Phenotypic and molecular characteristics of biofilm and other virulence genes in *E. coli* and *K. pneumoniae* isolates from healthy dairy cow, human and environmental sources

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Abstract

Escherichia coli and *Klebsiella pneumoniae* for ones role in opportunistic infections and pathogenic methods in veterinary and human medicine, including the formation of biofilms. 33 *E.coli* and 8 *K. pneumoniae* isolates of these micro-organisms isolated from cow's milk, stools and utensils. Identification were done by biochemical reaction and confirmed by Polymerase Chain (PCR). These isolates exhibit biofilms formation, as strong, moderate, weak capacities. The expression of the *fimA* gene. The percentage (84.8%)28/33 of *E.coli* was able to produce biofilm while (100%)8/8 in *K. pneumoniae* The virulence factors of all bacterial isolates were also studied to determine *hylA and bla-CTX-M* genes in *E.coli* and, *magA and bla-CTX-M* genes in *K. pneumoniae* were results presence extended spectrum β- lactamases (ESBLs). *bla-CTX-M* gene in *E. coli* and *K. pneumoniae* (78.7% , 87.5%) It also emerged that not all isolates carry other virulence genes for this study

Key words : biofilm, *virulence genes, E. coli , K. pneumoniae*

Introduction

Microbial adhesion onto, surfaces and therefore the biofilm formation are considered serious, regarding their economical and public health consequences in many sectors for humans. The presence of pathogenic microorganisms on food sector facilities represents a severe potential health risk to consumers. Contaminated food contact with surfaces promotes contamination of food products which leads to foodborne disease[1]. With this pathogen contamination, of bulk tank milk by Shiga toxin-producing *E. coli*. therefore, milk is at risk for contamination by any pathogen that is present in feces or farm environment[2] . Grass-fed cattle are the main reservoir of such *E. coli* strains. Some *E. coli* strains can produce toxins that induce serious human infections. cross-contamination of milk can occur in utensils contamination and during subsequent handling and preparation. Their feces might contaminate and thus act like a microbial carrier which might end up contaminating other foods such as milk, outbreaks due to *E. coli 0157*: H7 and *klebsiella spp.* have been associated primarily[1]. The ability to adhere to different materials and formed biofilms have been an important factor associated with *E. coli* and *klebsiella spp.* virulence additionally, resistance to antimicrobials in biofilm-forming isolates contributes to bacterial persistence which may lead to chronic infections and treatment problem[3,23,24] *Escherichia coli and Klebsiella pneumoniae* are not only constituents of the commensal gut flora but also common opportunistic pathogens in intestinal infection and implicated in the urinary tract and bloodstream infections. They frequently harbor ESBL- encoding genes. Broad-spectrum beta-lactamase production is associated with increased infections and mortality[4] virulence *E. Coli* capsule with polysaccharide. More than 80 different types of capsules, *E. Coli* capsules are categorized into four major classes according to the genetic organization of the capsule gene cluster and its biosynthesis and assembly mechanism[5]. To *K. Pneumoniae* usually expresses on its surface a smooth lipopolysaccharide (LPS with O antigen) and capsule polysaccharide (K antigen) both leading to the pathogenesis of microorganism diseases caused by this species[6-7]

Material and Methods

Samples (190) were collected from different locations (60 cows milk, 60 stool human and 70 utensils samples). The collected samples were immediately, delivered to the laboratory in a cool box and tested within 24 h.

All *E.coli* and *Klebsiellan pneumoniae* isolates grow readily on MacConkey agar and colonies typically appear large and pink, with pink pigment usually, diffusing into the surrounding agar indicating, fermentation of lactose. *Klebsiella peneumoniae* show the colonies mucoid on MacConkey agar. However *E. coli* colonies have a characteristic green metallic sheen on EMB agar. The API-20 E kit (bioMérieux – France), was used for confirmation the identification of isolates.

The method of DNA, extraction was done by using (Geneaid extraction Kit). The concentration of DNA, was estimated by using the nanodrop system[8]

Biofilm formation was assayed phenotypically by the ability of cells to adhere to the wells of 96-well microtiter plate[9]. Two hundred microliters of this bacterial culture were used to inoculate pre-sterilized 96-well polystyrene microtiter plates and later incubated for 48 hrs. at 37ºC, all wells were washed with sterile physiological saline for the elimination of unattached cells. Afterward, 200 µl of 1% crystal violet was added to each well. After 15 min. at room temperature, each well was washed with 200 µl sterile physiological saline. This process was repeated three times. The crystal violet bound to the biofilm was extracted later with 200µl of ethyl alcohol, and then absorbance was determined at 540 nm in an ELISA reader[10].

