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Virulence and Comparison of Methods for Detection of Biofilm Formation by Escherichia coli Isolated from Retail Meat in Tunisia

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Abstract

The virulent *Escherichia coli* strains are responsible for extraintestinal infections. However, no past studies have been undertaken via the presence of virulence genes, ability of biofilms formation and the frequency of *Escherichia coli* pathovars recovered from different butcheries and slaughterhouses in Tunisia. The aims of this study was to investigate 1-) the prevalence of pathogenic *E. coli* strains isolated from bovine, ovine, and poultry meat in Tunisia, 2-) to determinate the antibiotic resistance profiles, and 3-) to determine their biofilm-forming ability by three phenotypic methods .**Totally,** 36 *E. coli* isolates from meat of healthy animals (bovines, ovine, and chickens) collected from different butcheries and slaughterhouses were investigated by searching by PCR genes encoding virulence factors *(hlyA, stx1, stx2*, *fimH, papC, eaeAet papG allèle III*, *ibeA*, *iutA*, and antibiotic susceptibility testing. Biofilm production was detected by three phenotypic: Congo Red Agar (CRA) method, tube method (TM) and tissue culture plate (TCP) method. In addition, the genetic relationship of isolates was determined by PFGE.

Results: According to the occurrence of specific genes, the 36 isolates were classified as: 6 EHEC, 4 UPEC, 1 EPEC/EHEC. Therefore, 22 Extra-pathogenic *E. coli* and 3 Avian Pathogenic *E. coli* (APEC) were presented amongst our isolates. One *E. coli* isolate of bovine meat was showed biofilm-production detect by the CRA method. Two isolates from bovine and ovine meat were weakly adherent and weakly biofilm producer. A single isolate from ovine meat was strongly adherent and strongly forming biofilm using the TCP method. The isolates were unrelated by PFGE. Taken together, high diversity of pathovars which carried diverse combinations of virulence genes in healthy isolates was reported. In addition, it seems that the infections can transferred by eating undercooked meat. The TCA method is a superior technique for detection biofilm formation

Keywords: *Escherichia coli*; Biofilm; Pathovars; Virulence genes

Introduction

Gram-negative microorganisms are a significant cause of infection in both community and nosocomial settings. The increase, emergence, and spread of antimicrobial resistance among bacteria are the most important health problems worldwide. One of the mechanisms of resistance used by bacteria is biofilm formation, which is also a mechanism of virulence [1].

Use of antimicrobials in food animals is a public health concern because antimicrobial resistant (AMR) bacteria can emerge and be transmitted to humans through consumption and handling of foods of animal origin [2]. AMR bacteria may spread across borders via trade and travel and are therefore considered to be a global problem [3]. Commensal *E. coli* can be an important reservoir of AMR genes that may spread to pathogenic bacteria. In addition, some *E. coli* are also pathogenic to humans and animals [4].

In fact, the ability to form communities called biofilms embedded in an exopolysaccharide matrix is one of the mechanisms of resistance used by bacteria to survive in the presence of an antibiotic [5]. In this state, bacteria can be up to 1,000-fold more resistant to antibiotics than those in a planktonic state [6–8]. Several studies recommend combined antibiotic therapy as the treatment of choice in biofilm-associated infections caused by Gram-negative bacteria, with macrolides (erythromycin, clarithromycin, and azithromycin) being the main antibiotics chosen due to their high antibiofilm activity in vitro and in vivo. However, antibiotic treatment of biofilm-associated infections requires further study, since the selection of a specific treatment is difficult because of the wide variability of the microorganisms involved.

Several studies have demonstrated that low doses of certain antibiotics can induce biofilm formation indicating that biofilm regulation includes the presence of antibiotics.

However, the correlation between biofilm formation and antibiotic resistance is currently unclear and remains under investigation [5,6]. However, more studies are needed to elucidate this relationship.

