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Original Research

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Genes Contributed on Biofilm Forming Bacteria Incriminated in Various Disease Conditions in Cattle

Amira E. Lamey[1*], Amany O. Selim[2], Noha M.A. Atia[1], Rehab E. Mowafy[3]

[1]Bacteriology Department, Zagazig Province Laboratory, Animal Health Research Institute, ARC, Egypt.

[2]Bacteriology Department, Benha Province Laboratory, Animal Health Research Institute, ARC, Egypt.

[3]Pathology Department, Zagazig Province Laboratory, Animal Health Research Institute, ARC, Egypt.

***Correspondence**
Corresponding author: Amira E. Lamey
E-mail address: amera.lam33y@yahoo.com

Abstract

Biofilm production is a complex process that occurs as a series of molecular and physiological events; bacteria employ biofilms as a physical barrier to shield themselves from antimicrobial agents. In this study, biofilm-forming bacteria were isolated from the lung, heart, spleen, and liver of diseased cattle after slaughter. Out of 210 examined samples, 82 samples were positive for biofilm forming ability either by Cong red binding assay or by tube method. The 82 isolates were identified as 27 *E. coli* strains belonging to O146:H21, O26:H11, O55:H7, O111:H2, O127:H6, O124:H2, O153:H1, and O111:H4, 23 isolates were identified as *S. aureus*, 15 as *K. pneumoniae* belonging to K1(HVKP), K2 (HVKP), K1 (CKP), and K2 (CKP) and 3 strains as K. *oxytoca*. Besides, 14 strains were identified as *P. aeruginosa* belonging to O6 (G), O11 (E), and O2 (B). Results showed that *E. coli* and *P. aeruginosa* isolates were positive for all tested biofilm-related genes whereas *mkr*A and *spa*A genes were the most commonly detected genes among *K. pneumoniae* and *S. aureus* isolates, respectively. Isolates showed a high level of antimicrobial resistance (AMR) to most tested antimicrobial agents. Macroscopical examination of the affected organs showed that the lung lost its spongy texture; the spleen was darkened and enlarged in addition to congestion of the liver and heart. Microscopical findings showed that the liver and heart were congested with leucocytic cells infiltrations while the lung showed peribronchial hypercellularity, excessive interstitial and alveolar cellular infiltration with severe congestion and degeneration of alveolar epithelial cells, destruction of alveoli with lymphocytic cells infiltration and collapsed alveoli with fibrous tissue proliferation while spleen exhibited marked congestion with aggregation of bacterial colonies either focal or diffuse, necrosis was also detected.

KEYWORDS
Cattle, PCR, Biofilm bacteria, Pathology

INTRODUCTION

Biofilm has a great impact on veterinary medicine particularly in livestock leading to serious economic losses (Chakraborty *et al.*, 2018). Over the years, little concern has been paid to biofilm produced in animal products and edible tissues, with most of the attention being paid to human biofilm. The greatest issue in dealing with biofilm is its extraordinary capacity to resist most antibiotics (Abdullahi *et al.*, 2016).

A biofilm is a syntrophic consortium of microorganism's cells that cling to one another and become enveloped in a slime matrix consisting of extracellular polymeric substances (EPS) (Hall-Stoodely *et al.*, 2004). The viscoelastic properties of the EPS matrix, which has a mushroom-like structure, are responsible for the mechanical firmness of biofilms. In reaction to unfavorable environmental conditions, most microorganisms develop biofilms (Gebreyohannes *et al.*, 2019).

First colonist bacteria adhere to the surface initially by weak van der Waals forces which are reversible attachments followed by permanent and irreversible adhesion owing to some virulence structures such as pili or fimbriae (Briandet *et al.*, 2001; Takahashi *et al.*, 2010)

Many papers demonstrated that *S. aureus* attaches to biotic or abiotic surfaces forming biofilm allowing its adaptation to stressful environmental circumstances, nevertheless association between biofilm formation in *S. aureus* isolates and their genetic background was not well studied (Salehzadeh *et al.*, 2016).

Foodborne pathogens like *E. coli* use a variety of tactics to survive and stay in the environment, and various *E. coli* serogroups have a biofilm-forming capacity (Vogeleer *et al.*, 2015). The endemicity of *E. coli* is associated with biofilm formation aided by some virulence markers establishing its mechanical attachment as fimbria (Palmer *et al.*, 2007; Grakh *et al.*, 2022).

P. aeruginosa is recognized not only as a human pathogen but also as an animal pathogen causing different disease conditions such as mastitis and pneumonia but most studies in cattle are concerned with mastitic cases and lack of histopathological analysis (Park e al., 2014; Banerjee *et al.*, 2017).

Antimicrobials are known to be more effective against rapidly growing cells but in the case of biofilm-forming bacteria, the bacteria residing in the outer layers are affected while the bacteria living in the inner layers exhibited high resistant levels (Mah and O'Toole, 2001). More effort should be directed at interfering with biofilm growth rather than focusing on therapy, which

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is frequently difficult to perform, and this can be accomplished through increased research on the genetic mechanisms involved in biofilm development (Abdullahi *et al*., 2016).

Histopathological analysis of the biofilm-forming bacteria-affected organs revealed congestions, aggregations, and multinucleated hepatocytes in the liver, chronic inflammatory cell infiltration of the kidney tubule interstitial, coagulative necrosis of the kidney and spleen, as well as significant stromal fibrosis of the spleen. The degenerative change that occurs most frequently in cells is coagulative necrosis. The virulence factors that bacteria produced, such as those associated with biofilm formation, hemagglutination characteristics, and capsule development, were reflected in the lethality and pathological effects (Akinkunmi *et al*., 2014).

The significant and widespread clinical effects of bacterial biofilms have motivated us to conduct this research to better understand the genetic basis of biofilm formation. To do this, we isolated bacteria from cattle and examined them histopathologically to look for signs of biofilm-related virulence genes and their effects on antibiotic resistance.