Specific primer sequences in PCR assays, and the predicted size of the amplified products, for the different pathogenic gene, coding regions were employed .(Table 1). The reaction volume for *magA, hylA, fimA* and *bla-CTX-M* genes was 20 µl , while the amplification program was 3 at 95 C for denaturation , 30 cycles for denaturation 95 C (45 Sec.) ,annealing 60 C (45 Sec.) and extension 72 C for 50 Sec. with final extension 72 for 10 Min.

Table 1 Sequences and predicted lengths of PCR amplification, products of the oligonucleotide primers used

Primer	Primer sequence (5-3)	Product	Source
fimA	F 5 AGTTAGGACAGGTTCGTACCGCAT-3 R 5- AAATAACGCGCCTGGAACGGAATG-3	316bp	11
bla-CTX-M	F5"TCTTCCAGAATAAGGAATCCC"3 CCGTTTCCGCTATTACAAAC"3 R5"	909bp	12
magA	TAGGTCAGGCAGCTGTTGTG "3 F"5 GCTCCGTTGCAATATGACCG "3 R5"	312bp	13
hylA gene E. coli	"3 F-5" TGAAGTGTCAGGAGACGCTG "3 R- 5" ATGGAGAATGCGTTCCTCAAC	156bp	27

Results

The raw milk, stool and utensils samples displayed the presence of *E.coli* and *klebsiella pneumonia* contamination .The raw milk samples were polluted with the fecal coliform according to the results of fermentative growth in MacConky. Out of 190 samples (milk, stool and utensils). were collected from different sources, 33 samples (17.36%) were contaminated by *E.coli isolates*. While eight samples (4.21%) *Klebsiella pneumoniae* were confirmed. All *E. coli* 33 isolates were produce biofilm (100%) . Out of 33 isolates 14 (42.42), were strong biofilm producer . The high percentage of isolates with strong biofilm produce were in stool isolates 10/19 (52.63%). The moderate biofilm produce isolates were 8/33(24.24%), While the weak biofilm produce isolates were 11/33 (33.33%). Also all *K. pneumoniae* ,isolates were able to produce biofilm (100%). Out of a 8 tested *Klebsiella pneumoniae* isolates by using pre-sterilized 96-well polystyrene microtiter plates (Fig.1) 5/8 (62.5%) of isolates were moderate biofilm producers and the remaining isolates 3/8 (37.5%) were weak biofilm, producer