E. coli isolated from animals with multiple antibiotic-resistant phenotypes have been reported in Tunisia and worldwide [5,6]. Pathogenic *E. coli* can be divided into two groups diarrheagenic *E. coli* (DEC) and extra-intestinal pathogenic *E. coli* (ExPEC), which can cause urinary tract infection (UTI) and septicemia [5]. DEC includes entero pathogenic *E. coli* (EPEC), entero invasive *E. coli* (EIEC), entero toxigenic *E. coli* (ETEC), entero hemorrhagic *E. coli* (EHEC), entero aggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) based on the virulence genes present, which include *bfp* (EPEC), *eae* (EPEC/EHEC), *invE* (EIEC), *elt* and *est* (ETEC), *stx* (EHEC), *eagg* (EAEC), and *daaD*(DAEC). In poultry farms, another pathotype of ExPEC avian pathogenic *E. coli* (APEC) strains can cause colibacillosis, which responsible for the mortality of 3%–4% of the animals on a farm, and for a 2%–3% reduction in egg production [5,7]. Among ExPECs, uropathogenic *E. coli* (UPEC) is one of the most important pathogens causing community-acquired UTI [6], and AMR UPEC has become a major public health concern [7]. For food safety, it is important to continuously monitor the contamination of bovines, ovine and chicken meat by AMR bacteria and their possession of virulence genes in retail chicken, bovines and ovine meats sold in Tunisia. Antimicrobials are widely used as a growth promoter in the production of livestock including poultry [8-10], which has resulted in the emergence of AMR bacteria. Consumption of chicken has been rapidly growing globally including Tunisia. These AMR bacteria could enter the food chain, resulting in a major impact on public health [9,10]. Most of the biomass of microorganisms on the planet live in biofilms, and more than 75% of all infections are caused by biofilms [11]. A biofilm is a heterogeneous and structured community of aerobic or anaerobic microorganisms adhering to each other and to an inert or biological surface [12]. In the natural environment and in the human host, bacteria fluctuate between two forms: mobile cells and cell-forming biofilms, the latter being the most common mode [13]. Living bacteria within the biofilm are less susceptible to antibiotics and disinfectants than planktonic cultures of the same organisms [14]. Biofilm formation is commonly associated with infections of medical devices and is considered to be relevant in some spontaneous diseases, such as native valve endocarditis, periodontitis, chronic prostatitis, otitis media, and bronchopulmonary sepsis in patients with cystic fibrosis [15]. Biofilm production in *E. coli* promotes the colonization and leads to increase rate of UTIs, and such infections may be difficult to treat as they exhibit multidrug resistance (MDR) [16]. Although the ability of *E. coli* to form biofilms is widely recognized, the frequency of this trait in

strains causing clinical infection and its relationship to other clinical or bacterial characteristics is still unknown. Classical studies suggest that the magnitude of bacteria as determined by quantitative blood cultures may be related to mortality independently of the underlying disease's severity [14-16].

In this regard, it would be interesting to isolate biofilm-forming bacteria from meat. This situation has resulted in a need for more epidemiological information on the prevalence of resistance to various antibiotics and their relevant genes, such as virulence gene combinations in animal isolates.

Thus, the aims of this study were to determinate the frequency of the occurrence of potentially pathogenic *E. coli* strains isolated from bovine ovine and poultry meat in Tunisia, and to detect their virulotypes and to analyze biofilm-forming bacteria by three methods that is, Congo Red Agar (CRA) method , tube method (TM) and tissue culture plate (TCP) method. This could be used as a model for studying anti-biofilm activities in future studies.

Materials and methods

Bacterial isolates collection

A total of 36 *E. coli* isolates from meat of healthy animals (bovines, ovine, and chickens) were recovered from different butcheries and slaughterhouses located in five different governorates in Tunisia between March and April 2016. These isolates were recovered from meat from: beef (n = 15), ovine (n = 13), and poultry (n = 8). From each sample, 25 grams of meat were homogenized for 2 minutes with 225 mL of buffered peptone water (Bio-Rad, Marnes la Coquette, France). Then, 1 mL was seeded onto MacConkey agar plates and incubated for 24 hours at 37°C. Isolates with typical *E. coli* morphology were selected (one per sample), and the presumptive identification was confirmed by classical biochemical methods and by the API20E system (BioMerieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing by disk diffusion

Antimicrobial susceptibility was determined using the standard disk diffusion method based on the Clinical and Laboratory Standards Institute (CLSI)'s 2012 guidelines [17]. The following antibiotics were tested: amoxicillin, amoxicillin/clavulanic acid, ceftazidime, cefotaxime, imipenem, colistin, streptomycin, tetracycline, trimethoprim-sulfamethoxazole, sulfonamide, nalidixic acid, ciprofloxacin, norfloxacin, and gentamicin (Oxoid, Madrid, Spain). *E. coli* ATCC 25922 was used as ESBL negative and *Klebsiella pneumoniae* 700603was used as ESBL positive reference strain.