MATERIALS AND METHODS

Sample collection

A total of 210 fresh random tissue specimens of different edible offal of bovine carcasses were collected directly after slaughtering and evisceration from different slaughterhouses and street vendors in different districts in Sharkia and Kalyobia provinces. Samples were collected during March to October 2022 from apparently healthy animals exposed to anti-mortem inspection. The specimens were collected from condemned parts or from street vendors with signs of disease such as hepatized lungs, enlarged spleen or livers, or brown atrophy of the heart. Samples including (liver, heart, spleen, and lungs) were collected under complete aseptic conditions, inoculated in brain heart infusion broth then immediately transported to the laboratory for further examination.

Ethical approval

Verbal consent was obtained from each of the cattle owners who had sent animals for slaughter at the abattoir after explaining the purpose and importance of the study before data collection.

Clinical samples were collected from freshly slaughtered animals from private farms. The animal study was endorsed by the committee of Animal Welfare and Research Ethics, Animal Health Research Institute, ARC, Egypt.

Use of organs or other material from animals slaughtered as part of routine commercial food (sourced from butchers, or abattoirs) Examples of when AEC approval is not required.

Phenotypic characterization of biofilm-forming ability

Qualitative Detection of Biofilm forming bacteria

Biofilm formation was detected using the Congo red agar (CRA) method, color of bacterial colonies was checked as black colonies with dry crystalline consistency indicating a positive result, weak slime producers usually remained pink. The absence of a dry crystalline colonial morphology and a darkening of the colonies indicated an ambiguous outcome (Pramodhini *et al*., 2012).

Quantitative assessment of biofilm formation (Tube method)

Assessment of biofilm-forming quantitatively by using the tube method and according to results laid down by Christensen *et al*. (1982). Bacteria were classified into weak, moderate, and strong biofilm producers.

Isolation and identification of biofilm-forming bacteria

Biofilm-forming bacteria were picked up from CRM and cultivated on blood agar, McConkey agar, EMB, Mannitol salt agar, Paired parker, and Cetramide agar. Pure colonies were identified morphologically according to shape, arrangement, and staining reaction. Members of Enterobacteriaceae, *Pseudomonas*, and *Staphylococci* were biochemically identified (Markey *et al*., 2013).

Serological identification of isolated bacteria

Serological identification of the recovered Gram-negative bacilli isolates including *E. coli*, *Klebsiella*, and *Pseudomonas* was done according to Kok *et al*., 1996; Edmonson and Cooke 1979; Glupczynski *et al*., 2010, respectively in Food Analysis Center, Faculty of Veterinary Medicine, Benha University, Egypt.

Genotypic detection of biofilm virulence-related genes

DNA extraction was carried out by Bioneer DNA extraction kit (Cat. N: K-3032) according to the manufacturer instructions. Amplification of target genes using designed pair of specific primers (Table 1), the primary denaturation was done at 94 °C for 5 min. for all cycles, amplification cycling protocol for each gene was illustrated in Table 1. Amplified PCR products were visualized on agarose gel electrophoresis.

Antibiotic susceptibility testing

The disc diffusion method was used to conduct an AMS test on bacterial isolates to determine whether they were resistant to 10 different antibiotics (Markey *et al*., 2013). The CLSI guidelines' criteria were used to interpret the sizes of the inhibition zones (CLSI, 2019). Multidrug-resistant isolates were those that were resistant to more than three distinct antibiotic classes.

Histopathological examination of tissue samples

Most parenchymatous organ tissue samples, including those from the liver, heart, spleen, and lung, were collected, fixed in 10% neutral-buffered formalin, and then embedded in paraffin wax. According to Suvarna *et al*. (2013)'s description, sections were cut, stained with hematoxylin and eosin, and then analyzed microscopically.

RESULTS

The recovery rate of biofilm-producing bacteria

In the current study, out of 210 different examined samples for biofilm-forming ability, 82 isolates were considered biofilm producers (39.02%). Further identification of isolates revealed the recovery of 27 *E. coli*, 23 *S. aureus*, 18 *Klebsiella*, and 14 *Pseudomonas* isolates. The obtained isolates were classified by the tube method into strong, moderate, or weak biofilm producers. Results were shown in Table 2.

Table 1. Oligonucleotide primers and cycling protocols for biofilm related genes in isolated bacteria.

Target Gene	Primer Sequence (5'-3')	Secondary Denaturation	Annealing	Extension	PCR Product (bp)	Reference
*adr*A	F:ATGTTCCCAAAAATAATGAA R:TCATGCCGCCACTTCGGTGC	94˚C /30 sec	50˚C /1min	72˚C /1min	1113	Bhowmick *et al*. (2011)
*fim*H	F:TGCAGAACGGATAAGCCGTGG R: GCAGTCACCTGCCCTCCGGTA		50˚C /40sec	72˚C /45sec.	508	Ghanbarpour and Salehi (2010)
crl	F: TTTCGATTGTCTGGCTGTATG R: CTTCAGATTCAGCGTCGTC		50˚C /30sec	72˚C /30sec.	250	
*pap*C	F:TGATATCACGCAGTCAGTAGC R: CCGGCCATATTCACATAA		58˚C /40sec	72˚C /45sec.	501	Wenjie *et al*. (2008)
*fim*A	F: CGGACGGTACGCTGTATTTT R:GCTTCGGCGTTGTCTTTATC		55˚C /40sec	72˚C /45sec.	436	Alcántar-Curiel *et al*. (2018)
*mkr*A	F: CGGTAAAGTTACCGACGTATCTTGTACTG R: GCTGTTAACCACACCGGTGGTAAC		55˚C /40sec	72˚C /45sec.	475	
*ecp*A	F: GCAACAGCCAAAAAAGACACC R: CCAGGTCGCGTCGAACTG		55˚C /40sec	72˚C /45sec.	477	
*pel*A	F: CATACCTTCAGCCATCCGTTCTTC R: CGCATTCGCCGCACTCAG		60˚C /40sec	72˚C /45sec.	786	Ghadaksaz *et al*. (2015)
*psl*A	F: TCCCTACCTCAGCAGCAAGC R: TGTTGTAGCCGTAGCGTTTCTG		60˚C /40sec	72˚C /45sec.	656	
*fli*C	F:TGAACGTGGCTACCAAGAACG R: TCTGCAGTTGCTTCACTTCGC		56.2˚C /30sec	72˚C /30sec.	180	
*ica*A	F: CCT AAC TAA CGA AAG GTA G R: AAG ATA TAG CGATAA GTG C		49˚C /60sec	72˚C /1min.	1315	Ciftci *et al*. (2009)
bap	F: CCC TAT ATC GAA GGT GTA GAA TTG R: GCT GTT GAA GTT AAT ACT GTA CCT GC		62˚C /40sec	72˚C /1min..	971	Cucarella *et al*. (2001)
*spa*A	F: TCA ACA AAG AAC AAC AAA ATG C R: GCT TTC GGT GCT TGA GAT TC		55˚C /30sec	72˚C /30sec.	226	Wada *et al*. (2010)

