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RESEARCH ARTICLE

INSIGHTS INTO VIRULENCE AND ANTIMICROBIAL RESISTANCE PLASMID ASSOCIATED GENES OF ESBL *ESCHERICHIA COLI* ASSOCIATED WITH ARTHRITIS IN CHICKENS IN EGYPT.

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Abstract

Increasing the incidence of extended spectrum beta-lactamase (ESBL) among Enterobacteriaceae especially avian pathogenic *Escherichia coli* (APEC) is of great concern to poultry industry. This study aimed to detect the prevalence and resistant profile of isolates showed ESBL activity among 100 *E. coli* isolates collected from hock joints in chickens (14-70 days old) with arthritis symptoms, from different chicken farms at EL-Gharbia Governorate in Egypt, in addition to, detect the incidence rates for selections of some virulence and antibiotic resistance plasmid associated genes using conventional PCR. A total 40 isolates were phenotypically identified as *E. coli*, with predominant (50%) O125 serotypes. All (100%) *E. coli* strains demonstrated a high level of multidrug resistance (MDR). Meanwhile, only 5 (12.5%) isolates were identified as ESBL producers based phenotypically on double disc synergy test and genotypically on screening for presence of β-lactamases genes. The ESBL isolates showed high resistance rates to β-lactam, aminoglycoside, tetracycline and quinolones antimicrobials, which could be explained by the presence of (100%) bla_{TEM}, (100%) *aad*A, (80%) *tet*A and (20%) *qnr*S resistance genes, respectively. Regarding virulence genes, the result showed that only *iss* and *omp*A genes were detected in all isolates showed ESBL activity. The co-occurrence of these antibiotic resistance and virulence plasmid associated genes confirmed the acquisition of antimicrobial resistance genes by ESBL producing *E. coli*, which associated with increased virulence. Thus, urgent intervention is needed to successfully control contamination with MDR isolates in Egypt especially in poultry and its products.

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Introduction:-

Escherichia coli is normal inhabitant in intestine of chicken but certain strains of avian pathogenic *Escherichia coli* spread into different internal organs causing colibacillosis, which is characterized by septicemia with multiple organ

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lesions. Arthritis is the most common forms of colibacillosis lead to economic losses due to decrease in body weight, emaciation and deaths between infected chicken (Oh et al. 2011).

Antimicrobial resistance assessment of APEC at molecular level may be a useful tool for understanding the role of genetic elements in developing, dissemination and persistence of resistance in bacteria (Alekshun and Levy 2007). Recently, drug resistance has increased dramatically all over the world, especially, the broad-spectrum cephalosporins resistance which is very important in the care of human health, which conducted mainly to the spread of genes encoding extended spectrum beta-lactamases (Su et al. 2008). One of the main concerns about ESBL genes located in mobile genetic elements that can easily be transferred between and within bacterial species, in addition, it can carry different virulence and antibiotic resistance genes (Hawkey and Jones 2009). Therefore, it is strongly recommended that ESBL detection is carried out systematically for all bacteriaceae. The most common types of plasmid borne β-lactamases in resistant *E. coli* isolates in Egypt are *TEM*-, *SHV*- and *OXA*-type (Ahmed et al. 2015).

The ESBL producing members are resistant to penicillins, cephalosporins, and aztreonam, and frequently resistant to aminoglycosides, trimethoprim-sulfamethoxazole, and quinolones. Thus, resistance caused by these enzymes increase the frequent of treatment failures in humans (Dutil et al. 2010). Recently, WHO (2014) reported high resistance rates to third generation cephalosporins in Egypt.

Therefore, the current study aimed primary on the prevalence of ESBL isolates among multidrug resistant *E. coli* isolates associated with hock joint infections between chickens in Egypt, in addition to, detect the incidence rates for selections of some virulence and antibiotic resistance plasmid associated genes using conventional PCR.

