Archive of SID

International Journal of
Enteric
Pathogens

Int J Enteric Pathog. 2019 May;7(2):37-43

Original Article

http://enterpathog.abzums.ac.ir

doi 10.15171/ijep.2019.10

Antibiotic Resistance Profiling and Molecular Phylogeny of Biofilm Forming Bacteria From Clinical and Non-clinical Environment in Southern Part of Bangladesh

Zulkar Nain, Md. Ariful Islam, Mohammad Minnatul Karim*

Department of Biotechnology and Genetic Engineering, Faculty of Applied Science and Technology, Islamic University, Kushtia-7003, Bangladesh

***Corresponding Author:**
Mohammad Minnatul Karim,
Tel: 8801620191993,
Email:
mkmicro.du@gmail.com

Published Online March 11, 2019

Keywords: Antibiotic Resistance, Biofilm Formation, *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Cronobacter sakazakii*

Abstract

Background: Biofilm is a surface adhered extracellular polymer matrix produced by bacteria. The establishment of biofilms is considered as an important pathogenic trait in many chronic infections and antibiotic resistance.

Objective: The present study was intended to evaluate biofilm forming potency and antibiotic resistance (AR) pattern in clinical and non-clinical bacterial isolates, and their phylogenetic characterization.

Materials and Methods: A total of 82 bacterial isolates were obtained from clinical settings and animal farms from southern (Kushtia-Jhenaidah) region of Bangladesh. Biofilm forming potentials and AR profile were evaluated by standard biofilm assay and Kirby-Bauer disk diffusion method, respectively. Further, antibiotic exposure was assessed by multiple antibiotic resistance (MAR) value indexing. Furthermore, statistical methods were applied to estimate the relationship between AR and biofilm formation. Finally, selected isolates were characterized by morphological and biochemical tests, as well as 16S rRNA gene sequencing.

Results: Clinical isolates showed higher biofilm formation (OD_{595}=1.17±0.03) than non-clinical isolates (OD_{595}=0.68±0.03). Among all, *Pseudomonas* isolates produced the highest amount of biofilms (OD_{595}=2.08±0.02). The AR profiles fell within 46.67-86.67% and MAR index ranged from 0.47 to 0.87. Moreover, a significant positive correlation ($P<0.05$) was found between biofilm formation and AR. Eventually, heavy biofilm producers with ≥60% resistance profile were characterized and identified as *Escherichia coli, Cronobacter sakazakii, Pseudomonas aeruginosa, Staphylococcus sciuri*, and *Staphylococcus aureus*.

Conclusion: In general, biofilm formation and MAR were highly correlated regardless of the source, type, and environment of the isolates. Therefore, a rigorous evaluation of both biofilm formation and AR is demanded to minimize AR and associated problems.

Received October 17, 2018; **Revised** February 14, 2019; **Accepted** March 4, 2019

Background

Bacteria reign over this planet have still remained in power after more than four billion years. The formation of biofilm, as a surface-attached sessile microbial community is viewed as one of their key survival strategies.[1] In addition, it is well-known to play a significant role in chronic infection and antibiotic resistance (AR).[2] For example, dental plaque is a common biofilm-mediated problem which contains more than 700 bacterial species.[3] Moreover, resistant to antibiotics is more prevalent in bacteria within biofilm compared to their free-living counterparts due to the inaccessible extracellular polymer matrix present in the biofilm structure.[2,4] Further, the growing AR is attributed to the overuse and misuse of antibiotics in clinical setting and feedstocks preparation for therapeutic reasons and growth promotion, respectively.[5,6] Furthermore, AR complicates and can jeopardize the treatment of serious bacterial infections in both animals and humans.[7]

In recent years, biofilm formation by a group of highly pathogenic bacteria, known as ESKAPE (i.e., *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*) has drawn special attention.[4] For example, *P. aeruginosa* and *S. aureus* cause several important nosocomial infections and, have already become resistant to last-line of antibiotics.[8-10] Biofilm forming *P. aeruginosa* and *S. aureus* and their resistance to antibiotics are reported earlier.[11,12] Additionally, multidrug resistant (MDR) *Escherichia coli* was observed in non-

© 2019 The Author(s); Published by Alborz University of Medical Sciences. This is an open-access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

www.SID.ir

Archive of SID

clinical sources.[13] The genetic flexibility and adaptability of *E. coli* allow them to develop AR mechanisms based on the changes in their environments.[14] Therefore, biofilm formation and its relation with AR need to be studied for bacteria from both clinical and non-clinical sources.