Table 2. Recovery rate of isolated biofilm forming isolates.

Bacteria	Biofilm			Total	
	Strong	Moderate	Weak	No.	%
E. coli	25	2	0	27	32.9
S. aureus	21	2	0	23	28.04
Klebsiella	18	0	0	18	21.95
P. aeruginosa	13	1	0	14	17.07

Serological identification

E. coli, *Klebsiella*, and *Pseudomonas* isolates were serotyped revealing the prevalence of different serogroups as presented in Table 3.

Antimicrobial susceptibility test

Results of antimicrobial resistance (AMR) were presented in Table 4 and revealed that *E. coli* isolates showed the highest susceptibility to colistin (CT) and ciprofloxacin (CIP), elevated resistance levels to ampicillin (AMP), erythromycin (E), and cefotaxime (CTX), and variable resistance levels to the rest of the antibiotics, whereas *Klebsiella* isolates were mostly sensitive to CIP, gentamycin (CN) and chloramphenicol (C) and variable susceptibility to other antibiotics. *P. aeruginosa* was only sensitive to CIP and CN, and highly resistant to other tested antibiotics. *S. aureus* isolates exhibited variable resistance levels to antibiotics. Most isolates showed a high level of MDR.

Detection of virulence genes

Molecular detection of biofilm-related genes was performed on some of the culturally positive bacterial isolates. PCR results showed positive amplification of the target genes in all *E. coli* and *P. aeruginosa* isolates and in some but not all *K. pneumoniae* . and *S. aureus* isolates (Fig. 1- 4).

A correlation between microorganisms, biofilm ability, and virulence genes' existence was presented in Table 5.

Table 3. Serological identification of bacterial isolates.

Bacterial isolates	Serodiagnosis	NO. of isolates
E. coli	O146:H21	4
	O26:H11	6
	O55:H7	3
	O111:H2	2
	O127:H6	4
	O124	2
	O153	3
	O111:H4	2
	Untyped	1
Klebsiella	*K. pneumoniae* K1 (HVKP)	5
	K. pneumoniae K2 (HVKP)	3
	K. pneumoniae K1 (CKP)	4
	K. pneumoniae K2 (CKP)	3
	K. oxytoca	3
Pseudomonas	*P. aeruginosa* O6 (G)	4
	P. aeruginosa O11(E)	4
	P. aeruginosa O2 (B)	6

Postmortem examination

Lung lost its spongy texture. Darkened and enlarged spleen was also detected with congestion of the liver and heart.

