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**Research Article** **Open Access**

# Investigation of Virulence Factors in Microbial Organisms that Associated with Public Health Risk Isolates from Different Environmental Regions

Suhad A. Abid[1], Sarah Naji Aziz[1], Noor Al-Huda Ali A.H Saeed[1], Shaimaa N. Mizil[1], Israa M.S. Al-Kadmy[1,*], Nadheema H. Hussein[1], Nadal Al-Saryi[1], Susan A. Ibrahim[1], Jumaah D. Hussein[2]

[1]Department of Biology, College of Science, Mustansiriyah University, POX 10244, Baghdad, IRAQ.
[2]Ministry of Health, Baghdad, IRAQ.

*Correspondent contact: israaalkadmy@gmail.com

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## ABSTRACT

Infectious diseases caused by infected tools in the environments are threaten to the safety and public health. Transmission sources of these infectious diseases are unknown, but it is thought that non-living materials called fomites, are the major source of acquired infections. Three hundred and one swabs were taken from different sources and cultured on blood agar to study heamolysis ability of isolated bacteria. In this study, MacConkey agar was used to isolate Gram-negative bacteria and Sabouraud agar (SDA) to isolate fungi. The biofilm formation test was done by Congo red plate assay. 41 (13.6%) bacterial isolates were obtained and (18.27%) of fungi were isolated on Sabouraud agar (SDA). *Staphylococcus aureus* was the more frequent bacterial species that isolated in this study. 29% of samples showed hemolysin activity on blood agar and 32%of the isolates were biofilm- producer. Results revealed that (7.9%) of Gram-negative bacteria harbored the *fimH* gene, (9%) harbored the *icaA* were Gram-positive and 6.3 % of fungal samples had *HWP1* gene. Furthermore, (9.3%) from the total samples are bacterial samples harbored *hla* gene belong to *Staphylococcus* spp. Furthermore, (5.07%) of tested samples possessed *hlyA* gene were Gram-negative bacteria. We found in our study that infectious organisms can be transmitted from one individual to another by fomites responsible for acquired infection.

**KEYWORDS**: pathogen; disease transmission; fomites.

## الخلاصة

الامراض المعدية المتسببة بالادوات الملوثة في البيئة تهدد السلامة والصحة العامة، المصادر الناقلة لهذه الامراض المعدية غير معروفة. ولكن يعتقد انها تنتقل بواسطة مواد غير حية تسمى مواد معدية وهي المصار الرئيسية للاصابات المعدية. ثلاثمائة و واحد هو عدد العينات التي اخذت من مصادر مختلفة وتمت زراعتها على وسط آكار الدم لدراسة قابلية البكتريا المعزولة على تحليل الدم. وعلى وسط الماكونكي لعزل البكتريا السابة لصبغة جرام و وسط السبارود لعزل الفطريات. اختبار تكوين الاغشية الحيوية اجري بواسطة احمر الكونغو على وسط الاكار. ضهرت (13.6%) من العزلات البكتيرية و (18.27%) من العزلات ضهرت فطريات على وسط السبارود. *Staphylococcus aureus* كانت الاكثر تكرارا من بين بقية الانواع التي ضهرت اثناء العزل. 29% من اعلينات المعزولة اضهرت تحلل الدم على وسط اكار الدم لخمس انواع من البكتريا المعزولة و32% من العزلات انتجت اغشية حيوية. (7.9%) من العزلات تخفي *fimH* جين وهذه العزلات تعود الى البكتريا السالبة لصبغة جرام. (9%) منها تخفي جين *icaA* الذي يعود لبكتريا الموجبة لصبغة جرام. % 6.3 من العزلات لديها جين *HWP1* والتي تمثل العزلات الفطرية. (9.3%) من العزلات البكتريا كانت تعود لبكتريا *Staphylococcus* spp التي تخفي جين hla. اضافة الى ذلك (5.07%) من العينات كانت تملك جين *hlyA* والتي تعود لبكتريا السالبة لصبغة جرام. خلاصة ما تم التوصل اليه في هذا البحث هو ان الكائنات تلمعدية من الممكن ان تنتقل من شخص الى اخر بواسطة المواد المعدية المسؤولة عن الاصابات المكتسبة.

