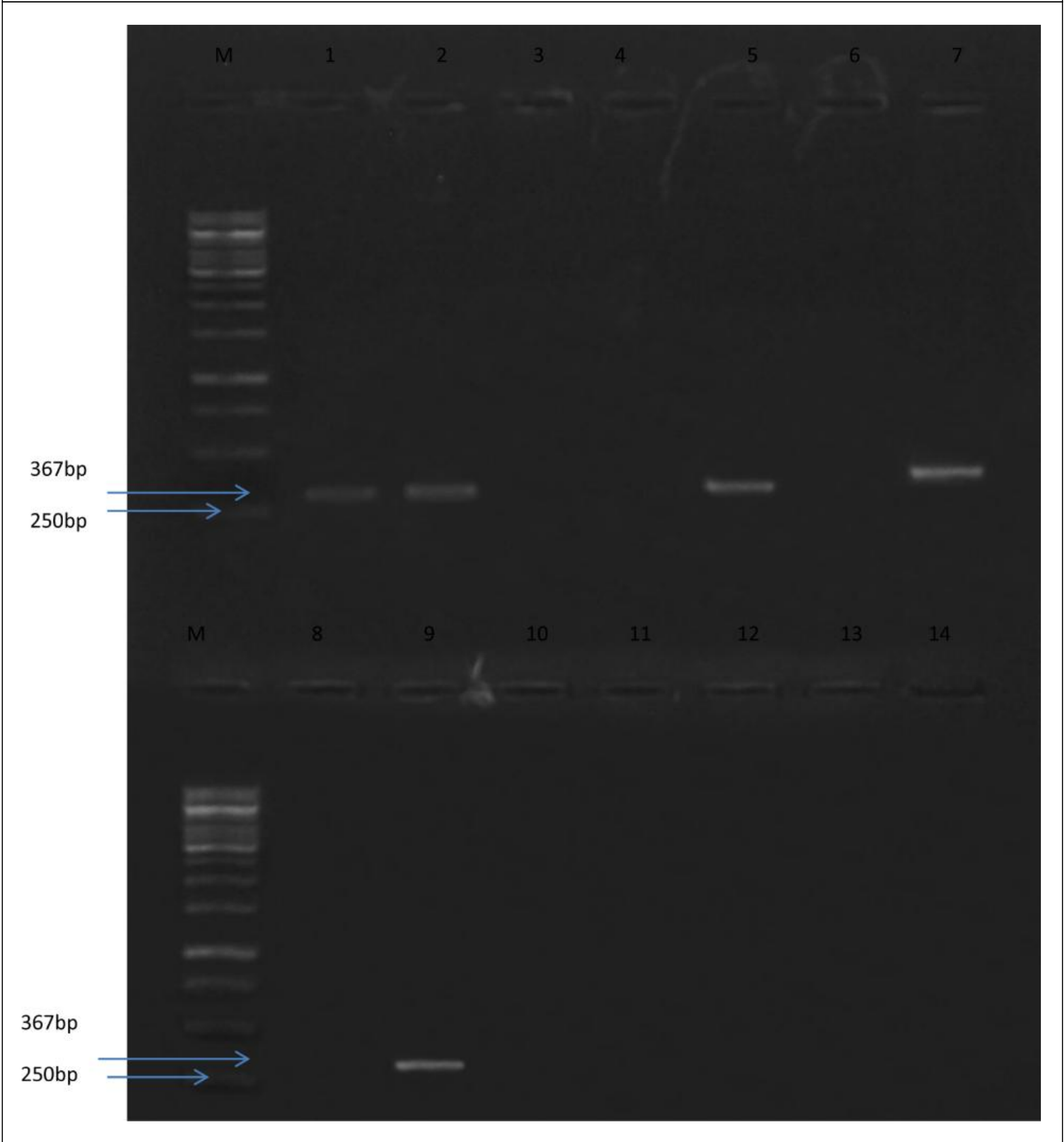
Figure 8: PCR for Detection of aphA1, M Abbreviated to Marker (1 Kb), Samples 1, 2, 3, 4, 5, 6 Showing Negative Results, Sample 7 Showing Positive Result (600 bp)



Half (50%) of trimethoprim resistant isolates harbored *dhfrla* gene (Figures 7 and 8). In other previously studies it was reported that percentage of *E.coli* isolates resist to trimethoprim 40%, 33%, 23% for diseased cattle, poultry and swine respectively (Seputien *et al.*, 2010).

Among the aminoglycosides resistant isolates, kanamycin encoding genes were detected in 80% of kanamycin resistant *E.coli* isolates (Figure 9). In other studies *aphA1* was 63% (Karczmarczyk *et al.*, 2011). None of the ciprofloxacin resistant isolates carried *qnrA*

Figure 9: PCR for Detection of dhfrIa, M Abbreviated to Marker (1 Kb), Samples 3, 4, 6, 8, 10, 11, 12, 13, 14 Showing Negative Results, Sample 1, 2, 5, 7, 9 Showing Positive Result (367 bp)



resistance gene. The later finding suggests other possible resistance mechanism for quinolone resistance.

In conclusion, the intensive use of antibiotics would increase the number of antibiotic resistance and integron prevalence which could be a significant public health importance and can transfer antibiotic resistance genes to other bacteria and the *E.coli* in this study can be considered a reservoir of resistance genes and

highlight the need for the abundant use of antimicrobials in animal husbandry.

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