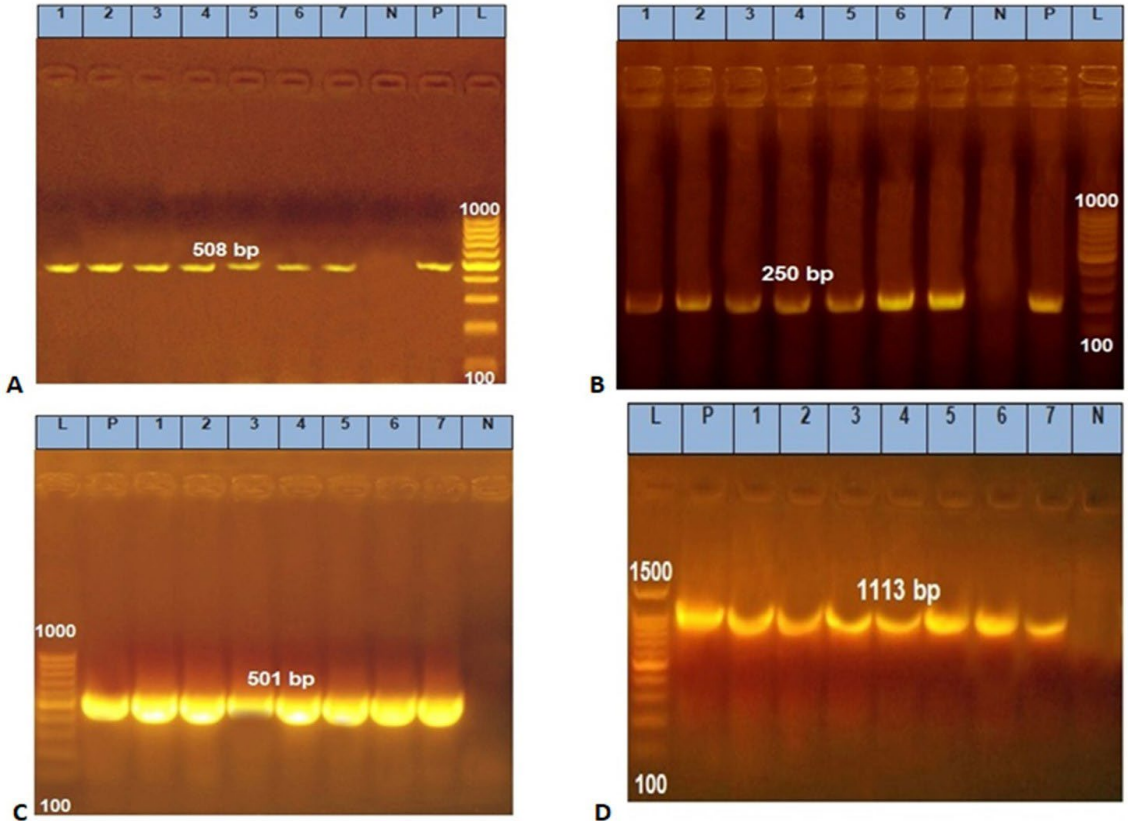

Fig. 1. (A, B, C, D): Agarose gel electrophoresis of *fim*H, *crl*, *pap*C, and *adr*A genes, respectively. Lanes (1-7): positive amplification of target genes at 508, 250, 501, and 1113 bp, respectively. M: 100 bp DNA marker. P: positive control. N: negative control.

Table 4. Antibiotic resistance profile of isolated bacteria.

AMA / symbol	Potency (µg)	*E. coli* (n=27)		*Klebsiella* (n=18)		*S. aureus* (n=23)		*P. aeruginosa* (n=14)	
		R	%	R	%	R	%	R	%
Ampicillin (AMP)	10	24	-88.8	14	-77.7	15	-65.2	13	-92.8
Ceftriaxone (CTX)	30	25	-92.59	16	-88.8	16	-69.5	14	-100
Chloramphenicol (C)	30	19	-70.37	6	-33.3	11	-47.8	12	-85.7
Ciprofloxacin (CIP)	5	6	-22.2	3	16.6	2	-8.69	5	-35.7
Colistin (CT)	25	1	-3.7	-	-	-	-	-	-
Doxycycline (DO)	30	20	-74	8	-44.4	12	-52.17	12	-85.7
Erythromycin (E)	15	25	-92.6	17	-94.4	8	-34.78	14	-100
Gentamycin (CN)	10	7	-25.9	4	-22.2	4	-17.39	3	-21.4
Lincomycin (MY)	15	18	-66.6	-	-	23	-100	13	-92.8
Imipenem (IMP)	10	-	-	1	-5.5	-	-	1	-7.14
Vancomycin	30	-	-	-	-	1	-4.34	-	-
Sulfamethoxazole +Trimethoprim (SXT)	25	17	-62.9	9	-50	4	-17.39	11	-78.5

AMA: Antimicrobial agents; Number in parenthesis are percentage

Microscopical findings

The main aim of the histopathological study was to follow the pathology after infection of some organs with a high dose of some biofilm-forming bacteria in examined tissue samples such as in the liver, lung, spleen, and heart. Among the previously examined organs liver and heart were less affected with mild lesions restricted to congestion with leucocytic cell infiltrations while the lung and spleen were the most affected organs suffering severe lesions. Obtained lesions included lung with peribronchial hypercellularity (Fig. 5a). Excessive interstitial and alveolar cellular infiltration with severe congestion (Fig. 5b), degeneration of alveolar epithelial cells, destruction of alveoli with lymphocytic cells infiltration and collapsed alveoli with fibrous tissue proliferation were also seen, while spleen exhibited marked congestion with aggregation of bacterial colonies (Fig. 5c) either focal or diffuse. Necrosis was also detected which mainly infiltrated with primary bacterial colonies formation in most cases (Fig. 5d, e). Diffuse primary bacterial colony formations were noticed in the spleen of most examined tissues (Fig. 5f).

DISCUSSION

In the veterinary field, few reports focus on biofilm's direct role in infection with most studies in literature focusing on in vitro characterization of bacterial biofilm and not on their role in vivo (Pedersen *et al.*, 2021). Unfortunately, the bacteria isolated from cattle in the existing study fall under this category (zoonotic

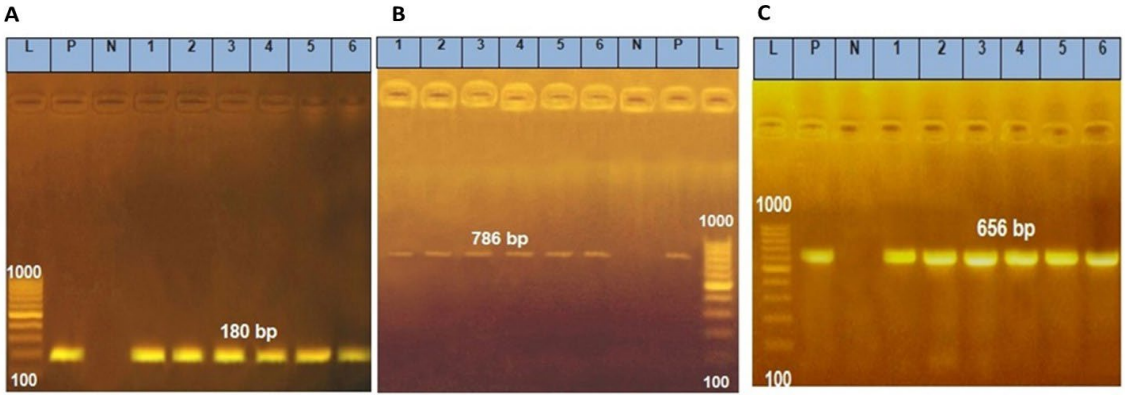

Fig. 2. (A, B, C): PCR product electrophoresis of *psl*A, *pel*A, and *fli*C genes of *P. aeruginosa* isolates. Lanes (1-6): positive amplification of target genes at 656, 786, and 180 bp, respectively. L: 100-bp marker (ladder). P: positive control. N: negative control.