## INTRODUCTION

Trillions and trillions of microorganisms are found in the environment: the oceans, polar ice, in the depth of earth's crust, the human body, animals and plants. Few of them are harmful to humans (pathogenic) and the non-pathogenic types are widely spread in the environment [1, 2]. Infectious diseases can threat public health from infected tools in environment. One of the transmission sources of

the infectious diseases was formerly unknown, but it is apparently the handles of toilet doors because of the frequent use, therefore, they fill with microorganisms. Recently, the presence of pathogens has been studied on non-permeable surfaces like toilet surfaces, floor surfaces, door handles and kitchen surfaces. Recently, the major threat to public health is increasing antibiotic resistance microorganisms. Therefore, reducing infectious diseases is required by improving hygiene standards [3, 4].

Infectious organisms can be transmitted from one individual to another by non-living materials called fomites, which are the major source of acquired infections in hospitals as well as contribute to the transmission of infection among patients. General cleanliness, frequency of use and presence of moisture are several factors that affect the contamination rate of fomites, which in turn transmit the infection through toilet seats, knobs of conveniences, door handles, chairs, tables, sinks, lockers, and showers [5].

Microorganisms are especially found in public places, restrooms, hotels, hospitals, and restaurants. In general, large transition of normal flora and other microorganisms can be spread out of toilets and bathrooms by the users. Exposure to microorganisms, frequency of site contamination, host excretion grade of pathogen, pathogen virulence, personal hygiene and person's immuno-efficiency in contact determine disease transmission risk via fomites. Furthermore, Methicillin resistant *Staphylococcus aureus* (MRSA) can persist after hard hand washing after using toilets and bathrooms, which leads to outbreaks especially in high prevalence areas. Furthermore, fomites act as microbial dams via aerosolization and direct transfer from hand to fomite surface [5-7]. Thus, the aim of our study was to investigate microbes from the environment in Baghdad and genetic detection of virulence factors that are associated with these microbes.

# MATERIALS AND METHODS

### Samples collection

Three hundred and one swabs were taken from different sources in Baghdad City from March to June 2018. The swab samples were cultured on blood agar, MacConkey agar and Sabouraud agar (SDA), diagnosed microscopically by light microscope, biochemical tests and confirmed by VITEK 2.

### Biofilm formation

Biofilm formation test was by Congo red plate assay using Congo Red Agar (CRA) medium, which was made by dissolving sucrose (36 gms/L), Brain hart infusion (BHI) broth (30 gms/L) and agar -agar (18gms/L) in 900 ml D.W. 100 ml of Congo red dye (0.8 gms/L) was prepared and filtered. After autoclaving and cooling the agar to 55°C, dye added. The prepared medium was poured and used to detect biofilm producing bacteria. Single colony of each isolate was straked on agar plates and incubated at 37°C for 24hours. Appearance of black colonies indicated the positive results.

White or pink colonies indicated non-biofilm producing isolates.

### Detection of virulence genes

The PCR detection of *fimH*, *icaA*, *HWP1* and Hla genes were performed. Genomic DNA was isolated by boiling method to use it as DNA template to detect these genes. The primers sequence and product sizes are listed in Table 1.

**Table 1.** Displays the primers sequences and product sizes of this study.

| Genes | Sequence 5'→3' | Product size (bp) | Annealing temperature | Reference |
|---|---|---|---|---|
| *fimH* | F- TGCAGAACGGATAAGCCGTGG<br>R- GCAGTCACCTGC CCT CCG GTA | 508 | 63 °C | [8] |
| *icaA* | F-AATCTTTGTCGGTACACGATATTCTTCACG<br>R-CGTAATGAGATTTCAGTAGATAATACAACA | 108 | 57 °C | [9] |
| *HWP1* | F-GCTACCACTTCAGAATCATCATC<br>R-GCACCTTCAGTCGTAGAGACG | 941 | 58 °C | [10] |
| *Hla* | F-CTGATTACTATCCAAGAAATTCGATTG<br>R-CTTTCCAGCCTACTTTTTTATCAGT | 209 | 59 °C | [3] |
| *hlyA* | F-AACAAGGATAAGCACTGTTCTGGCT<br>R-ACCATATAAGCGGTCATTCCCGTCA | 1177 | 63 °C | [11] |

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# RESULTS AND DISCUSSIONS

## Isolation and Identification of microorganisms

The swabs obtained were cultured on blood and Macckoncy agar plates for bacteria and Sabouraud agar (SDA) for fungi for initial identification. 41(13.6%) samples showed a bacterial growth on MacConkey agar and blood agar after overnight incubation at 37°C, and final identification by biochemical tests and VITEK II system. 55 (18.27%) of samples showed a fungal growth on Sabouraud agar (SDA) at 25 °C after 7 days and identified by fungus morphology and microscopic examination. Table 2 displays the results of bacterial and fungal growth on MacConkey, blood agar and Sabouraud agar (SDA). Figure 1 demonstrates the types of microorganisms were identified in this study.