Materials and Methods:-

Bacterial isolates

A total of 100 samples were collected from hock joints in chickens (14-70 days old) with arthritis symptoms, from different chicken farms at EL-Gharbia Governorate in Egypt. All samples were subjected for isolation and biochemical identification of *Escherichia coli* according to Quinn et al. (2002). For further confirmation, API20E system (BioMérieux, France) was used for identification of all biochemically identified isolates. The biochemically identified *E. coli* isolates were serotyped according to Edward and Ewings (1972), using agglutination test in serology unit in animal health research institute, Dokki, Egypt.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed for all *E. coli* identified isolates according to the Kirby - Bauer disk diffusion method (Finegold and Martin 1982), using Mueller Hinton agar using discs (Oxoid) of different antibiotic groups; β-lactam (amoxicillin; AML 10 µg), tetracycline (doxycycline; DO 30 µg), aminoglycoside (gentamycin; CN 10 µg, and streptomycin; S 10 µg) and quinolones (ciprofloxacin; CIP 5 µg). The results were interpreted according to the criteria recommended by CLSI (2011). Each isolate was examined in triplicate. Isolates showed resistant to more than two different antibiotic groups were considered as MDR isolates.

ESBL isolates and their antibiotic resistance and virulence plasmid associated genes

The screening for ESBL activity was applied for all confirmed multi-resistant *E. coli* strains by double disc synergy test using cefotaxime and ceftazidime disks with and without clavulanic acid (Mast Diagnostics, Merseyside, UK) (CLSI 2014). The DNA from phenotypically identified ESBL isolates was extracted using QIAamp DNA mini kit (Cat. no. 51304; Qiagen). Screening for the presence of β-lactamases genes (*bla*TEM, *bla*SHV, and *bla*CTX-M), in addition to a selection of some virulence and antibiotic resistance plasmid associated genes was carried out by conventional PCR. The primers and probes used for amplification of different genes and size of PCR amplicons used in the current study were presented in Table 1. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used negative and positive quality control strains, respectively.

Results:-

Isolation and identification of *E. coli*

Based on phenotypic characters, 40 *E. coli* isolates were preliminary identified from 100 samples obtained from hock joints of chicken showing arthritic symptoms. The preliminary identification was based on their colonial morphology; pink colonies on MacConkey agar and characteristic green metallic sheen colonies on Eosin Methylene

Blue agar. The identical biochemical characters showed positive reaction with indole test and negative reaction with Simmon´s Citrate agar, in addition to yellow slant and butt with gas formation on Triple Sugar Iron agar medium, while, negative results were obtained with urease test with no H_2S production. Furthermore, all identified isolates were confirmed as *E. coli* by API 20E system. The serotyping of isolates revealed two predominant O serotypes; (50%) O125 and (20%) O27. In addition, O86a, O127 and O78 serotypes (10%, each) were detected.

Antimicrobial susceptibility testing

The antibiotic resistance profiles of the 40 *E. coli* isolates demonstrated high rates of resistance to amoxicillin and streptomycin (100%, each), followed by doxycycline (80%) and ciprofloxacin (70%). The lower resistant rate was detected with gentamycin (35%). Regarding MDR, All (100%) All *E. coli* strains exhibited resistance to three antibiotics at least, thus, showed high level of multi-drug resistance.

ESBL isolates and their virulence and antibiotic resistance plasmid associated genes

Out of 40 MDR *E. coli* isolates, 5 (12.5%) isolates showed ESBL activity based on double disc synergy test. Regarding β-lactamase genes, these isolates harbored only *bla*TEM gene, Figure 1. The ESBL isolates showed 60%, 60%, 80%, 100% and 100% resistance rates for ciprofloxacin, gentamycin, tetracycline, amoxicillin and streptomycin, respectively. The phenotypic resistance to β-lactam, aminoglycoside, tetracycline and quinolones antimicrobials could be explained by the presence of (100%) *bla*TEM, (100%) *aad*A, (80%) *tet*A and (20%) *qnr*S resistance genes, respectively, among the isolates showed ESBL activity. Regarding virulence genes, the result showed that *iss* and *omp*A genes were detected in all isolates showed ESBL activity, while none of *eae*A and *CFA*/I genes were detected (Table 3).