The emergence of MDR bacteria is a growing concern for public and animal health, particularly in Bangladesh due to the frequent uses of antibiotics.[15,16] Moreover, nearly 90% of veterinary medicines, which are commonly used in animal farming, are excreted from the animal feces.[6,17] These animal wastes, containing antimicrobial residues and resistant bacteria, are further used as fertilizer in agriculture.[18] For instance, approximately 50% and 10% of the total daily-produced poultry wastes in Bangladesh are directly used in fish culture and crop production, respectively.[19] The prevalence of MDR bacteria in raw milk and associated public health problems are well-documented as well.[13,20] Accordingly, threats to public health can emerge from transferring pathogens or their resistance genes to other bacterial genera and the human.[21]

Objectives

The present study aimed to assess the biofilm forming potency, AR profile, and molecular phylogeny of bacterial isolates obtained from clinical settings (i.e., Kushtia General Hospital and Islamic University Medical Center) and animal farms in nearby cities, Kushtia, and Jhenaidah, Bangladesh.

Materials and Methods

Sample Collection and Isolation of Bacterial Strains

Seventy-two clinically relevant samples were directly received from the environment (i.e., clinical wastes and dusts) and patients (i.e., nasal mucus, human stools, and urine catheter) admitted to the Kushtia General Hospital and the Islamic University Medical Center. In addition, twelve non-clinical samples (i.e., poultry litter and raw cow milk) were obtained from commercial animal farms in Jhenaidah. After processing, samples were selectively cultured on Eosin Methylene Blue Agar (EMB, HiMedia, India), Cetrimide Agar (CEA, HiMedia, India), and Mannitol Salt Agar (MSA, Scharlau, Spain) in order to isolate *Escherichia*, *Pseudomonas*, and *Staphylococcus*, respectively.

Evaluation of Biofilm Forming Potential

Tube Adherence Method

Biofilm production was detected by a quantitative assay proposed by Christensen et al.[22] Briefly, a loopful of the isolate was inoculated into a glass tube containing 5 mL of Tryptic Soy Broth (TSB, HiMedia, India) and incubated at 37ºC for 48 hours. The tubes were decanted and dried after incubation and stained with 0.1% crystal violet (CV). Subsequently, the tubes were gently washed and placed upside down for drying. The visible lining of the wall and bottom of the tube by a film was considered as positive. Further, the optical density (OD) of the stained adherent bacterial biomass was measured at 595 nm (OD_{595}) with UV-VIS spectrophotometer (Shimadzu, Australia). Sterile TSB tubes were used as the negative control.

Microtiter-Plate Method

The formation of biofilm was performed in a standard 96-well microtiter plate as described by Stepanović et al.[23] Generally, after grown in TSB at 37°C for 36 hours, non-adherent bacterial cells were removed by an aspirator. However, the adherent cells were stained with 0.1% CV solution for 50 minutes after which the CV solution was removed. Furthermore, the excess stain was rinsed off by dH_2O two times through gentle shaking for 20 minutes. Biofilm production was quantitatively measured at OD_{595} with Tecan GENios Microplate Reader (Tecan Group Ltd., Switzerland), and the wells containing TSB broth only served as negative control. Moreover, the mean OD_{595} of the negative control was subtracted from the mean OD_{595} of inoculated wells in order to correct the background staining of CV. The adherence ability was classified based on cut-off absorbance value according to Stepanović et al.[23] Finally, a microscopic portion of the adhered biofilms was visualized in bright field microscopy as well.

Antibiotic Resistance and Multiple Antibiotic Resistance Value Index

Antibiotic susceptibility/resistance patterns towards fifteen clinically relevant antibiotics (included in eight classes) were examined by the Kirby-Bauer method according to the standard of Clinical and Laboratory Standards Institute.[24] The following antibiotic discs (HiMedia, India) were used.

Penicillin G (P, 10 µg), tetracycline (TE, 30 µg), co-trimoxazole (COT, 25 µg), erythromycin (E, 15 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg), ciprofloxacin (CIP, 5 µg), ceftazidime (CAZ, 30 µg), azithromycin (AZM, 30 µg), nalidixic acid (NA, 30 µg), colistin (CL, 10 µg), polymyxin B (PB, 300 µg), doxycycline (DO, 30 µg), amoxicillin (AMX, 30 µg), and ceftriaxone (CTR, 30 µg). Multidrug resistance was defined as the acquired non-susceptibility to at least one agent in 3 or more antimicrobial categories.[25] The MAR index was calculated as the ratio of the number of antibiotics to which the isolate displayed resistance to the number of antibiotics to which the isolate was evaluated for susceptibility.[26]