**Table 2.** Places where swabs obtained and numbers of bacterial and fungal species isolated.

| Specimen | Bacteria | Fungi |
|---|---|---|
| Door of W.C | 16 | 7 |
| Elevator | 20 | 7 |
| ATM machine | 4 | 7 |
| Mobile phone | 0 | 0 |
| Door of Fitting room | 5 | 3 |
| Shopping cart handle | 19 | 7 |
| Door of refrigerator | 42 | 41 |
| Door of bus | 18 | 7 |
| Restaurant table | 9 | 9 |
| Donation fund | 0 | 0 |
| Electric stairs | 3 | 4 |

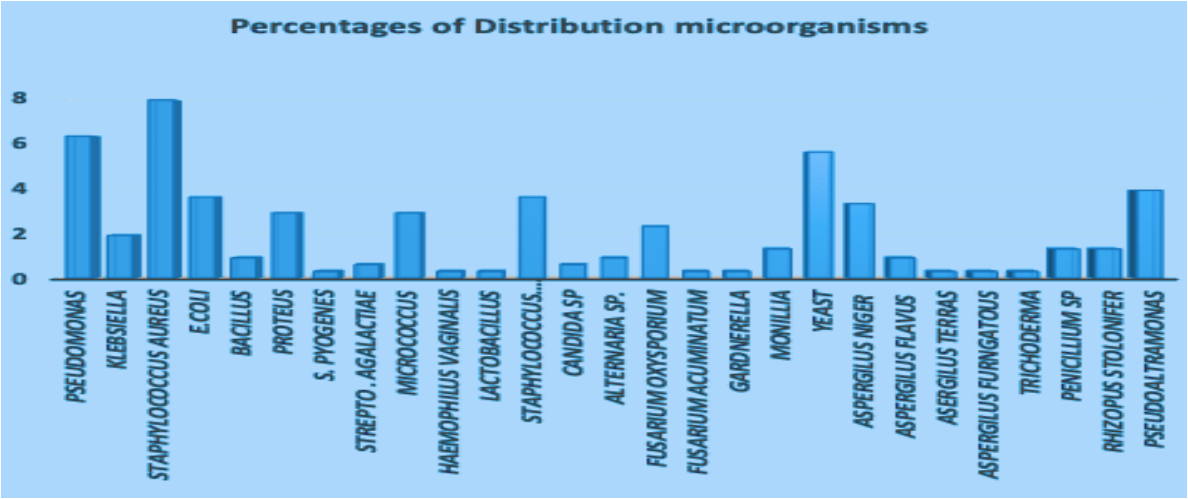


**Figure 1.** Percentages of bacterial and fungal species.

## Detection of hemolysin production and biofilm ability of the isolated bacteria

On blood agar plates, 29% of bacterial isolates showed hemolysin production for five types of bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus*). In addition, the ability to form biofilm was tested on Congo red agar plates. black-colored colonies were observed in (32%) of bacterial types and considered as biofilm producer, whereas negative colonies were appeared as pink as demonstrated in Figure 2.

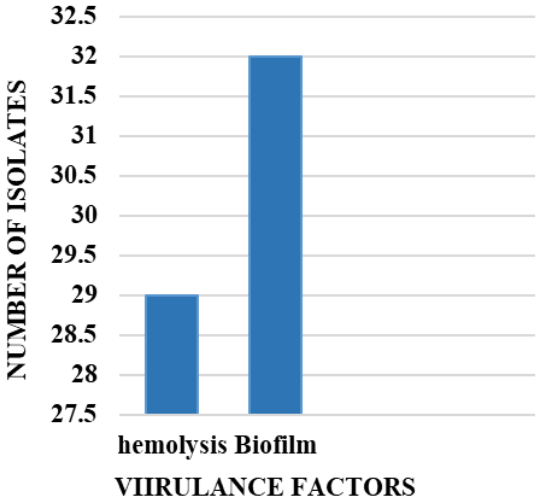


**Figure 2.** percentages of heamolysis and biofilm formation isolates.

**Molecular genes detection for biofilm formation and hemolysin**

The samples were investigated for the presence of some virulence of biofilm formation and hemolysin genes by PCR. Results revealed that (7.9%) of the samples harbored the *fimH* gene and these samples were all belong to Gram-negative bacteria, including E. coli and P. aeruginosa. (9%) of the isolates harbored *icaA* gene included Gram-positive S. aureus bacteria. The fungal samples were (6.3%) harboring the *HWP1* gene, which was of Candida species only. A percentage of (9.3%) from the total samples that harbored the Hla gene included S. aureus. Furthermore, (5.07%) of tested samples harbored the *hlyA* gene, where Gram-negative bacteria included E. coli as shown in Table 3.