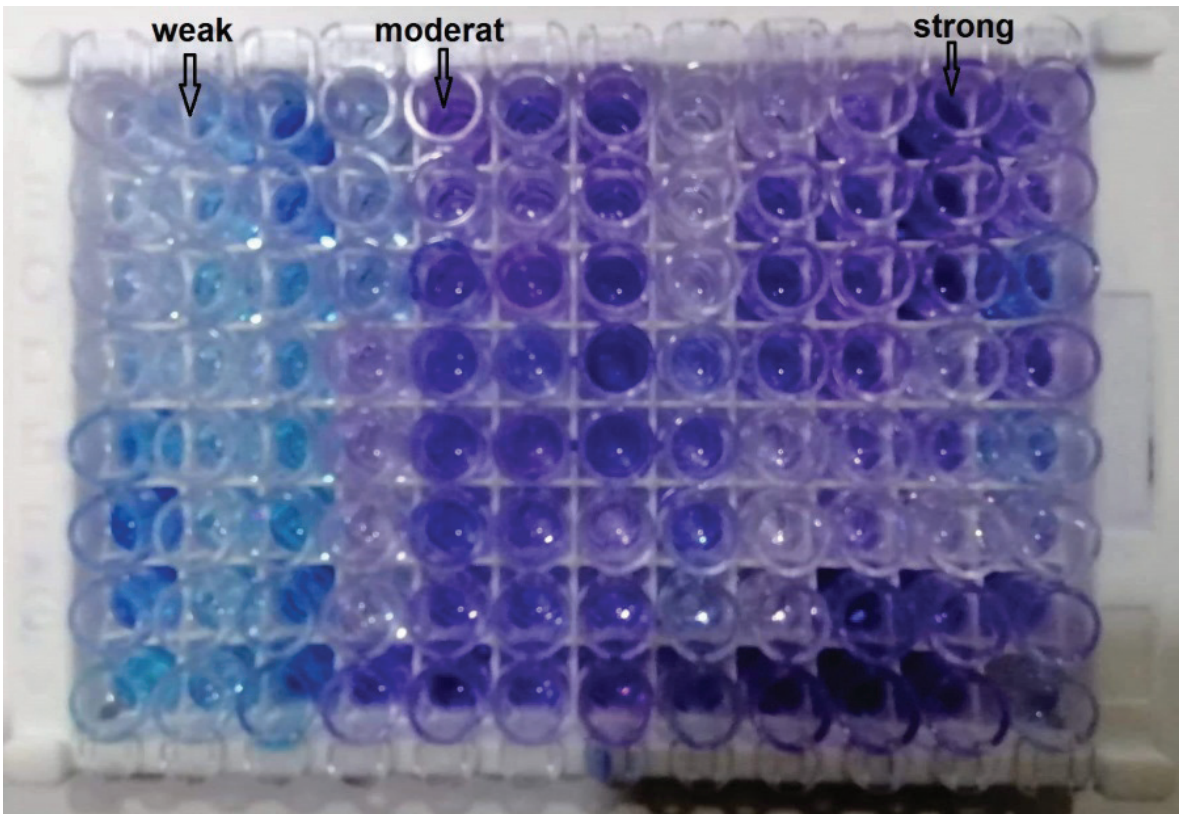

Figure 1 :pre-sterilized 96-well polystyrene microtiter plates biofilm production .

From 33 *E.coli* isolates . 28(84.8%) were carried the genes responsible on biofilm formation *E. coli* and 8 (100%) *k. pneumoniae* were had *fimA* gene responsible for biofilm formation (Table 2, Fig 2). The detection of *hylA* gene in *E.coli* and *magA* gene in *k. pneumoniae* while *bla-CTX-M* in both . The results showed that *hylA* gene and *magA* gene were not found an all *E. coli* and *K. pneumoniae* isolates respectively, while *CTX-M* gene was found in 26/33 (78,7 %) of *E. coli* isolates, while in *K. pneumoniae* isolates was found in percentage 7/8 (87.5%), table 3, Fig 3.

Table 2 distribution of *fimA* gene in *E.coli* and *K.pneumoniae* isolates

Sample	E. coli		K. pneumoniae	
	NO.	fimA NO./ %	NO.	fimA NO./ %
Stool	19	16/84.2	3	3/100
Milk	11	9/ 81.8	5	5/100
Utensils	3	3/100	0	0/0
Total	33	28/84.8	8	8/100

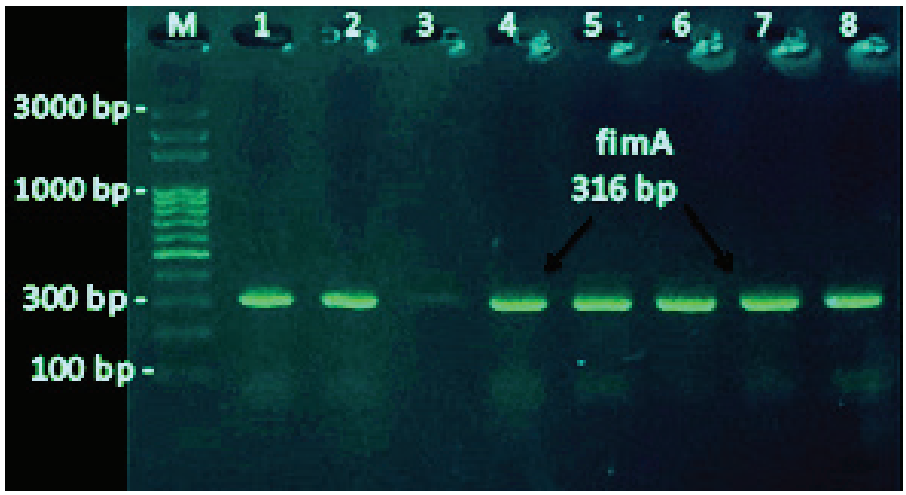

Figure (2): Gel electrophoresis, of amplified *fimA*. gene, using conventional PCR. . Agarose, 2%, and TBE(1X), at (75 V/cm for 90 min., stained with RedSafe™ and visualized on a UV transilluminator. Marker (M): (100-3000bp), Lanes:(1-8) Milk Samples

Table 3 : Distribution of *bla-CTX-M* gene in *E.coli* and *k.pneumoniae* isolates

Sample	E. coli		K. pneumoniae	
	NO.	bal-CTX-M NO. /%	NO.	bla-CTX-M NO./%
Stool	19	15 / 78.9	5	4/80
Milk	11	9 / 81.8	3	3/100
Utensils	3	2 / 66.6	0	0/0
Total	33	26 / 78.7	8	7/87.5