Detection of virulence genes and pulsed-field gel electrophoresis (PFGE)

The presence of 9 virulence genes encoding toxins (*hlyA, stx1, stx2*), adhesins (*fimH, papC, eaeAet papG allèle III*), invasins (*ibeA*) and the siderophores (*iutA)* were analyzed by PCR (Table 1) [7,17]. Details regarding and description/functions of virulence genes and their corresponding *E. coli* pathotypes as well as the clinical manifestations and diseases in human and animal associated were illustrated in Table 2. Pathovars were determined according to the occurrence of specific virulence genes: ExPEC (*fimH*, *hlyA*, *papC*, and *papG* allele III), UPEC (*iutA*), EHEC (*stx1* and/or *stx2*), EPEC or EHEC (*eaeA*), APEC (*ibeA*) (Table 2) [17, 18]. Control strains were those reported previously by Kilani *et al.*[17]. For Pulsed Field-Gel Electrophoresis (PFGE), chromosomal DNA was prepared as previously described using the restriction enzyme *XbaI* (Amersham Life Sciences, Uppsala, Sweden) (Kaufmann, 1998) [20]. DNA fragments were separated by electrophoresis in 1.2% agarose gels (pulsed-field agarose certified; Bio-Rad, Hemel Hempstead, United Kingdom) and 0.5X Tris-borate-EDTA buffer using a contour-clamped homogeneous electrified (CHEF-DRIII system; Bio-Rad) under the following electrophoresis conditions: 12◦C at 6V/cm for 27h with pulse times ranging from 10 to 40 s. Clonal relationships were established following Tenover criteria [19].

PCR / (Genes)	Initial Denaturation	Denaturation	Hybridation	Elongation	Final Elongation	cycle Number
PCR 1 (*fim*H,*be*A)	95°C : 5mn	94°C : 30s	63°C : 30s	72°C : 3mn	72°C: 10 mn	25
PCR 2 (*eae*A,*Stx*1,*Stx*2	94°C 5mn	94°C : 30s	59°C : 30s	72°C : 1mn	72°C : 10mn	30
PCR 3 (*Hly*A,*PapGallele*III,*pap*C,*Iut*A)	95°C : 5mn	94°C : 30s	63°C : 30s	72°C : 3mn	72°C: 10 mn	25

Table 1: PCR amplification Condition of virulence genes

Virulence Gene	Pathovar (s)	Clinical syndrome
fimH, papC, papGallèle sIII, cnf1, hlyA, fuyA, iutA	ExPEC UPEC	Méningite/ Infection de tractus urinaire/ Diarrhée Cystite, pyélonéphrite, bactériémie, septicémie
AstA, aggC	EAEA	Diarrhée aqueuse aigüe et persistante
eaeA, exhA	EPEC EHEC	Diarrhée aigüe, colite hémorragique, syndrome hémolytique et urémique (SHU)
stx1, stx2	EHEC	Diarrhée aigüe, colite hémorragique, syndrome hémolytique et urémique (SHU) chez l'homme/ Diarrhée chez les jeunes veaux
ipaH	EIEC	Dysenterie/ Diarrhée de l'adulte
ibeA	ExPEC ; APEC	Colibacillose aviaire

Table 2: Virulence genes associated with pathovars of E. coli in relation to human and animal diseases (Mainil, 2003 ; Wu et al., 2007; Dahbi et al., 2013 ; Dadie et al., 2014)

Detection of biofilm

Qualitative study

Congo Red Agar method (CRA): The 36 strains were incubated for 16 hours at 37°C in 3 ml of Tryptone Soy Broth (TSB) medium. Then they were sub cultured on Red Congo agar. After incubation at 28°C for 96 hours, colony morphology was determined. This test was carried out by varying the pH of 5, 7 and 11; as well as the NaCl concentration of 0%, 3.5% and 7%. The slime production of the isolates was evaluated by the Congo Red Agar method (CRA), according to the protocol of Freeman et al. (1989) [20]. This method enables the differential detection of slime-forming strains (black colonies on the red agar) and non-slime-forming strains (red-colored colonies).

In vitro test

Quantitative study

This study was carried out according to two approaches;

Tube method (TD): The ability of strains to produce a biofilm in the LauriaBertani (LB) nutrient medium was studied. Glass tubes filled with 5 ml of salt-free LB broth were inoculated with a loop of a pure culture of the 36 isolates. The biofilm formation was visualized after incubation for 96 h at 28°C in LB medium. The formation of the biofilm is observed as a film at the air-broth interface, in the LB medium [21,22].

Tissue culture plate (TCP): Crystal violet assay.

For the biofilm formation assay, strains from fresh agar plates were inoculated in 5 mL of Tryptic Soy Broth and incubated for 24 hours at 30°C. Biofilm formation was conducted in 96-well flat bottom plates (with lid) [22,23] (Mathur et al., 2006).