**Table 3.** Displays the target primer, producing microorganism, their percentages, and the functional characteristics.

| Target gene | Type of microorganism | Percentage | Responsible characteristics |
|---|---|---|---|
| *fimH* | Gram negative bacteria (*E. coli* and *P. aeruginosa*) | 7.9 % | regulate the production of Exopolysaccharide (ESP) in biofilm |
| *icaA* | Gram positive bacteria (*S. aureus*) | 9 % | regulate the production of Exopolysaccharide (ESP) in biofilm |
| *HWP1* | *Candida* species | 6.3 % | Regulate the adhesion and biofilm |
| *Hla* | Gram positive bacteria (*S. aureus*) | 9.3 % | producing hemolysin |
| *hlyA* | Gram negative bacteria (*E. coli*) | 5.07 % | formation of toxin and cause damage in tissue and promote inflammatory cytokines |

# DISCUSSIONS

In the past, extensive study of miscellaneous microorganisms on human hands or surfaces focused on pathogens persistence [12]. In this study, various public places were investigated for bacterial and fungal contamination. According to results stated in table (1), about 16 bacterial and 7 fungal types isolated from toilet doors, that's belongs to the fact that toilets are human disposal, urine, faeces, vomit, or menstrual waste. Besides, toilets act as serious source of different infections. Toilet doors are contaminated by users especially with the poor personal hygiene for faecal and urinal contamination like, *E. coli*, *Proteus*, *Candida*, yeast or monilia, or by individuals suffer from infections by pathogens like, *Pseudomonas*, *Klebsiella*, *Bacillus*, *Micrococcus*, *Streptococcus*, *Aspergillus*, *Penicillium* or *Rhizopus*. Some of these microorganisms are normal flora on skin like *S. epidermidis* and *S. aureus*. All of these microorganisms can be transferred through touching bath points through entrance and exit leading to picking up pathogens to others [13, 14]. The other public investigated places like fitting room doors, elevators, restaurant tables donation food, electric stairs and doors of buses, some types of bacteria and fungi were detected. Human hands can be a source for the microorganism isolated, transference of pathogens by hands among people occurs by touching the same objects. Microbes survival time in the environment assist in spread of contamination [1]. Furthermore, the previous studies demonstrated that the dust plays an important role in transport microbes, so microbial communities increase with the presence of moisture and dust. For that, it is predictable that dirty surfaces have more microbes than clean surfaces [15]. ATM machine and shopping carts handles contamination belong to exposure of costumers to enteric and other microbes especially by using shopping carts for grocery for example, raw chicken and meat, fish, fresh vegetables and fruits and frozen food provide moisture and nutrition for microorganism's growth. The results showed the appearance of pathogens, which indicates the contact of unsanitary conditions and poor hygiene conditions of shopping carts with public circumstances, which play a good role in transmission of pathogens from one person to another leading to an increase the risk of infections [4, 16]. Contamination of refrigerator handles by bacteria was shown in 42 samples as mentioned in Table 1 and 41 samples by fungi, which belong to many factors which play an important role in refrigerator contamination, like persistence of microorganisms on the refrigerator handles, cleaning of refrigerator, dirty hands, unwashed raw food or through opening of refrigerator [11, 17]. By noticing in Figure 1, the most frequent bacterial

type isolated was *S. aureus*. This result is agreed with other studies because this bacterium is a normal flora of skin, throat, and nostrils, which is responsible for boils, wound infections, toxic shock syndrome, abscesses, and pimples [6, 14, 17]. The following bacteria after *S. aureus* is *P. aeruginosa*, which is most common in soil, multidrug resistant pathogen coming from air dust and/or soil dirt [4].