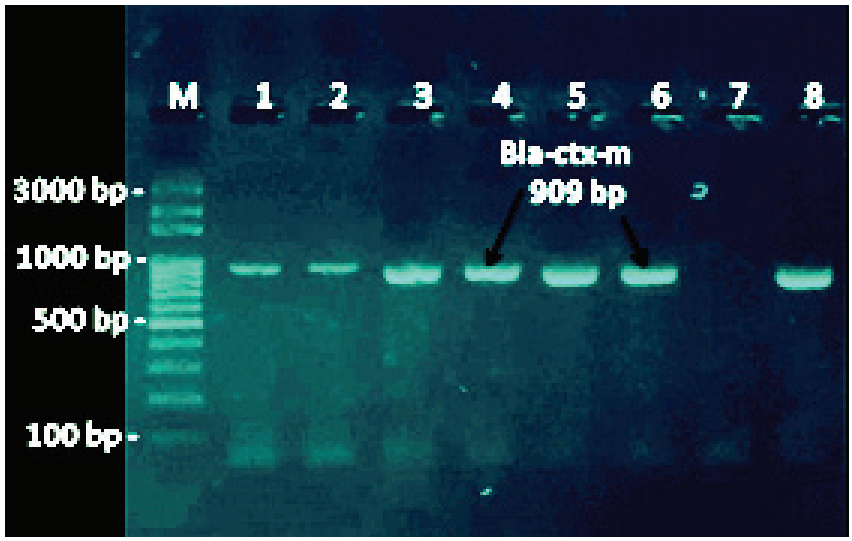

Figure 2 : Gel electrophoresis, of amplified *Bla-ctx-m* gene, using conventional PCR. . Agarose, 2%, and TBE(1X), at (75 V/cm for 90 min., stained with RedSafe™ and visualized on a UV transilluminator. Marker (M): (100-3000bp), Lanes:(1-8)stool samples

Discussion

In this study a high percentage of agreement, was observed for a phenotypes of isolates, determined by microtitir plate applicability, reliability and high reproducibility of the microtitir plate were previously verified for bacterial biofilm[15]. The isolated *E. coli* and *K. pneumoniae* were evaluated for biofilm formation, by using phenotypic microtiter plate screening as well as molecular, detection of, genes. Microtiter plate (MTP) showed that 33 isolates of *E.coli* were biofilm produce , 42.2 % of isolates were strong, 24.2 % were moderate and 33.3 % were weak biofilm producers. this study consistent with results of previous study by[32] . while eight isolates, of *K. pneumoniae,* were able to form biofilm percent 62.5% moderate and 37.5 weak. These data are in accordance with those reported by[17]

E.coli tends to be the most important cause of infections associated with mastitis. However, more precise investigation of individual farms revealed a farm-specific infection pattern where a single Gram-negative bacterial species. perhaps there is subclinical mastitis or intra mammary infection[18]

The *fimA* gene is responsible for the formation of biofilms. Type 1 fimbriae are encoded by *fimA* gene, which can facilitate bacterial adhesion and biofilm formation[26] . This study was detected production of *fimA* gene in 84.8 % of *Escherichia coli* isolated from stool ,milk and utensils where as found in Table (4) **.** Result in this study is an agreement with a result of a study done by[20]. Another study had been noticed less percentage in study done by[21]. Who founded 76%. This study was revealed that all *K. pneuomoniae* isolates wich was all have been positive for the presence of *fimA* gene in 8/8100%. This result agreement with result of[21] .who showed the presence of *fimA* and *fimH* genes in 100 %.

The investigation and detection of *blaCTX-M* gene in *E.coli* isolates revealed in this study the presence of this gene in percentage 78.7% (26/33) this result near from study done by[22]

In *K. pneumoniae* the result presence of a *blaCTX-M* gene was found in percentage 80 % . But this result was less than the result by[25] in China who mentioned the percentage was 87.62%. But it was higher than the result reported by[24]. who reported 30%. the other hand the *hylA* gene was not detected in all *E.coli* that similar to studies findings were obtained by[30] . While disagree with the result reported by[20] . while was *magA* gene in all were negative for *K. pneumoniae* isolates this result agreement with result reported [27] but was differ from the result reported in AL-diwaniyah city, Iraq who recorded *magA* gene in percentage 92% by[22] . Variations in the geographical distribution of Serotypes of *K. pneumoniae*, were identified by [28] .

The presence of multiple genotypes and a high degree of genetic variation in *K. pneumoniae* isolates has also been confirmed by[29] . A study reported by[31] that there are plasmid-carried genes that can express capsular polysaccharide synthesis (cps) genes.

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Conflict of Interest: The author declare there is no conflict of Interest

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq. All experimental protocols were approved under the College. while experiments were carried out in accordance with approved guidelines

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