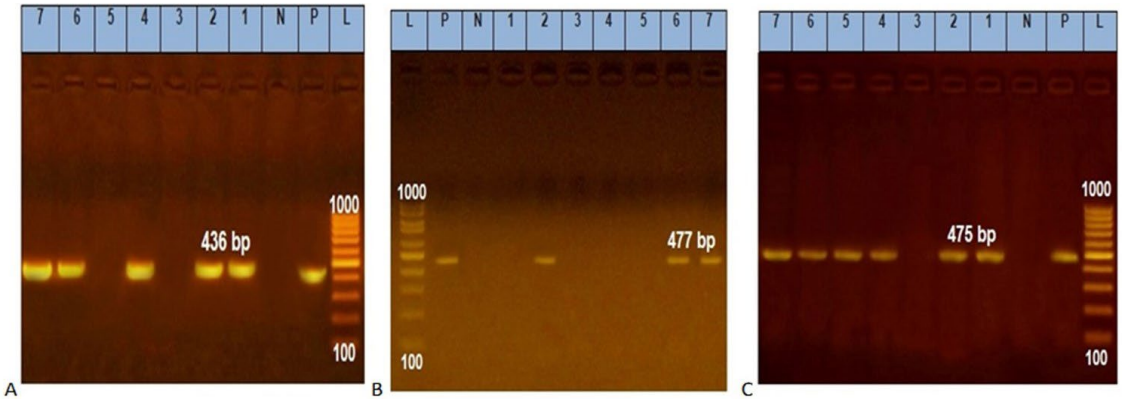

Fig. 3. (A, B, C): Electrophoretic banding pattern of *K. pneumoniae* isolates. Lanes (1, 2, 4, 6, and 7), Lanes (2, 6, and 7), Lanes (1, 2, 4, 5, 6, and 7): positive amplification of *fim*A, *ecp*A, and *mkr*A genes at 436, 477, and 475 bp, respectively. M: 100 bp DNA marker. P: positive control. N: negative control.

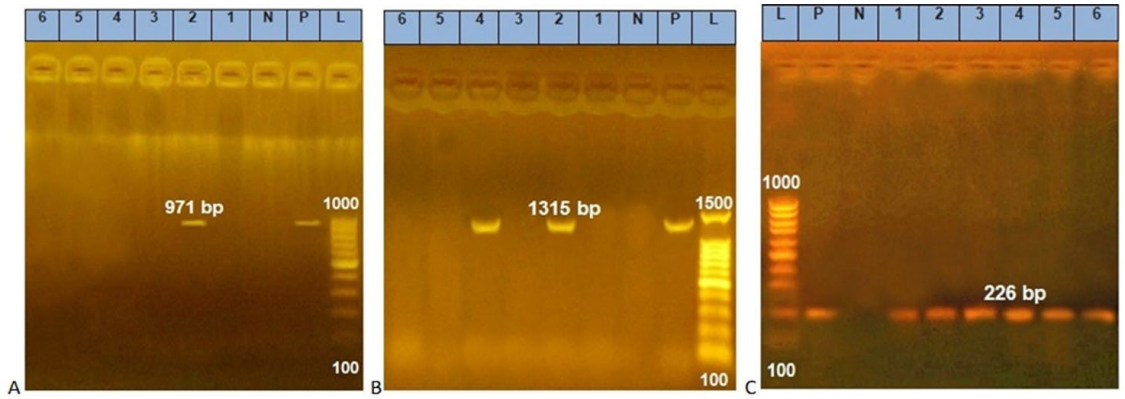

Fig. 4. Agrose gel electrophoresis in *S. aureus* isolates. A: Lane 3: positive amplification of *bap* gene at 971 bp. B: lane (2, 4): positive amplification of the *ica*A gene at 1315 bp. C: Lanes (1-6): positive amplification of *spa*A gene at 226 bp. L: 100-bp marker (ladder). P: positive control. N: negative control.

pathogens) in addition to their biofilm-forming ability and multidrug resistance, both features can complicate the condition and made them difficult to treat.

Biofilm formation by bacteria is a widespread, common, and dynamic phenomenon (Gebreyohannes *et al*., 2019). Biofilm is considered an important example of bacterial adaptation as it allows its growth as a sessile, exopolymer-hidden community leading to persistent infections (Jefferson 2004). The genetic basis for biofilm production and its association with antibiotic resistance is yet to be addressed, so in the existing study, we tried to highlight the role of some not all genes implicated in biofilm formation in each bacterium concerning their antimicrobial resistance.

Results revealed that 82 isolates (39.02%) produce black and rough colonies on CRA indicating positive test results, interpretation of results varied among studies as some considered black colonies as a positive result and red colonies as a negative result (Jain and Agarwal, 2009) whereas Cucarella *et al*. (2004) considered rough colony as a positive result whatever its color. Such discrepancy is accepted since the test itself was not originally designed for investigating special M.O. therefore further studies are needed to fully clarify this assertion (Bissong and Ateba, 2020).

Obtained isolates were cultured on specific media and further identified biochemically. *E. coli* was the most identified bacterium (27) followed by *S. aureus* (23), *Klebsiella* (18), and *P. aeruginosa* (14), this is in accordance with Mahmood and Abdullah (2019) who stated that the most frequent biofilm producing coliforms are *E. coli* and *K. pneumoniae* with strong biofilm forming capacities. Biofilm production was assessed quantitatively based on criteria laid down by Christensen via the tube method (Christensen *et al*., 1982) and the results showed that most isolates were strong biofilm producers.