Discussion:-

Avian pathogenic *E. coli* is one of major causes of morbidity and mortality in chicken and associated with economic losses (Smith et al. 2007). The result showed high prevalence rate of (40%) E. coli isolates, which indicated its potential role in avian pathogens associated with arthritis in Egypt. Similar result was recorded in Bangladesh (Rahman et al. 2004). However, higher level of prevalence was reported in (60%) Egypt (Tawfik et al. 2016), while, lower prevalence rate was reported in (7.8%) Iraq (Rasheed 2011). The variation in prevalence rates were might be conducted to many factors, including birds; age, breed, immune status and medication during sample collection, environment and hygienic measure inside farms (El Tawab et al. 2015), in addition to status of affected joint during sampling if closed or opened (Tawfik et al. 2016). Also, the sampling scheme and detection protocol might play a role (Ammar et al. 2015).

In the last century, Orskov et al. (1977) reported a serotyping system for *E. coli* strains based on O- antigen. This system has been the basis for distinguishing strains during outbreaks, in addition for surveillance. In the current study, serological identification of *E. coli* isolates revealed five different O-serotypes (O86a, O127, O125, O78 and O27), were detected, and the predominant serotype was (50%) O125. Similar observation was reported previously in Egypt (Tawfik et al. 2016). Thus, they might confirm their role particularly in adaptation and involvement in extra intestinal colibacillosis infections, especially, in arthritis infection in chicken farms in Egypt. On the other hand in USA, Gomis et al. (2001) reported that the predominant serotypes were O1, O6, O8, O15 and O78. Also, Abd El-Mongy et al. (2018) detected seven serotypes; O2, O26, O78, O127, O1, O91 and O153. These variations reflect that *E. coli* serotypes are country specific or area specific. However, the health status of the birds, management strategies and climatic conditions might be playing a role in a specific serotype occurrence and its role in disease production (Srinivasan et al. 2013).

Intensive use of antimicrobial drugs especially in the poultry industry is causing increased resistance to antibiotics commonly used and increased incidence of multi-drug resistant strains (WHO 2014). In the current study, *E. coli* isolates showed high rates of resistance to streptomycin and amoxicillin followed by doxycycline and ciprofloxacin. In accordance, many studies in Egypt reported high level of resistance rates among APEC against different groups of antibiotics (Eid and Erfan 2013; Ammar et al. 2015; Shaza 2016), which attributed to the regular usage of these antibiotics for infection control in poultry industry in many district in Egypt. Moreover, antimicrobials are also administered at sub-therapeutic doses to prevent infections or assist in stress management or promote growth. Thus, the discrepancies in rates of *E. coli* resistance between countries appear to be due to differences in the level of dependence on certain groups of antimicrobial and management practices in poultry production, as well as differences in legislation that guide the use of antimicrobials from country to another (Sahoo et al. 2012).

In the current study, the high levels of antibiotic resistance were detected, indicating the increase in multidrug resistance frequencies in *E. coli* isolates. Similar results were reported in Egypt (Eid and Erfan 2013). Meanwhile, six different antibiotic resistance patterns were detected in the current study. In accordance, several studies demonstrated several resistance patterns among their *E. coli* isolates in Egypt (Ammar et al. 2015) and Zimbabwe (Saidi et al. 2012). These results highlight the importance of establishing rules and legislations to regulate the excessive use of antibiotics in Egypt.

Out of 40 MDR *E. coli* isolates, 5 (12.5%) isolates were phenotypically identified based on double disc synergy test as ESBL producers. Then, they were screened for beta lactamase genes, where, only *bla*TEM gene was detected in all isolates had ESBL activity. The incidence rate of different beta lactamase genes in *Enterobacteriaceae* was varied in several studies in Egypt; in septicemic broilers (El-Shazly et al. 2017), mastitic cows (Ahmed and Shimamoto 2011), and humans (Al-Agamy 2013). The epidemiology of ESBL genes is changing rapidly and shows marked geographic differences (Hawkey and Jones 2009). In the current study, the ESBL producing *E. coli* strains were detected in the diseased chickens of two farms, which assuming either ß-lactam antibiotic pressure or initial farms contamination due to improper cleaning and disinfection (Laube et al. 2013; Blaak et al. 2014).