Identification of High Biofilm Forming Multidrug Resistant Isolates

Bacterial isolates with the highest profile in both biofilm formation and AR were characterized and identified using standard biochemical and molecular techniques. Morphological and biochemical characteristics were evaluated following standard methods described in Benson's Microbiological Applications Lab Manual for tentative identification.[27]

www.SID.ir

Archive of SID

Molecular Characterization

Each selected isolate was subjected to 16S rRNA gene sequencing for species-level identification. The genomic DNA was extracted using the automated DNA extractor with Maxwell DNA Kit (Promega, USA). The processed DNA was then applied for polymerase chain reaction (PCR) using Gene Atlas (Astec, Japan). Two universal bacterial PCR primer pair 27F and 1492R [Forward (5′-AGA GTT TGA TCC TGG CTC AG-3′)] and [Reverse (5′-TAC GGH TAC CTT GTT ACG ACT T-3′)] were used to amplify a partial segment of bacterial ribosomal DNA.[28] For amplification, a 25 µL mixture containing 12 µL Hot Start Green Master Mix (dNTPs, Buffer, $MgCl_2$, Taq Polymerase, Promega, USA), 1 µL of T-DNA (25-65 ng/µL), 1 µL of each primer (10-20 pMol), and 10 µL of water was employed. The thermal cycle involved 2-minute pre-heating at 95°C before 30 cycles including 30 seconds at 95°C (denaturation), 30 seconds at 48°C (annealing), and 1.5 minutes at 72°C (extension). Finally, a 5-minute extension at 72°C was performed, followed by a final hold of overnight at 4°C. The amplified PCR products were confirmed and purified by electrophoresis (CBS Scientific, USA) through 1% agarose gel in TBE buffer and stained with EtBr solution (Promega, USA). Ultimately, the purified rRNA gene was ligated to vector pGEM-cz and sequenced by the automated sequencer (Applied Biosystems, USA).

Phylogenetic Analysis

The quality of sequences was assessed by ChromasPro, version 1.7. The forward and reverse sequences were assembled in Lasergene SeqMan software (DNASTAR, Inc.) and then the complete consensus sequences were compared against GenBank database of the National Center for Biotechnology Information using Basic Local Alignment Search Tool. For each sequence, eight sequences with >90% similarity and one reference sequence as the outgroup, as suggested by Hall, were curated from GenBank database and subjected to a phylogenetic tree construction using neighbor-joining methods in MEGA6 with 1000 bootstrap replications.[29-31] In addition, *P. aeruginosa* PAO1 was considered as the legitimate outgroup for *E. coli* and *C. sakazakii* while *E. coli* ATCC 43893 was counted for *P. aeruginosa*. On the other hand, *Bacillus cereus* NC7401 was used as a legitimate outgroup for *Staphylococcal* isolates. The complete 16S rDNA sequences were deposited to GenBank database.

Statistical Analysis

All experiments were performed in triplicates and the obtained data were presented as mean ± standard deviation (SD) where appropriate. The relationship between biofilm formation and AR was estimated using Pearson's correlation analysis, and intergroup differences were calculated with one-way analysis of variance (ANOVA). The statistical analysis was performed in Microsoft Excel™ 2013 and considered significant when *P* value was less than 0.01, 0.05, and 0.001. Graphical illustrations were prepared using Origin Pro, version 8.0 (OriginLab Corporation, USA) as well.