In order to increase biofilm formation [21,22], culture strains were diluted with a fresh medium (which commonly contains 0.25% (w/v) glucose) supplemented with 2.25% glucose [26] until a final OD600nm of 0.1 in each well was reached (Table 3). The polystyrene microplate wells were filled with 200 µL of diluted culture, whereas only broth with glucose served as a control to check the sterility of the medium and non-specific attachment. The *Escherichia coli* (*E. coli*) laboratory strain DH5α is a non-biofilm-forming bacterium and has been used as a negative control [23], whereas three strains *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa*, M1) and *Staphylococcus aureus* ATCC33581 (*S. aureus*, M2) have been used as positive controls.

Results

Among the 36 isolates of *E. coli* studied, 18 were resistant to amoxicillin, 15 to tetracycline, 13 to ciprofloxacin, 9 to streptomycin, 8 to nalidixic acid, 6 to trimethoprim/ sulfamethoxazole, and 9 to sulfonamides. Some isolates were susceptible to all antibiotics: 7 isolated from ovine meat, as well as one isolate for bovine and poultry meat, respectively. Fifteen isolates were multidrug resistant. No resistance to imipenem, ceftazidime, cefotaxime or gentamicin was observed (Table 3). ESBL production was not detected in any isolate.

Gene encoding the production of toxins *stx1 was* detected in 6 isolates, with absence of *stx2*and *hlyA*. The adhesin-encoding gene *fimH* was detected in 31 isolates, and the *eaeA* (3 isolates), whereas *papC*, and *papG* allele III genes were not detected in our isolates. For the invasins, the *ibeA* gene was detected in 3 isolates, whereas the siderophores were manifested by the presence ofgene *iutA* in 4 isolates (Table 3).

In total, 6 types of genes combination were detected: *fimH+ stx1* (n = 6);*fimH +iutA*(n = 2); *fimH, ituA, eaeA*(n = 1); *FimH+eaeA* (n = 1); *ituA, eaeA* (n = 1); *IbeA , fimH* (n = 1). Based on the occurrence of specific genes or combinations, the 36 isolates were classified as 6 EHEC (18,33%), 4 UPEC (11,11%), and 1 EPEC/EHEC (2,7%). Therefore, 22 ExPEC (61,11%) and 3 APEC (8,33%) were detected among the isolates (Table 3). Thus the NTEC and EAEC were no detected in our stains. Genetic relatedness by PFGE, showed that all strains presented unrelated PFGE patterns and thus were considered not clonal (Figure 1).

Among the 36 strains of *E. coli*, a single strain of bovine meat (EC 1) was pushed on the Congo red medium formed rough and black colonies and considered as slime-producing strain (Figure 2). The others isolates were classified as non-producers (red colonies) (Figure 2). The tube test was negative in all strains. According to the TCP test, which was used to detect microbial attachment to an abiotic surface, 18 strains have an OD value of less than 0.120 therefore non-adherent and non biofilm-forming. Two strains (EC1 and EC26) which have a value between 0.120 and 0.240, therefore these strains were weakly adherent and weakly forming biofilm. A single strain (EC25) which has a value greater than 0.240 therefore strongly adherent and strongly forming biofilm (Table 3). After 24 h of incubation, 3 isolates formed a biofilm; two low formation from bovine and ovine and one from ovine showed a strong formation of biofilm (Table 4).

Discussion

Antibiotic resistance was one of the most serious threats to global health, food security and development today, and it can affect anyone, at any age, in any country. In the present work, the study of antibiotic resistance was carried out on a collection comprising 36 strains of *E. coli*. The results showed high levels of resistance to amoxicillin and tetracycline, moderate levels of resistance to streptomycin, ciprofloxacin, and nalidixic acid, and low levels of resistance to trimethoprim-sulfamethoxazole and Amoxicillin-