On the other hand, ESBL producing *E. coli* strains showed high rate of resistance to amoxicillin, streptomycin (100%, each), doxycycline (80%), ciprofloxacin and gentamycin (60%, each). In accordance, the high degrees of antimicrobial resistance were reported in Egypt in chickens (El-Shazly et al. 2017) and humans (Khater and Sherif 2014). Based on previous data, the antimicrobial resistance rates are widespread in Egypt. Furthermore, isolates showing ESBL activities are usually play a role in cross resistant to other antibiotics due to the presence of different resistance genes on the same mobile genetic elements (Machado et al. 2005). The molecular investigations of resistance mechanisms in the current study revealed that resistance of ESBL producing *E. coli* isolates to amoxicillin, streptomycin and doxycycline antimicrobials could be explained by the presence of *bla*TEM, *aad*A and *tet*A in the tested isolates, respectively. While in case of ciprofloxacin, the plasmid mediated quinolone resistance (*qnr*S) gene was detected only in 20% of resistant strains. Also, gentamicin resistance rate was 60% in spite of presence of *aad*A gene in all ESBL producing isolates. This might be conducted to presence of another resistance mechanism responsible for a significant loss of antibiotic susceptibility (Davin-Regli et al. 2008).

Also, in the current study, some of plasmid associated genes of ESBL producing *E. coli* isolates were investigated. It was proposed that the iss gene can be used as a marker for distinguishing between APEC and commensal strains (Kwon et al. 2008). In the current study, all tested strains of ESBL *E. coli* were positive for iss gene. Similar results were reported in Egypt (100%) (Ammar et al. 2015) and Kora (100%) (Jeong et al. 2012). Yaguchi et al. (2007) reported that iss gene is most frequently detected in *E. coli* strains isolated from chicken. The *omp*A gene is an essential constituent of outer membranes, thus is required for the cell surface structural integrity (Chen et al. 1980). The *omp*A gene was detected in all tested strains in the current study. In accordance, Johnson et al. (2008) detected *omp*A gene in all APEC isolates, while, Abd El-Mongy et al. (2018) detected *omp*A gene in all isolated *E. coli* serotypes except O153. Meanwhile, eae and *CFA*/I genes could not be detected in all tested ESBL producing *E. coli* strains. This might be due to (*CFA*/I) fimbriae are prevalent among ETEC strains than extra-intestinal strains (Nataro and Kaper 1998), in addition, the eae gene is responsible for intimate adherence on epithelial cells, which typical for EHEC and EPEC strains (Frankel et al. 1998).

Our results showed that all ESBL *E. coli* strains had two virulence genes with antibiotic resistance genes. Thus, the co-occurrence of these antibiotic resistance and virulence plasmid associated genes confirmed that acquisition of antimicrobial resistance genes by ESBL producing *E. coli*, which associated with increased virulence (Ammar et al. 2015).

Based on the current findings, it can be clearly demonstrated that E. coli is a major pathogen associated with arthritis infection of poultry in Egypt. Meanwhile, the high levels of multidrug resistant were detected. These might be attributed to a combination of antibiotic resistant genes, which could cause colibacillosis treatment failure, in addition to multiple virulence determinants in plasmids of ESBL producing *E. coli* isolates

Whatever, studding of virulence and resistance genes is very important to know the pathogenicity of the present microorganisms, but more investigation about other mechanism of resistance is necessary. Based on the reported data herein, the high incidence rate of virulent multidrug resistant *E. coli* strains among chicken flocks requires

urgent intervention approaches to control spread of these strains to humans through poultry and its products in Egypt.

Disclosure Statement
No competing financial interests exist.