Serological identification of *E. coli*, *Klebsiella*, and *Pseudomonas* were performed for each bacterium. The ability to differentiate commensal *E. coli* from extraintestinal pathogenic *E. coli* is important for risk assessment and pathogenic potential based on its source and serotype as commensal *E. coli* are untyped. In 1987, WHO recognized EPEC to comprise strains of 12 O serogroups (O26, O111, O86, O114, O119, O125-128, O142, and

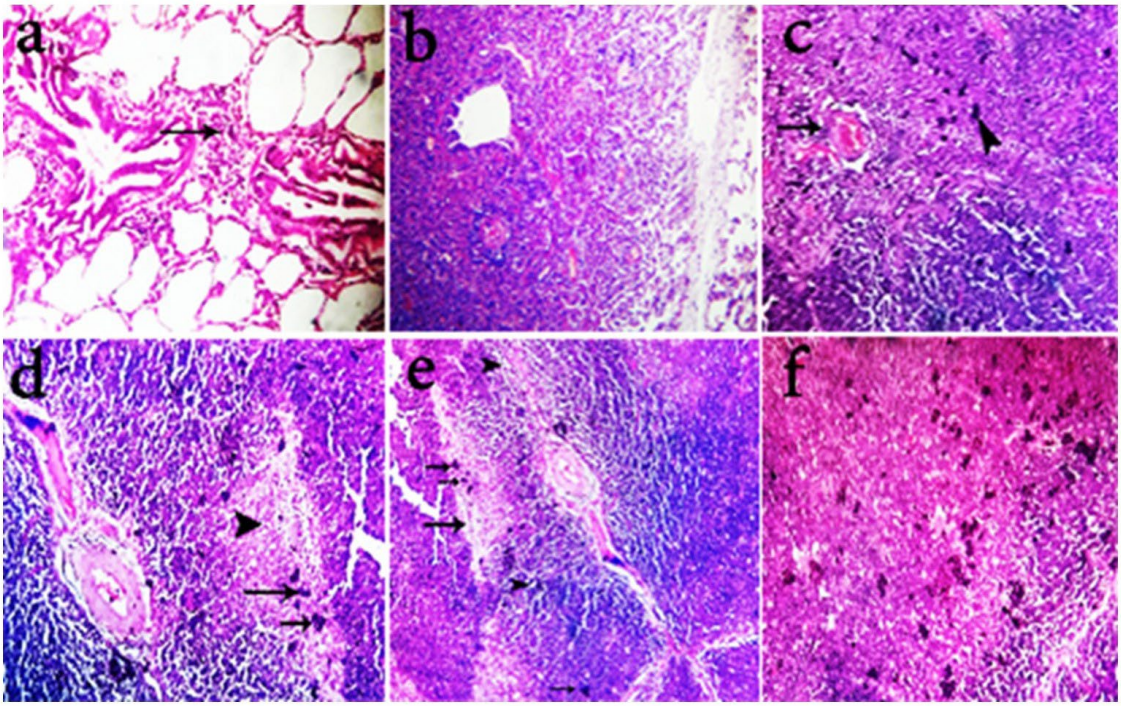

Fig. 5. Photomicrograph of lung and spleen of infected cattle showing (a): lung with peribronchial hypercellularity (H&E x400). (b): lung with excessive cellular infiltration with severe congestion (H&E x200). (c): spleen with marked congestion (arrow) and primary aggregation of bacterial colonies (arrowhead) (H&E x200). (d&e): spleen with the necrotic area (arrowhead) infiltrated with primary bacterial colonies formation(arrow) (H&E x200 &100) (f): spleen with diffuse primary bacterial colonies formation (H&E x100).

O158) (WHO, 1987). Serological identification of *K. pneumoniae* is important as it possesses a pronounced capsule described as K type which provides mucoid phenotype to the isolate and acts as a virulence marker (Derakhshan *et al*., 2016). Herein *K. pneumoniae* isolates were pathogenic as they belonged to K1 and K2 serotypes. Serotyping of *P. aeruginosa* according to the International Antigenic Typing Scheme (IATS) has classified it into 20 different serotypes (O1 to O20) based on the structure of their O-polysaccharide which plays an important role in the virulence of *P. aeruginosa* (Liu *et al*., 1983). In the present study, the recovered isolates belonged to O6, O11, and O2. This agrees with Bert *et al*. (1996) who proposed these serotypes to account for 65% of *P. aeruginosa* infections.

Several studies have led to the identification of a plethora of genes associated with biofilm development, but sorting out the role of all these genes is an exceedingly complex task (Jefferson, 2004).

Adhesive organelles such as curli and type 1 fimbria play a major role in *E. coli* attachment and mediate biofilm formation (Wood, 2009). Curli fibers maintain cell-to-cell and cell-to-surface interactions and are encoded by the csg operon (Sharma *et al*., 2016). The *adr*A gene encodes diguanylate cyclase that regulates cellulose production, and it needs a functional *crl* gene for its expression (Gualdi *et al*., 2008). The *fim*H is a major determinant of the adhesive subunit of type 1 fimbriae with a major role in pathogenesis as it allows bacteria to attach to mannosylated receptors on the luminal surfaces of mammalian epithelial cells (Sharma *et al*., 2016).

Herein PCR test was applied on 7 *E. coli* biofilm-producing isolates belonging to different serogroups for the detection of 4 genes (*fim*H, *pap*C, *crl*, and *adr*A) that were assumed to be mostly associated with biofilm formation according to the above-mentioned data. Results revealed the detection of all tested genes in all isolates and that supposed that there was a strong correlation between biofilm-forming ability and the 4 studied genes. This finding agreed with previous studies that showed an association of the *fim*H gene to biofilm formation in *E. coli* (Bronzato *et al*. 2017). Naves *et al*. (2008) showed that strong biofilm-producing *E. coli* had a high frequency of *pap*G, and *pap*C. Additionally, *fim*H and *pap* genes were detected in biofilm *E. coli* isolates from mastitic milk emphasizing its role in biofilm formation (Van Houdt and Michiels, 2005). A significant correlation between biofilm formation and the presence of the *fim*H gene in extraintestinal *E. coli* (Cargole-Novella *et al*., 2015). The *fim*H and *crl* genes were detected in all MDR biofilm-producing *E. coli* isolated from mastitic milk samples (Garcia *et al*., 2015).