Reference of isolate	Origin/source	Region	Resistance profile	Virulotype	Pathotype	PFGE
EC-1		Ariana	Amx, Nal, Cip	*FimH+eaeA*	**EPEC\ EHEC**	**P1**
EC-2	Healthy bovine/Meat	BenArous -Mourouj 5	Cip	*FimH*	**ExPEC**	**P2**
EC-3		BenArous-Fouchena	Amx, S, Tet	*FimH*	**ExPEC**	**P3**
EC-4		Tunis- Zahrouni	Amx, S, Tet, Cip	*FimH*	**ExPEC**	**P4**
EC-5		Ben Arous	Amx, Tet, Nal, Cip	*FimH*	**ExPEC**	**P5**
EC-6		BenArous -Zahra	Nal, Tet, Cip	*FimH+ stx1*	**EHEC**	**P6**
EC-7		Tunis-IbnouSina	Sensible	*FimH + stx1*	**EHEC**	**P7**
EC-8		Tunis-Megrine	Tet, Cip	*FimH*	**ExPEC**	**P8**
EC-9		Manouba	S, Tet, Nal, Cip	*FimH*	**ExPEC**	**P9**
EC-10		Tunis-Centre-Ville	Amx, S, Tet, Sxt	*FimH*	**ExPEC**	**P10**
EC-11		BenArous- -Mourouj 3	S, Tet, Nal, Cip,	*FimH*	**ExPEC**	**P11**
EC-12		BenArous - Rades	S,Tet	*FimH*	**ExPEC**	**P12**
EC-13		BenArous-Mourouj 1	Amx, Cip, Sxt	*FimH*	**ExPEC**	**P13**
EC-14		Tunis-Bardo	Amx, Amc, Cip	*IbeA*	**APEC**	**P14**
EC-15		BenArous-Mourouj 2	Amx, Amc	-	**ExPEC**	**P15**
EC-16	Healthy ovine/Meat	Tunis- Bardo	Sensible	*FimH*	**ExPEC**	**P16**
EC-17		BenArous-Hammem-Linf	Sensible	*FimH+stx1*	**EHEC**	**P17**
EC-18		Ariana	Amx	*FimH*	**ExPEC**	**P18**
EC-19		Tunis- Megrine	Amx, Amc	*IbeA*	**APEC**	**P19**
EC-20		BenArous-Mourouj 5	Sensible	*FimH*	**ExPEC**	**P20**
EC-21		BenArous -Rades	Tet	*FimH*	**ExPEC**	**P21**
EC-22		Ben Arous	Sensible	*FimH + stx1*	**EHEC**	**P22**
EC-23		Tunis -IbnouSina	Sensible	*FimH + stx1*	**EHEC**	**P23**
EC-24		BenArous Hammem-chat	Amx, Amc, Nal, Cip, Sxt	*FimH+ stx1*	**EHEC**	**P24**
EC-25		BenArous Mourouj 1	Amx, S, Tet, Sxt	*FimH*	**ExPEC**	**P25**
EC-26		Tunis- Centre-Ville	Tet	*FimH*	**ExPEC**	**P26**
EC-27		BenArous -Morneg	Sensible	*FimH*	**ExPEC**	**P27**
EC-28		Manouba	Sensible	*FimH*	**ExPEC**	**P28**
EC-29	Healthy chickens /Meat	Nabeul-Béni khalled	Sensible	*FimH*	**ExPEC**	**P29**
EC-30		Nabeul- Grombalia	Amx, S, Sxt	*fimH, ituA, eaeA*	**UPEC**	**P30**
EC-31			Amx, Amc, Cip	-	**ExPEC**	**P31**
EC-32		Nabeul-Menzel bouzelfa	Amx	*ituA, eaeA*	**UPEC**	**P32**
EC-33			Amx	*IbeA, fimH*	**APEC**	**P33**
EC-34			Tet, Cip, Nal	*FimH*	ExPEC	P34
EC-35		Tunis	Amx, Tet, NA	*fimH +iutA*	**UPEC**	**P35**
EC-36		Tunis	Amx, S, Tet, Nal, Sxt	*fimH+ iutA*	**UPEC**	**P36**

Nal: Acidenalidixique; **Sxt:** Triméthoprime/Sulfaméthoxazole; **Tet:** Tétracycline; **Amx:** Amoxicilline; **Amc:** Amoxicilline/ac.clavulanique; **Cip:** ciprofloxacine; **S:** Streptomycine;

ExPEC: Extra intestinal pathogenic *E. coli*; **EHEC:** Enterohemorrhagic *E. coli*; **EAEC:** Enteroaggregative *E. coli*; **APEC:** Avianpathogenic *E. coli*; **UPEC:** Uropathogenic *E. coli*; **EPEC:** Enteropathogenic *E. coli;* a Virulence-associated genes shown in bold face are the genes characteristics of EPEC, UPEC, EAEC, EHEC, APEC, and ExPEC pathovars

Table 3: The characteristics of *E.coli* isolates

OD values	Adherance	Biofilm formation
>0.120	No adherent -	non-biofilm producers
0.120-0.240	Weakly adherent +/-	Low formation of biofilm
<0.240	strongly adherent ++	strong biofilm producers

Table 4: Classification of bacterial adhesion using tissue culture plate TCP method

A

B

C

Figure 1: Appearance of *E.coli* after transplanting on Congo Red Agar (CRA) method**; (A)** Non- biofilm; (-) producing strain; **(B)** Black strain; (+) producing biofilm; **(C)** Variable phenotype strain with red outline and black center; (+/-) producing biofilm