Results

Isolation of Bacterial Isolates

A total of 82 bacterial isolates (i.e., 72 clinical + 10 non-

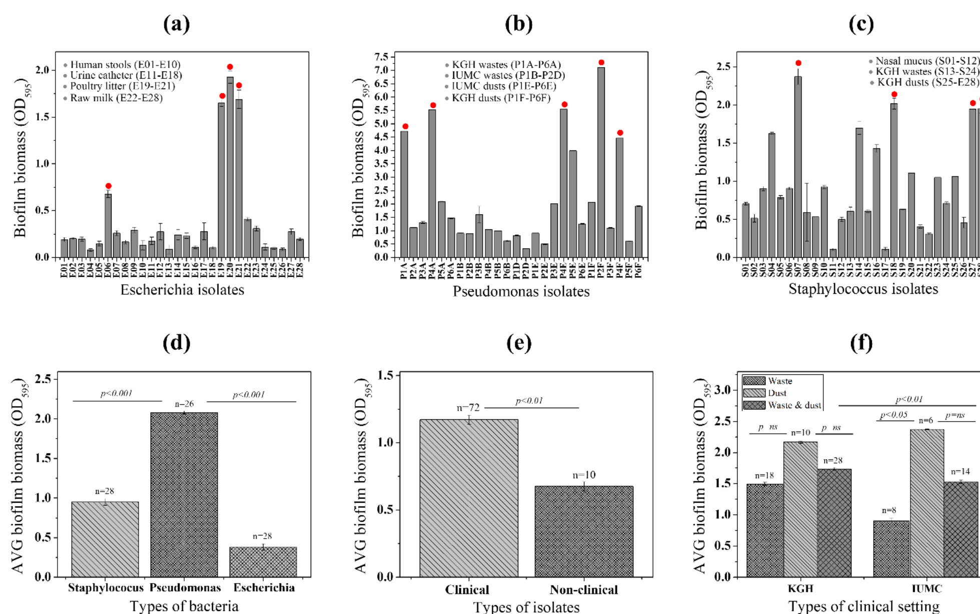

Figure 1. The Evaluation of Biofilm Production by Different Isolates. Mass screening for biofilm formation by **(a)** *Escherichia*, **(b)** *Pseudomonas*, and **(c)** *Staphylococcus* isolates, and individual group-dependent study based on the type of **(d)** bacteria, **(e)** isolates, and **(f)** clinical settings. The selected heavy biofilm producers and the number of organisms tested are marked by 'red circle' and 'n', respectively. The level of significance is indicated by *P* value while 'ns' denotes lack of any significance.

www.SID.ir

Archive of SID

clinical) were obtained in the present study. The highest percent of bacteria was isolated from clinical wastes (26, 31.71%), followed by clinical dust (16, 19.51%), nasal mucus (12, 14.63%), human stools (10, 12.20%), urine catheter (8, 9.76%), raw cow milk (7, 8.54%), and poultry litter (3, 3.66%).

Biofilm Formation and Selection of Heavy Biofilm Producers

As shown in Figures 1a-c, the qualitative and quantitative evaluation suggest that all the isolates produced biofilms at a different extent. The average biofilm biomass produced by *Pseudomonas*, *Staphylococcus*, and *Escherichia* isolates is 2.08 ± 0.02, 0.95 ± 0.04, and 0.38 ± 0.06, respectively (Figure 1d). Based on the data illustrated in Figure 1e, the clinical isolates produce higher average biofilm biomass (1.17 ± 0.03) compared to non-clinical isolates (0.68 ± 0.03). As regards the waste and dust samples, Kushtia General Hospital (KGH) isolates show higher biofilm formation (1.74 ± 0.02) compared to the University Medical Center (IUMC) isolates (1.53 ± 0.03), the details of which are depicted in Figure 1f. High biofilm yielding isolates (i.e., red circle), displayed in Figures 1a-c, from each bacteria group are selected for further investigation.

Antibiotic Resistant Pattern and Multiple Antibiotic Resistance Value Index

The AR profile ranged between 46.67-86.67%. Most of the isolates were resistant to P, TE, COT, E, NA, K, S, CAZ, and AMX and susceptible to CL, DO, PB, CIP, AZM, and CTR (Table 1). Among all, nonclinical isolates (i.e., E19, E20, and E21) exhibited the highest AR profiles (53.33%-86.67%). Considering the clinical setting, KGH isolates (i.e., E06, P1A, P4A, S07, and S18) showed 46.67%-73.33% AR while IUMC isolates (i.e., P4E, P2F, P4F, S27, and S28) provided 46.67%-66.67%. Further, clinical isolates demonstrated 100% resistance to P and NA while non-clinical isolates were 100% resistant to P, E, NA, CAZ, and AMX. Furthermore, both clinical and non-clinical isolates represented complete susceptibility to PB. However, all the selected isolates were resistant to more than four antibiotic classes and were classified as multiclass antibiotic resistant. The multiple antibiotic resistance (MAR) index ranged from 0.47 to 0.87 (Table 1). The isolate E20, E21, P4A, P2F, S07, and S18 (≥60% resistance) were selected for phylogenetic analysis.

The Relationship Between Biofilm Formation and Antibiotic Resistance

Pearson's correlation analysis was used to assess the interrelation between biofilm production and AR. A positive correlation (r= 0.59, P<0.05) was observed between biofilm formation and AR (Figure 2).

Identification and Phylogenetic Analysis of Bacterial Strains

The Basic Local Alignment Search Tool result of 16S rDNA sequences from isolates E20, E21, P4A, P2F, S07, and S18 rendered a close relationship with *Escherichia coli, Cronobacter sakazakii, Pseudomonas aeruginosa, P. aeruginosa, Staphylococcus sciuri*, and *S. aureus*, respectively. Moreover, the neighbor-joining phylogenetic tree further confirmed the position of the bacterial strains in their respective lineage (Figure 3). Finally, 16S rDNA sequence was deposited to GenBank database and a unique accession code was received for each strain, *viz.*, *Escherichia coli* strain MZ20 (MG693099), *Cronobacter sakazakii* strain MZ21 (MG735676), *Pseudomonas aeruginosa* strain MZ4A (MG735679), *P. aeruginosa* strain MZ2F (MG735678), *Staphylococcus sciuri* strain MZ07 (MG735680), and *S. aureus* strain MZ18 (MG735703).