Referring to figure 2, 32% of isolates can form biofilm on the solid surfaces. Carbohydrate-binding proteins represented by (Lectins) represent a specific class that differs from enzymes and antibodies, which are contained in various organisms and are responsible for cell–cell interactions. Lectins are involved in the bacterial adhesion process [18, 19] by attaching the gram-negative microorganism to abiotic surfaces, the compositions of proteins in the outer membrane are altered, while fimbriae play a role in nonspecific adhesion [20]. Moreover, the gram-positive microorganisms contain teichoic acid which composes of ribitol with either α- or β-glycosidically linked N-acetyl glucosamines residues [21]. The Genes regulating biofilm are *icaA* and *fimA* in gram positive and gram-negative bacteria. These genes are common in bacteria and responsible to produce Exopolysaccharide (ESP) in biofilm. The EPS applies the intercellular cohesion of the bacteria and protect microorganisms from antibiotics treatment and host immune system. The *icaA* operon fundamentally participates in the production of capsular polysaccharides and is functionally important for cell-to-cell adhesion. The deletion of *icaA* gene leads to loss the capacity to produce polysaccharide intercellular adhesion (PIA) and compose biofilm *in vitro*. To comprehend the molecular mechanism of biofilm production, we explored the detection of *icaA* gene, because the *icaA* are considered as indispensable operators for adhesion. these genes are only important for the forming of the multilayer in gram positive bacteria in the production of biofilm and PIA. However, these genes actuality related to both biofilm formation and slime [9, 22, 23]. The capacity of bacteria to produce biofilm is considered one of the main virulence and immune defense mechanisms. Gram negative bacteria have developed abundant virulence factors that are responsible for serious life threatening infections [1]. several adhesions participate in the pathogenesis of infection by biofilm producer bacteria. Interestingly, all the G-ve isolates which appeared *fimH* could form strong biofilms, also this gene located in the same chromosome with different genes like *cnf1, afa/draBC, cnf2 and csgA* may be responsible of biofilm formation [8, 11].

The important adhesion in *Candida* sp. which expressed on the hyphal surface and germ tube in hyphal wall protein in biofilm formation and coding gene is *HWP1*. There are studies on the function of adherence proteins, like *HWP1* gene in Candida *sp*., but a study in 2016 in Iran proofed the role of *HWP1* gene in adhesion proteins in *Candida sp*. by analyzing sequences of nucleotides [24].

Among 301 isolated samples, 29% of them caused blood hemolysis on blood agar. that's belongs to the hemolysin, which is the most important bacterial virulence factor [22]. Furthermore, transmission of infectious organisms is the main cause of prevalence of antibiotic resistance [8, 23, 25]. The acquisition of virulence factors can occur through horizontal transfer mechanisms, is the consequence of a process of physical selection through the microbes existing in a host [26], for this reason, they succeed in adapting and surviving. The emergence of multidrug resistant virulent strains is a vital threat, which can be a potentially critical clinical issues for treating infections caused by multidrug bacteria [27].

The α-hemolysin is a cytotoxic and cytolytic toxin that participates in serious infections like sepsis, pneumonia, and skin. In this study, 9.3% of gram-positive isolates were carrying the hemolysin gene *hla*. This gene was showed to have a significant association with drug resistance and with other virulence factors and pathogenicity [28]. The *hla* expression is stimulated over the post exponential-early stationary phase of growth, and toxin production is regulatory dominance by various regulators, including the *Agr* and *SarA* [29]. These two regulators have an important role in the formation of biofilm and quorum sensing. Hemolysin indirectly assists in protection of bacteria from drugs [30, 31]. However, the precise effect of hemolysis remains to be discovered as our results *in vitro* but *in vivo* study is required. The *hly* gene is considered a key for increasing formation of toxins in gram negative causing tissue damage and promoting inflammatory cytokines, like induce IL-8 &IL-6. The mechanisms of the *hly* gene regulatory need more investigation, but the best suggestion is that the transcription of *hly*A

is regulated by *hly*U and *Fur* genes at the mid-logarithmic growth phase. However, many unknown regulators may repress their transcription [32].

## CONCLUSIONS

Infectious organisms can be transmitted from one individual to another by fomites, which are non-living materials responsible for acquired infection. Several factors affect the contamination rate of fomites, like general cleanliness, frequency of use and presence of moisture. Toilets act as a serious source of different infections since users contaminate their doors, especially with the poor personal hygiene for fecal and urinary contamination. The dust plays an important role in transporting microbes therefore microbial communities increase with the presence of moisture and dust. The most frequent bacterial type was found in this study *Staphylococcus aureus* that's belongs to normal flora of skin, throat, and nostrils.

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