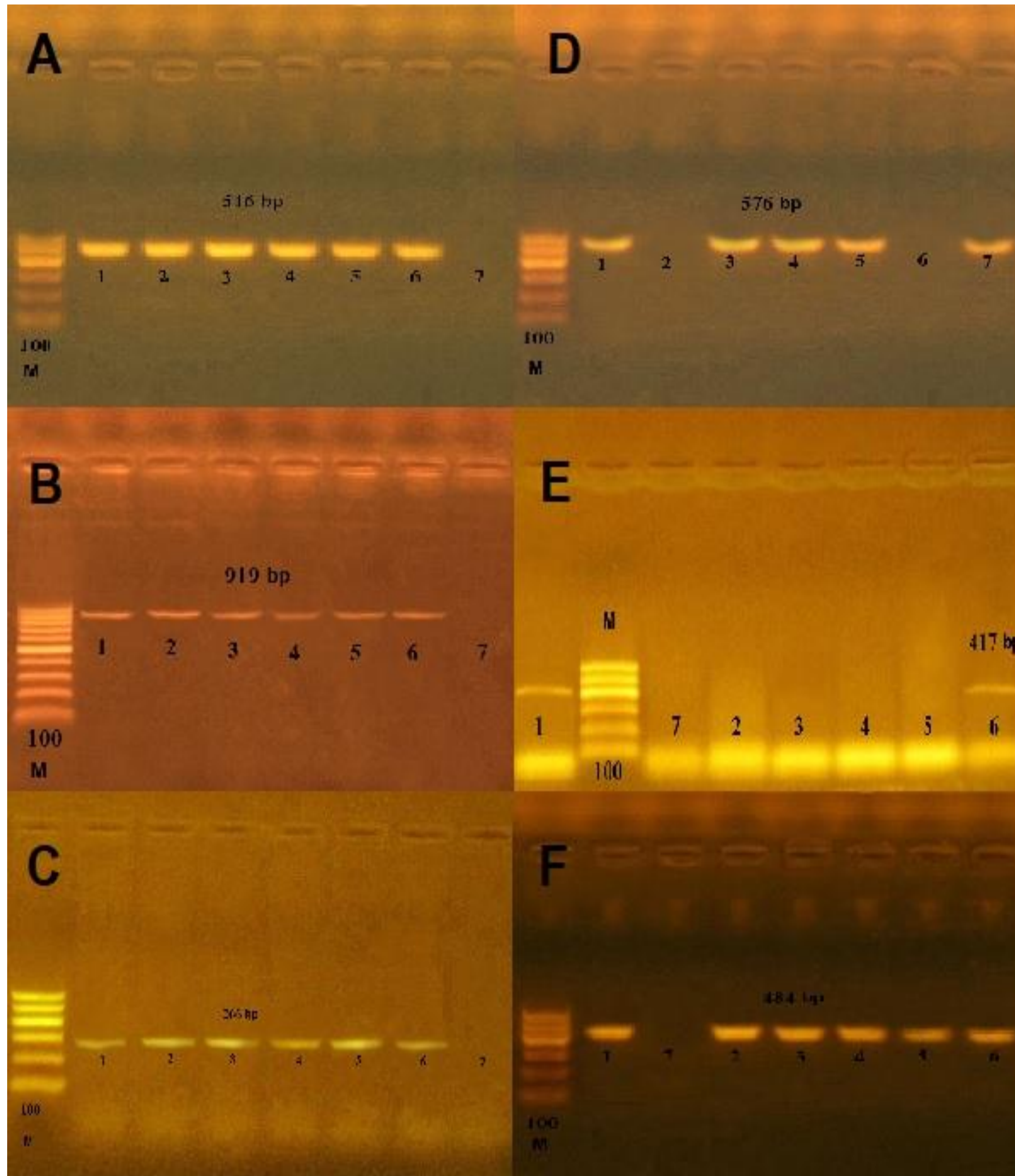

Figure 1:-Agarose gel electrophoresis pattern for amplification of antibiotic resistance and virulence plasmid associated (A; *bla*TEM, B; *omp*A, C; *iss*, D; *tet*A, E; *qnr*S, F; *aad*A) genes of identified ESBL *E. coli* isolates; 2-6; positive isolates, 7; Negative control, 1; Positive control, M: DNA molecular size marker (100 bp)