Discussion

In this study, biofilms were developed in glass-tube, as well as in microtiter-plate. The biofilm formation in microtiter-plate was significantly higher (P<0.05) and different from

Table 1. Resistance Pattern and MAR Value Index of 13 Isolates

Isolates (n=13)	Source and Isolate Specific Antibiotic Resistance Profiles				
	Resistance Pattern	**Resistance No. (%)**	**MAR Index**	**Clinical (n = 10); Disk (%)**	**Non-clinical (n = 3); Disk (%)**
E06	P-TE-COT-E-S-CAZ-NA	7 (46.67)	0.47	P (100%), TE (90.0%), COT (90.0%), E (90.0%), K (50.0%), S (90.0%), CIP (10.0%), CAZ (70.0%), AZM (40.0%), NA (100%), CL (30.0%), PB (0.0%), CTR (10.0%), DO (30.0%), AMX (50.0%)	P (100%), TE (66.7%), COT (100%), E (100%), K (66.7%), S (66.7%), CIP (66.7%), CAZ (100%), AZM (33.3%), NA (100%), CL (66.7%), PB (0.0%), CTR (66.7%), DO (66.7%), AMX (100%)
E19	P-COT-E-K-CAZ-NA-CTR-AMX	8 (53.33)	0.53		
E20	P-TE-COT-E-K-S-CIP-CAZ-NA-CL-CTR-DO-AMX	13 (86.67)	0.87		
E21	P-TE-COT-E-S-CIP-CAZ-AZM-NA-CL-DO-AMX	12 (80.00)	0.80		
P1A	P-TE-COT-E-S-NA-AMX	7 (46.67)	0.47		
P4A	P-TE-COT-E-K-S-CAZ-NA-CL-DO-AMX	11 (73.33)	0.73		
P4E	P-TE-COT-E-K-S-CAZ-NA	8 (53.33)	0.53		
P2F	P-TE-COT-E-K-S-CAZ-NA-DO-AMX	10 (66.67)	0.67		
P4F	P-TE-COT-K-S-NA-AMX	7 (46.67)	0.47		
S07	P-TE-COT-E-S-CAZ-AZM-NA-CL	9 (60.00)	0.60		
S18	P-TE-COT-E-S-CAZ-AZM-NA-CL-CTR-AMX	11 (73.33)	0.73		
S27	P-E-K-CIP-CAZ-AZM-NA-DO	8 (53.33)	0.53		
S28	P-TE-COT-E-S-AZM-NA	7 (46.67)	0.47		

Abbreviation: MAR, multiple antibiotic resistance.

www.SID.ir

Archive of SID

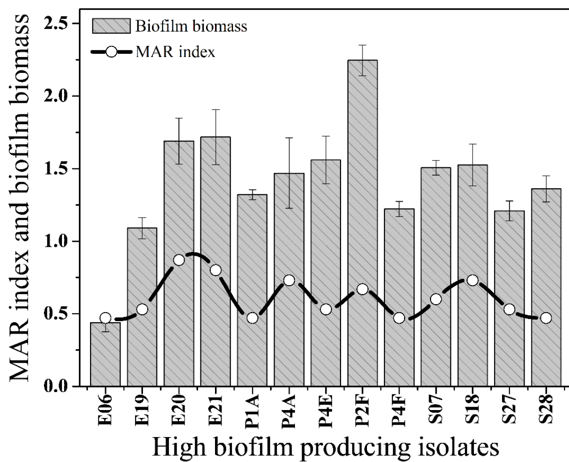

Figure 2. The Correlation Between Antibiotic Resistance and Biofilm Formation.

biofilms in glass-tube. All the isolates produced biofilms and showed AR at a different degree (Figure 1 and Table 1). *P. aeruginosa* is well-known for their extensive colonizing ability regardless of the surfaces.[32] Additionally, individual group-dependent study demonstrated that *Pseudomonas* isolates produced a significantly higher amount of biofilm (P<0.001) compared to other bacterial isolates (Figure 1d). It is known that biofilm formation varies with the type of bacteria.[33] Further, biofilm production by *S. aureus, E. coli*, and *C. sakazakii* was evident in the current study which coincides with the findings of the other studies.[34-36] Furthermore, clinical isolates provided significantly more biofilm biomass (P<0.01) compared to non-clinical isolates (Figure 1e). Interestingly, biofilm production was higher in isolates which belonged to dusts, wastes, and poultry litter samples. These are the accumulated reservoir of thriving bacterial strains from various sources. However, the present study particularly emphasized on the biofilm producers from the dust and waste in clinical environments because of their pathogenic potentials and the comparison of the inter-difference between the two clinical settings. In the case of wastes and dusts, KGH isolates harbored better biofilm-forming pathogens compared to IUMC isolates (Figure 1f). The waste and dust gathered in a public health institute like KGH which may come from different sources can lead to the accumulation of competent bacteria.