P. aeruginosa is an increasing global problem of public health concern posing many therapeutic challenges (Panwar *et al*., 2020). Studies on *P. aeruginosa* infection in cattle are mainly concerned with mastitic cases and lack histopathological studies (Tosaki *et al*., 2018). The *psl*A and *pel*A genes play a major role in the formation of the carbohydrate-rich structure of the biofilm matrix (Ghadaksaz *et al*. 2015). The *fli*C gene encoding flagellin subunit (FliC) has an important role in initial attachment and detachment during the dispersal stage of biofilm formation. (Suriyanarayanan *et al*., 2016).

Conventional PCR was carried out for the detection of *pel*A and *psl*A and *fli*C genes as they are considered the most fundamental genes implicated in biofilm according to before mentioned data. Results revealed the existence of these genes with 100%, these data matched with other studies conducted on biofilm-forming P. aeurginosa isolated from the human and avian host (Ghadaksaz *et al*., 2015; El Demerdash and Bakry, 2020).

However, *Klebsiella* is a well-studied human pathogen with hypervirulent and MDR strains that emerged globally; there is a lack of knowledge on the persistence and characterization of this pathogen from a non-human host (Franklin-Alming *et al*., 2021). Adherence of *K. pneumoniae* to eukaryotic epithelial cells is attributed to 2 majors adhesive pilli (Type I and 3 pilli), Type 1 are firm and filament-like projections encoded by the *fim* gene cluster) which consists of the *fim* A gene codes the major subunit *fim*A and the *fim*H gene codes the tiny tip adhesin *fim* H subunit (Struve *et al*., 2008). Type 3 pilli are spiral-like threads encoded by the mrkABCD gene cluster, which consist of major pillus (mrkA) and the minor adhesin tip (mrkD) (Paczosa and Mecsas, 2016), though it was suggested that mrkA is enough to facilitate bacterial interactions during biofilm development (Langstraat *et al*., 2001). ECP (*E. coli* common pillus antigen) has a principal role in cell adherence and biofilm formation (Alcantar-Curiel *et al*., 2013), it was revealed that a homolog of the *ecp*A gene is present in other members of Enterobacteriaceae other than *E. coli* mainly K. pneumoniae (Martnez-Santos *et al*., 2012).

Consequently, we choose *fim*A and *mkr*A and *ecp*A genes to be checked in 7 *K. pneumoniae* isolates and results showed pos-

Table 5. Correlation between M.O., biofilm ability and virulence genes existence.

Identified Bacteria	Isolate I.D	Identification	Biofilm ability	Virulence genes detected			
				*fim*H	*pap*C	*crl*	*adr*A
E. coli	1E	O146	Strong	+	+	+	+
	2E	O55	Strong	+	+	+	+
	3E	O153	Strong	+	+	+	+
	4E	O26	Strong	+	+	+	+
	5E	O111	Moderate	+	+	+	+
	6E	O124	Strong	+	+	+	+
	7E	O127	Moderate	+	+	+	+
				*mkr*A	*fim*A	*ecp*A	
Klebsiella sp.	1K	K1 (HVKP)	Strong	+	+	-	
	2K	K2 (HVKP)	Strong	+	+	+	
	3K	K2 (CKP)	Strong	-	-	-	
	4K	K2 (CKP)	Strong	+	+	-	
	5K	K2 (HVKP)	Strong	+	-	-	
	6K	K1 (HVKP)	Strong	+	+	+	
	7K	K2 (HVKP)	Strong	+	+	+	
				*psl*A	*pel*A	*fli*C	
P. aeruginosa	1P	O6 (G)	Strong	+	+	+	
	2P	O11(E)	Strong	+	+	+	
	3P	O2 (B)	Strong	+	+	+	
	4P	O2 (B)	Strong	+	+	+	
	5P	O11(E)	Strong	+	+	+	
	6P	O6 (G)	Moderate	+	+	+	
				*ica*A	*spa*A	*bap*	
S. aureus	1S	*S. aureus*	Strong	-	+	-	
	2S	*S. aureus*	Strong	+	+	+	
	3S	*S. aureus*	Moderate	-	+	-	
	4S	*S. aureus*	Strong	+	+	-	
	5S	*S. aureus*	Moderate	-	+	-	
	6S	*S. aureus*	Strong	-	+	-	

itive amplification of *mkr*A, *fim*A, and *ecp*A genes in 6, 5, and 3 isolates, respectively. The presence of *mkr*A and *fim*A genes at high frequency confirms the ubiquitous nature of these fimbriae in *K. pneumoniae* and are mostly related to biofilm formation as reported in previous investigations (Podschun and Ullmann, 1998; Alcántar-Curiel *et al*., 2013).

S. aureus expresses numerous virulence factors including icaABCD operon encoding biofilm components mainly *ica*A and *ica*D genes present at the *ica* locus and has a significant role in biofilm formation. In staphylococcal biofilms, the attachment to biotic or abiotic surfaces is facilitated via microbial surface components that recognize adhesive matrix molecules (MSCRAMMs) including (fibronectin-binding proteins and *spa*A) and the biofilm-associated protein (*bap*) which confers primary attachment and adhesion (Arciola *et al*. 2001; Otto, 2018).

Therefore, biofilm-forming *S. aureus* isolates were examined for the existence of *spa*A, *ica*A, and *bap* genes for their possible role in biofilm formation in this bacterium and results showed that all isolates amplify 226 bp products corresponding to *spa*A gene, moreover only 2 isolates amplify *ica*A gene whereas *bap* gene was detected in only 1 isolate.