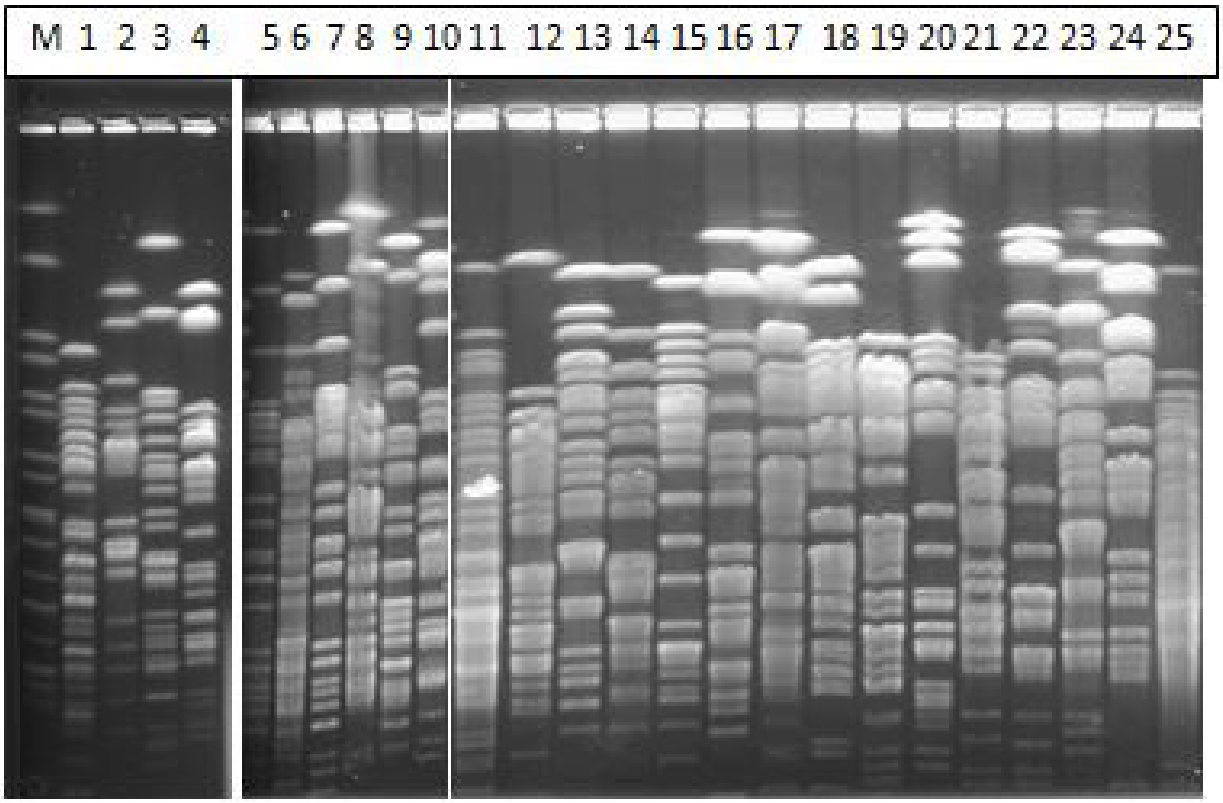

Figure 2: XbaI-PFGE profiles of thirty sex unrelated *Escherichia coli* isolates EC1–EC36. Lane M, XbaI-digested DNA of Salmonella enterica serovar Braenderup H9812 (size standard); **(A)** lanes 1–25:EC1-EC25; **(B)** lanes 26-36; EC26-EC36

clavulanic acid. Percentages similar to that obtained in this study have been reported in Tunisia [25,26]. The highest percentages of resistance were observed for streptomycin and tetracycline. Tetracycline is an old molecule used as the first-choice antibiotic in different animal species because of its broad spectrum of preventive activity for diarrhea and streptococcal infections [25-27]. In addition, high levels of antibiotic resistance reported in *E. coli* from food and clinical sources have also been observed in other studies [25-28]. The *stx1* gene that codes for toxin production was detected in 6 isolates; however, the *stx2* gene that was absent in our isolates. These isolates indicate the pathovar EHEC (STEC or VTEC) and one contained the gene encoding intimin (*eaeA*) specific for EHEC [17,27].