In this study, antibiogram results suggested that bacteria belonging to animal farms were more resistant (≥80%) compared to clinical environments (60-73.33%). For instance, *E. coli* MZ20 and *C. sakazakii* MZ21 from poultry litter showed 86.67% and 80% AR profiles, respectively (Table 1). This could be due to the frequent use of antibiotics in animal farms. The commonly used antibiotics in poultry farms are AMX, DO, E, and CL.[6] It was alarming that *E. coli* MZ20 and *C. sakazakii* MZ21 were resistant to all the studied antibiotics except for COT, K, CAZ, AZM, NA, and CIP. Nevertheless, polymyxin B was the most effective antibiotic against both clinical and commensal bacteria (Table 1). Further, multiple AR in *C. sakazakii* was previously reported.[37] On the other hand, clinical strain *P. aeruginosa* MZ4A and *S. aureus* MZ18 both represented 73.33% AR. Furthermore, higher AR was observed in KGH isolates compared to IUMC isolates. More antibiotic exposure to KGH patients than IUMC could be a plausible explanation. The percentage of AR exhibited by *S. sciuri* MZ07 (60%) was less compared to *S. aureus* MZ18 (73.33%). It could be due to their rare colonization and infection in humans.[38] The strain *S. sciuri* MZ07 of the current study was resistant to all penicillin,

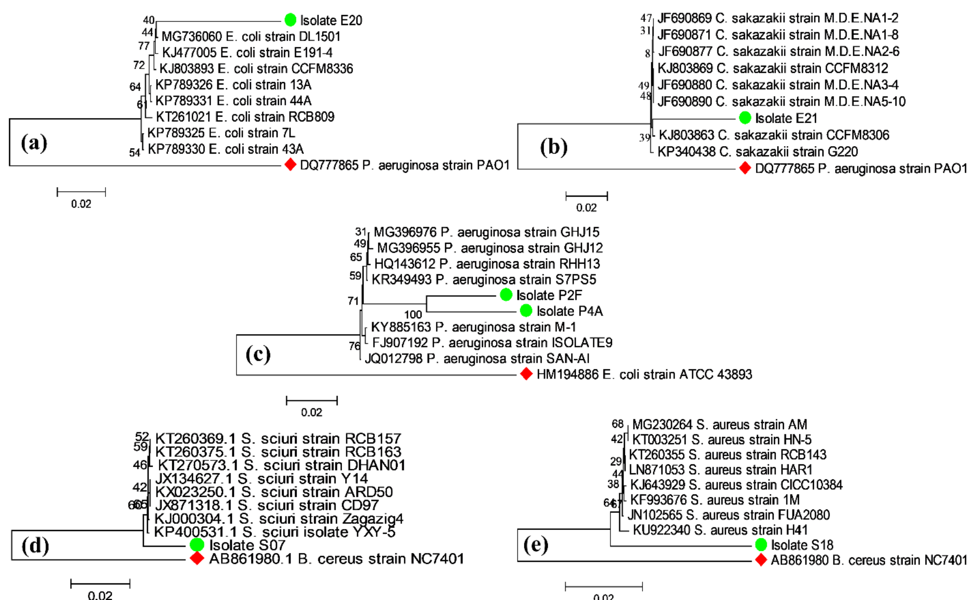

Figure 3. The Phylogenetic Positions of 6 Strains (Green Circle) Within Their Respective Neighbours and Outgroup Lineage (Red Square): (a) *Escherichia coli* MZ20, (b) *Cronobacter sakazakii* MZ21, (c) *Pseudomonas aeruginosa* MZ2F and MZ4A, (d) *Staphylococcus sciuri* MZ07, and (e) *Staphylococcus aureus* MZ18.

www.SID.ir

Archive of SID

tetracycline, and erythromycin classes of antibiotics. In a study by Couto et al, 21 strains of *S. sciuri* were found resistant to penicillin while susceptible to tetracycline and erythromycin. However, they indicated that only some strains could possess resistance to tetracycline and erythromycin.[39]

MAR indexing method is simple, rapid, and cost-effective compared to genotypic characterization and thus can be applied to differentiate bacteria from different sources.[26] Navon-Venezia et al reported that MAR index values larger than 0.2 indicate the high-risk source of contamination.[40] The higher MAR index more than 0.2 indicates that the isolates reported in the present study originated from high-risk sources of contamination where antibiotics are often used (Table 1). The observation was not surprising in the clinical setting and livestock preparations where antibiotics are used frequently.[5,18] Furthermore, the results of the current study revealed a significant correlation between AR and biofilm formation (Figure 2). In addition, the association of biofilms with AR was reported previously.[8]