*ica*A gene detection in only 2 isolates supported the hypothesis that there may be other *ica*-independent biofilm formation mechanisms (Li *et al*., 2012). The detection of the *bap* gene in only 1 isolate is in accordance with other studies that mentioned that this gene is rarely observed in *S. aureus* (Martins *et al*., 2015). However, others did not find the *bap* gene at all among *S. aureus* isolated from bovine mastitic cases (Achek *et al*., 2020).

Despite involving most parenchymatous tissues lung and spleen were the most affected organs that could be attributed to their direct involvement in blood and lymph drainage. Exhibited lesions in previously examined tissues suggested bacterial toxin involvement with its invasive nature (Liu, 2009) leading to destructive and degenerative changes in these tissues. These lesions vary in severity and pathogenicity. Bacterial colonization occurs at first due to the adhesion of pathogens to host tissues then damage to host tissues is mediated by its toxins (Fedtke *et al*., 2004). Several virulence factors expressed by bacterial cells demonstrate the severity of the pathological lesion (Akinkunmi and Lamikanra, 2012). Organ's congestion could be explained as a result of excessive intake or toxins circulation in the blood (Akinkunmi *et al*., 2014). Inflammatory leucocytic cells infiltrated the infected tissue as a result of immune reactions towards the infection either from bacterial cells or its toxin. Necrosis of the infected tissues refers to a spectrum of morphologic changes that follow cell death in living tissues (Proskuryakov *et al*., 2003). Necrosis also occurs in the setting of irreversible exogenous injury, pulmonary lesion detected was in partial agreement with Ahmad *et al*. (2012) who described granulomatous inflammation in the lung with narrowing of the pulmonary alveolar septa while our described lesion in the spleen was partially coordinating with Akinkunmi *et al*. (2014) who reported splenomegaly as the most

frequent macroscopic pathological alteration from some bacteria agents leading to spleen dysfunction in addition to other pathological disorders.

One accepted mechanism by which microbes tolerate antibiotics is biofilm production; the best line of defense in combating infection is an accurate diagnosis of the causative agent and performing antimicrobial susceptibility testing before deciding on a treatment plan (Brock *et al*., 2017). Previous studies suggested a mechanism by which biofilms became more resistant to antibiotics in that EPS of biofilm prevents the access of antibiotics to the bacterial cells (Stewart, 1996). Also, starvation of bacterial cells slows down their growth that generates phenotypic diversity accompanied by increased antibiotic resistance (Wentland *et al*., 1996).

Concerning results of the antibiotic susceptibility test *E. coli* isolates showed marked resistance to ampicillin, erythromycin, cefotaxime, and lincomycin, and relatively high resistance levels to Sulphamethoxazole-trimethoprim, doxycyclin, and chloramphenicol, whereas most isolates were susceptible to gentamycin, ciprofloxacin, and colistin. This result agreed with other investigations in India (Manjushree *et al*., 2019). Moreover, Olowe *et al*. (2019) attributed high susceptibly to these antibiotics to that they were not commonly prescribed in animal husbandry, although different resistance levels were detected in the previous study (Raheel *et al*., 2020)

In this regard, *S. aureus* isolates exhibited the highest resistance to AMP and CTX; moderate resistance to C and DO; and high susceptibility to VA, IMP, CIP, and CN. Regarding the high resistance to these antibiotics, this agreed with previous studies globally (Ateba *et al*., 2010; Marques *et al*., 2017). Furthermore, some studies attributed elevated resistance levels either to the transfer of multidrug resistance plasmid or due to the long-term use of some antibiotics in agriculture and healthcare settings (Moon *et al*., 2007; Bissong and Ateba, 2020). On the contrary, this resistance level was higher than what was detected before (Aslantas and Demirt 2016

Herein *P. aeruginosa* isolates displayed high resistance levels to all tested antibiotics except for CIP, CN, and IMP, this finding was in harmony with Ghadaksaz *et al*. (2015). Aminoglycos, fluoroquinolones, and carbenes can be considered as drugs of choice for this microbe (Tosaki *et al*., 2018). But generally, we can say that there is consensus among studies that *P. aeruginosa* displayed a very high level of multidrug resistance and that can be attributed to the low permeability of its outer membrane lipoprotein and multidrug efflux transport system pump encoded in its genome (Li *et al*., 1995).

K. pneumoniae exhibited high resistance levels for CTX, E, and AMP with exception of these antibiotics isolates showed relatively low resistance levels, these levels were higher than what was reported by Abebe (2020) but lower than that reported in previous studies conducted in Egypt by (Ammar *et al*., 2020)

It must be considered that resistance rates vary greatly among studies depending on geographic location or are affected by long-term and random antibiotic consumption in different localities (Aslantas and Demirt, 2016). A high level of multidrug resistance in most examined isolates was also detected. Increasing rates of antibiotic resistance are obvious in the existing study and that may be attributed to the overuse of antibiotics in animal production in addition to the ease of purchasing them freely without prescription. Another remark of interest is that some antibiotics showed a great effect on isolates regardless of bacterial species and it can be noticed that those antibiotics are rarely used in animal agriculture either because they are newly synthesized drugs such as imipenem (Ammar *et al*., 2020) or because they are already forbidden to use to be saved for human consumption as quinolones.

CONCLUSION

The present study revealed elevated emergence of MDR and biofilm forming bacteria, herein Identified bacteria were *E. coli*, *K. pneumoniae*, *S. aureus* and *P. aeruginosa* in which all of them are zoonotic pathogens in addition to their biofilm forming ability and multidrug resistance pattern and that can exaggerate condition leading to serious infection in cattle in addition to their public health hazards. Obviously, there is correlation between histopathological findings and various etiological agents. Studying genetic basis of biofilm formation was performed by detecting genes with a known role in biofilm development and that can provide an important basis for its control. Further studies are needed to find out novel and appropriate strategies to prevent, control and combat biofilm formation as its treatment by antibiotics is often diffi¬cult to be attained.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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