Table 1:-Primers used for pcr amplification of virulence and antibiotic resistance plasmid associated genes of ESBL *E. coli*

Target genes	Primers sequences (5`→3`)	Size bp[1]	°C[2]	Reference
***bla*$_{TEM}$**	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	**50**	Colom et al. (2003)
***bla*$_{CTX-M}$**	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYSAGCGG	592	**55**	Mulvey et al. (2003)
***bla*$_{SHV}$**	TTATCTCCCTGTTAGCCACC GATTTGCTGATTTCGCTCGG	795	**55**	Dierikx et al. (2010)
***omp*A**	AGCTATCGCGATTGCAGTG GGTGTTGCCAGTAACCGG	919	**58**	Ewers et al. (2007)
***eae*A**	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248	**51**	Bisi-Johnson et al. (2011)
iss	ATGTTATTTTCTGCCGCTCTG CTATTGTGAGCAATATACCC	266	**54**	Yaguchi et al. (2007)
***CFA*/I**	GCTCTGACCACAATGTTGA TTACACCGGATGCAGAATA	364	**50**	Ghosal et al. (2007)
***Tet*A**	GGTTCACTCGAACGACGTC CTGTCCGACAAGTTGCATGA	576	**50**	Randall et al. (2004)
***qnr*S**	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417	**55**	Robicsek et al. (2006)
***aad*A**	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATTT	484	**54**	Randall et al. (2004)

[1] bp; Base pair, [2] Annealing Temperature, *qnr*S; plasmid mediated quinolone resistance gene, *omp*A; outer membrane protein A gene, *eae*A; intimin *E. coli* attaching and effacing gene, *iss*; increased serum survival gene, *aad*A; streptomycin/spectinomycin adenylyltransferase gene, *bla*; β-lactamase genes, *tet*A; tetracycline resistance gene A, *CFA*/I; colonization factor antigen I

Table 2:-Antibiotic resistance patterns of *E. coli* isolates

Pattern	Antimicrobial resistance pattern	No of *E. coli* isolates (%)
1	**AML - S - CN - CIP - DO**	**5 (12.5)**
2	**AML - S - CN - DO**	**5 (12.5)**
3	**AML - S - CIP - CN**	**4 (10)**
4	**AML - S - CIP - DO**	**15 (37.5)**
5	**AML - S - CIP**	**4 (10)**
6	**AML - S - DO**	**7 (17.5)**
Total		**40 (100)**

AML; amoxicillin, S; streptomycin, CN; gentamycin, CIP; ciprofloxacin, DO; doxycycline

Table 3:-Antibiotic resstant profiles and contribution of antibiotic resistance and virulence plasmid associated genes in Esbl *E.coli* isolates

Strain	Phenotypic resistant profile	Genotypic resistant profile	Virulence gene profile
1	AML-S–CN-CIP-DO	*bla*$_{TEM,}$ *aad*A, *tet*A	*iss, omp*A
2	AML- S-DO	*bla*$_{TEM,}$ *aad*A, *tet*A	*iss, omp*A
3	AML-S-CN-CIP	*bla*$_{TEM,}$ *aad*A,	*iss, omp*A
4	AML-S-DO	*bla*$_{TEM,}$ *aad*A, *tet*A	*iss, omp*A
5	AML-S-CN-CIP-DO	*bla*$_{TEM,}$ *aad*A, *qnrS, tet*A	*iss, omp*A

AML; amoxicillin, S; streptomycin, CN; gentamycin, CIP; ciprofloxacin, DO; doxycycline, *qnr*S; plasmid mediated quinolone resistance gene, *omp*A; outer membrane protein A gene, *iss*; increased serum survival gene, *aad*A; streptomycin/spectinomycin adenylyltransferase gene, *bla*$_{TEM}$; β-lactamase gene, *tet*A; tetracycline resistance gene.

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