Conclusion

In conclusion, most clinical and non-clinical isolates were found as strong biofilm producers and resistant to multiple antibiotics. Clinical environments infested with these bacteria may lead to increased morbidity and mortality since treatment against biofilm forming MDR pathogens is far more complicated. Therefore, higher MAR index and biofilm forming potential of the bacterial isolates from animal farms and and hospital settings suggest to rigorously monitor the transmission of these bacterial strains and their resistance genes in the environment.

Authors' Contributions

Study design and experimental work: ZN and MMK. Analysis and interpretation of the data: ZN. Manuscript writing: ZN, MAI, and MMK. Statistical analysis: ZN. Critical revision of the manuscript: ZN, MAI, and MMK. Study supervision: MMK.

Ethical Approval

The article contains no studies conducted on human or animal subjects, as a result, ethical approval and informed consent were unnecessary.

Conflict of Interest Disclosures

None of the authors has a financial or other relationship with other people or organizations that may inappropriately influence this work.

Financial Support

This work is financially (partially) supported by University Grant Commission research fund fiscal year 2016-17 (Grant No. 4829), and Ministry of Science and Technology, Government of the Peoples' Republic of Bangladesh through NST fellowship.

References

1. Van Acker H, Van Dijck P, Coenye T. Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. Trends Microbiol. 2014;22(6):326-333. doi:10.1016/j.tim.2014.02.001
2. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284(5418):1318-1322. doi:10.1126/science.284.5418.1318
3. Karim MM, Nagao A, Mansur FJ, et al. The periodontopathogenic bacterium *Eikenella corrodens* produces an autoinducer-2-inactivating enzyme. Biosci Biotechnol Biochem. 2013;77(5):1080-1085. doi:10.1271/bbb.130047
4. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Biofilm formation mechanisms and targets for developing antibiofilm agents. Future Med Chem. 2015;7(4):493-512. doi:10.4155/fmc.15.6
5. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T. 2015;40(4):277-283.
6. Economou V, Gousia P. Agriculture and food animals as a source of antimicrobial-resistant bacteria. Infect Drug Resist. 2015;8:49-61. doi:10.2147/idr.s55778
7. Hao H, Cheng G, Iqbal Z, et al. Benefits and risks of antimicrobial use in food-producing animals. Front Microbiol. 2014;5:288. doi:10.3389/fmicb.2014.00288
8. Babapour E, Haddadi A, Mirnejad R, Angaji SA, Amirmozafari N. Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. Asian Pac J Trop Biomed. 2016;6(6):528-533. doi:10.1016/j.apjtb.2016.04.006
9. Taj Y, Essa F, Aziz F, Kazmi SU. Study on biofilm-forming properties of clinical isolates of Staphylococcus aureus. J Infect Dev Ctries. 2012;6(5):403-409. doi:10.3855/jidc.1743
10. Valot B, Guyeux C, Rolland JY, Mazouzi K, Bertrand X, Hocquet D. What it takes to be a *Pseudomonas aeruginosa?* The core genome of the opportunistic pathogen updated. PLoS One. 2015;10(5):e0126468. doi:10.1371/journal.pone.0126468
11. Neopane P, Nepal HP, Shrestha R, Uehara O, Abiko Y. In vitro biofilm formation by Staphylococcus aureus isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. Int J Gen Med. 2018;11:25-32. doi:10.2147/ijgm.s153268
12. Sanjee SA, Hassan MM, Manchur MA. In vitro biofilm formation by multidrug resistant clinical isolates of *Pseudomonas aeruginosa*. Asian J Med Biol Res. 2018;4(1):105-116. doi:10.3329/ajmbr.v4i1.36828
13. Szmolka A, Nagy B. Multidrug resistant commensal Escherichia coli in animals and its impact for public health. Front Microbiol. 2013;4:258. doi:10.3389/fmicb.2013.00258
14. Tasnim UT, Islam MT. Pathogenic and drug resistant bacteria in raw milk of Jessore city: A potential food safety threat. Bangladesh J Vet Med. 2015;13(1):71-78.
15. Tabashsum Z, Khalil I, Nazimuddin, Mollah AKMM, Inatsu Y, Bari L. Prevalence of foodborne pathogens and spoilage microorganisms and their drug resistant status in different street foods of Dhaka city. Agric Food Anal Bacteriol. 2013;3(4):281-292.
16. Mason T, Trochez C, Thomas R, Babar M, Hesso I, Kayyali R. Knowledge and awareness of the general public and perception of pharmacists about antibiotic resistance. BMC Public Health. 2018;18(1):711. doi:10.1186/s12889-018-5614-3
17. Marshall BM, Levy SB. Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev. 2011;24(4):718-733. doi:10.1128/cmr.00002-11
18. Furtula V, Farrell EG, Diarrassouba F, Rempel H, Pritchard J, Diarra MS. Veterinary pharmaceuticals and antibiotic