There are two serotypes known for their exceptional pathogenicity: *E coli* O104: H4 and *E coli* O157: H7 found in such pathovar. *E. coli* O157: H7 responsible for hamburger disease. Contradictory results have reported which showing the prevalence of *stx2* gene compared to the *stx1* gene. In Belgium, in 2003, out of 1479 carcasses of cattle ten STEC O157 strains (0.74%) were presented, of which eight strains had the stx2 gene and the two*stx1* and *stx2* genes. Five STEC O157: H7 or H- strains were isolated from the 298 minced meat samples tested (1.68%), of which four had the stx2 gene and a combination of the two stx1 and stx2 genes in a single strain. Also in Belgium in 2004, 1337 beef carcasses were swabbed. Eighteen strains of STEC O157 were identified, giving a prevalence of 1.4%. Seventeen strains are positive for the *stx2* gene and one strain is positive for the *stx1* and *stx2* genes. Two STEC O157 strains (*stx2*, *eae* and *ehxA*) were found in beef cuts (2/244), a prevalence of 0.8%, but none in ground beef (0/234) [28, 29]. In Senegal, in 2013, a study realized in Ngaba on the search for *stx2* and eaeA genes in *E. coli* O157 strains isolated from meat products in local markets showed contrary to our results that 6.45% of the strains tested carried either the gene *stx1* is the *stx2* gene but never both at the same time,. The adhesins which are considered as essential virulence factors in *E. coli*, these adhesins are encoded by several genes, among them *fimH*, *eaeA* respectively detected in 31, 3 isolates with absence of *papGalléle3* and *papC* genes. The *fimH* gene has been detected in almost all of our isolates except three, which is consistent with the literature showing that this gene is the most frequently detected against genes encoding other adhesins and the rest of the virulence genes. In addition, it is generally associated with pathovars responsible for extra-intestinal infections (ExPEC) [18,28]. The eaeA gene encoding the intimin responsible for attachment and deletion is often linked to EPEC and EHEC pathovars [18,28,29]. The presence of the *ibeA* gene was detected in 3 strains. This virulence factor is known to be involved in the crossing of the blood-brain barrier in *E. coli* strains responsible for neonatal meningitis in humans. It has also been noted that the 3 strains harboring this gene are of avian, bovine and ovine origin, which proves that there is contact between poultry and cattle and sheep. Indeed, avian *E. coli* pathogenic isolates (Avian pathogenic *Escherichia coli* (APEC) are generally carriers of this gene [30].

A weak presence of the last group of virulence factors encountered is that of iron uptake systems which is manifested by the presence of *iutA* gene in four strains. These siderophores are generally very prevalent among UPECs responsible for urinary tract infections [18,29,31]. *hlyA* gene has not been detected in our collection. The average virulence score was between 1 and 2.

The great diversity of pathovars of *E. coli* wihch strains presented unrelated PFGE patterns; 7 enteropathovars [(Entero-hemorrhagic (n = 6) and Entero-Pathogen / Entero-hemorrhagic (n = 1)], 3 avian pathovar and 26 extra-intestinal pathovars including 4 uro-pathogens that have been classified according to the presence of genes virulence requires surveillance of *E. coli* of avian, bovine and ovine origin that can be transferred to humans via the food chain, to successfully identify the risk factors and main routes of contamination that determine control of infections associated with pathovars.

The present study is aimed to analyzed in vitro the detection of biofilm formation among *E. coli* strains isolated from bovine, ovine and poultry meat by three different methods and to correlate the biofilm production with antibiotic resistance pattern. We observed for the EC1 which was isolated from bovine was classified as EPEC\ EHEC and showed a resistance to amoxicillin, nalidixic acid and ciprofloxacine. In human EHEC infections, disease out comes can range from mild to bloody diarrhea (hemorrhagic colitis) to more serious complications, such as hemolytic uremic syndrome (HUS), and even death [18,31,32]. EPEC, including EPEC O103, do not carry *stx* genes; however, they possess *eae* and other virulence genes to cause attaching and effacing lesions that can result in mild to severe diarrhea, or even death, particularly in children [33]. For the EC26, isolated from ovine meat, was resistant to tetracycline. These two strains were weakly producers of biofilm. These results were according to the literature which confirmed

that the fluoroquinolones, cephalosporins, and aminoglycosides can reduce the amount of biofilm produced by *E. coli* for UTI [1]. cephalothin, ceftriaxone, ceftazidime, and ciprofloxacin reduce biofilm biomass in susceptible strains. For EC25, isolated from ovine meat, we observed a resistance to amoxicillin, streptomycin, tetracycline and trimethoprim-sulfamethoxazole. This strain was strongly biofilm producer and classified as ExPEC, this pathovar can be classified as uro-pathogens with acquisition of the virulence gene *iutA*. The biofilm producing bacteria can be responsible for many recalcitrant infections in humans and are difficult to eradicate. Biofilm production in *E. coli* promotes colonization and lead to increased UTI [15,34]. Such infections may be difficult to treat as they exhibit multiple drug resistance. Biofilm production in *E. coli* promotes the colonization and leads to increase rate of UTIs, and such infections may be difficult to treat as they exhibit multidrug resistance (MDR). Many studies showed that the prevalence of biofilm among UPEC ranges from 60% to 70% [15]. As well as Biofilm formation may result in the increased ability of strain causing prostatitis to recurrent UTI. Several studies observed that 50%–70% isolates collected from patient with relapse infections to be biofilm producer [15,34].