www.SID.ir

Archive of SID

resistance of *Escherichia coli* isolates in poultry litter from commercial farms and controlled feeding trials. Poult Sci. 2010;89(1):180-188. doi:10.3382/ps.2009-00198

19. Waste Concern. CDM project potential in the poultry waste management sector in Bangladesh. Final Report 2005
20. Lucey JA. Raw milk consumption: risks and benefits. Nutr Today. 2015;50(4):189-193. doi:10.1097/nt.0000000000000108
21. Lundin JI, Dargatz DA, Wagner BA, et al. Antimicrobial drug resistance of fecal *Escherichia coli* and *Salmonella* spp. isolates from United States dairy cows. Foodborne Pathog Dis. 2008;5(1):7-19. doi:10.1089/fpd.2007.0018
22. Christensen GD, Simpson WA, Younger JJ, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol. 1985;22(6):996-1006.
23. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40(2):175-179. doi:10.1016/S0167-7012(00)00122-6
24. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4):493-496.
25. Magiorakos AP, Srinivasan A, Carey RB, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268-281. doi:10.1111/j.1469-0691.2011.03570.x
26. Davis R, Brown PD. Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica. J Med Microbiol. 2016;65(4):261-271. doi:10.1099/jmm.0.000229
27. Benson HJ, Brown AE. Benson's Microbiological Applications: Microbiology. McGraw-Hill Higher Education; 2006.
28. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991;173(2):697-703. doi:10.1128/jb.173.2.697-703.1991
29. Hall BG. Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol. 2013;30(5):1229-1235. doi:10.1093/molbev/mst012
30. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4(4):406-425. doi:10.1093/oxfordjournals.molbev.a040454
31. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30(12):2725-2729. doi:10.1093/molbev/mst197
32. Sabaeifard P, Abdi-Ali A, Soudi MR, Dinarvand R. Optimization of tetrazolium salt assay for *Pseudomonas aeruginosa* biofilm using microtiter plate method. J Microbiol Methods. 2014;105:134-140. doi:10.1016/j.mimet.2014.07.024
33. de Souza EL, Meira QG, de Medeiros Barbosa I, Athayde AJ, da Conceicao ML, de Siqueira Junior JP. Biofilm formation by *Staphylococcus aureus* from food contact surfaces in a meat-based broth and sensitivity to sanitizers. Braz J Microbiol. 2014;45(1):67-75.
34. Vital-Lopez FG, Reifman J, Wallqvist A. Biofilm formation mechanisms of *Pseudomonas aeruginosa* predicted via genome-scale kinetic models of bacterial metabolism. PLoS Comput Biol. 2015;11(10):e1004452. doi:10.1371/journal.pcbi.1004452
35. Lee JS, Bae YM, Lee SY, Lee SY. Biofilm formation of *Staphylococcus aureus* on various surfaces and their resistance to chlorine sanitizer. J Food Sci. 2015;80(10):M2279-2286. doi:10.1111/1750-3841.13017
36. Kim H, Ryu JH, Beuchat LR. Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. Appl Environ Microbiol. 2006;72(9):5846-5856. doi:10.1128/aem.00654-06
37. Kilonzo-Nthenge A, Rotich E, Nahashon SN. Evaluation of drug-resistant Enterobacteriaceae in retail poultry and beef. Poult Sci. 2013;92(4):1098-1107. doi:10.3382/ps.2012-02581
38. Schwarz S, Kehrenberg C, Ojo KK. *Staphylococcus sciuri* gene erm(33), encoding inducible resistance to macrolides, lincosamides, and streptogramin B antibiotics, is a product of recombination between erm(C) and erm(A). Antimicrob Agents Chemother. 2002;46(11):3621-3623. doi:10.1128/AAC.46.11.3621-3623.2002
39. Couto I, Sanches IS, Sa-Leao R, de Lencastre H. Molecular characterization of *Staphylococcus sciuri* strains isolated from humans. J Clin Microbiol. 2000;38(3):1136-1143.
40. Navon-Venezia S, Ben-Ami R, Carmeli Y. Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. Curr Opin Infect Dis. 2005;18(4):306-313. doi:10.1097/01.qco.0000171920.44809.f0

www.SID.ir