The qualitative study showed that among the 36 strains of *E. coli* a single strain of bovine meat was pushed on the Congo Red medium Agar (CRA) while for the test tube was negative in all strains. As well as a single strain from ovine meat was classified as a strongly adherent and strongly forming biofilm for the microplate test which used to detect microbial attachment to an abiotic surface.

After 24 h of incubation, three isolates formed a biofilm; two low formation from bovine and ovine and one from ovine showed a strong formation of biofilm. The two strains (EC1 from bovine meat and EC26 from ovine meat) have a value between 0.120 and 0.240 and classifiated weakly adherent and weakly forming biofilm. We observed for the EC1wihch isolated from bovine meat a resitance to amoxicillin, nalidixic acid and ciprofloxacine for the EC26 which isolated from ovine meat a resitance to tetracycline. These two strains were weakly producers' biofilms. For EC25 which isolated from ovine meat we observed a resistance to amoxicillin, streptomycin, tetracycline and Trimethoprim-sulfametoxazole. This strain was strongly producer biofilm. However, there are reports regarding relationships between biofilm formation and resistance to specific antibiotics. Thus, the acquisition of quinolone resistance has been related to a decrease in biofilm production in both UPEC and Salmonella typhimurium [35,36]. In this study the three isolates which formed a biofilm were classified two ExPEC and one EPEC/EHEC. This ExPEC pathovar can contributes many diseases; a meningitis, an urinary tract infection, a Diarrhea, cystitis, pyelonephritis, a bacteremia, a sepsis and for the EPEC/EHEC pathovar the principal diseases in human and animal were an acute diarrhea, a haemorrhagic colitis, a haemolytic uremic syndrome (HUS).

In the present study, we also found this relationship between quinolone resistance and biofilm formation in *E. coli*, with the susceptible isolates showing a greater capacity to form biofilm than the resistant isolates. It was confirmed that *Escherichia coli* biofilm has been found to be resistant to a number of antibiotics, mostly accredited to putative multidrug resistance pump. The development of the extracellular matrix and the observed increased resistance to common antibiotics create a challenge to control the infections caused by *E. coli* biofilms.

According to Panda et al (2016), the CRA method shows erroneous results in the case of the biofilm formation in vitro of *Escherichia coli* [21]. In contrast, other researchers consider the method as a reliable and specific test for the detection of biofilm formation of *Escherichia coli* [32]. In fact, the appearance of black colonies is the result of metabolic changes in the dye, which promote the formation of a secondary product [21]. The CRA method is a qualitative test that can lead to false results, due to difficulties in differentiating between moderate biofilm-producing strains and those which do not yield biofilms. The quantitative study of biofilm with tissue culture plate (TCP) to the all strains showed a positive result for a single strain and more / less positive results for two strains. The low presence of biofilms in our strains can be explained by two hypotheses, the first of which is that certain amino acids (dextrogyrous) produced by bacteria (eg Bacillus) were capable of dispersing the biofilms produced by different Gram-positive and Gram-negative bacteria; In 2009, Davies and Marques identified a small signal molecule (cis-2-decenoic acid) produced by Pseudomonas aeruginosa and capable of inhibiting the development of a biofilm and inducing its dispersion. This molecule has also been shown to be effective against biofilms produced by other Gram-negative, Gram-positive and yeast bacteria [37]. While the

second hypothesis confirms that the formation of biofilms is present in ESBL-producing *E coli* strains, these strains are generally found in humans with urinary tract infection [21,35-37].

Taken together, these findings highlight the importance of *E. coli* isolated from different butcheries and slaughterhouses as reservoir of antibiotic resistance that certainly could be linked to the excessive use of antibiotic in bovine ovine and avian husbandry. This dramatically situation is certainly not specific to Tunisia. Therefore, this is worrisome for global human health, especially with the increasing consummation of poultry meat in Tunisia and in other part of the world owing to its relatively lower cost comparing to red meat. A reasonable and effective global interventions and studies are therefore of urgently needed. In conclusion, the acquisition of specific antimicrobial resistance can compromise or enhance biofilm formation in several species of Gram-negative bacteria. However, MDR strains did not tend to have greater biofilm production than non-multiresistant isolates. Further studies are needed to determine how the acquisition of antibiotic resistance affects biofilm formation.

Conflit of Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could in appropriately influence or bias the content of the paper.

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