Impact of Pipe Materials and Chlorination on Planktonic and Biofilm Cells of *Listeria monocytogenes*

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Abstract: This work aimed to determine the influence of different chlorine doses to inactivate of *Listeria monocytogenes* biofilm cells collected from six types of pipe materials, which are commonly used in household at different biofilm ages. A laboratory-scale simulated distribution system, that consists of a design based on six different pipe materials which are: polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE), iron (I), copper (Cu) and rubber (R). Six samples of *L. monocytogenes* biofilm were collected from the previous designed system at three different biofilm ages (10, 40 and 90 days-old). The collected biofilm samples were exposed to different chlorine doses (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/l) at 10 min, and then total bacterial counts using pour plate method was enumerated. The results showed that the log reduction of 90 days-old of *L. monocytogenes* biofilm formation, when exposed to 3.0 mg/l of chlorine dose, on six pipe materials; PVC, PP, PE, I, Cu and R was 3.96, 4.16, 4.21, 4.17, 4.32 and 4.03 CFU/cm², respectively with the initial count of biofilm cells was 10⁶ CFU/cm². While the log reductions of *L. monocytogenes* planktonic cells were: 6.20 CFU/ml (complete reduction) at the same dose of chlorine dose. The present study concluded that the biofilm cells are more resistant to chlorine dose than planktonic cells. Moreover, the biofilm of 10 days-old is more sensitive than 40 days-old followed by 90 days-old that grows in different pipe materials. In addition, chlorine effectiveness depends on its concentration, the exposure time, nature of pipe materials and the age of biofilm.

Keywords: Biofilm, pipe materials, drinking water, *Listeria monocytogenes*, chlorine.

INTRODUCTION

*Listeria monocytogenes* is a Gram-positive, facultative anaerobic, non-spore-forming and rod-shaped bacterium with an optimal growth temperature in a range of 30-37°C. *L. monocytogenes* is considered as a food-borne pathogen causing a listeriosis disease to human beings and animals by ingestion [1, 2]. It is widely distributed in different environments and it can be found in water, soil, animal fecal matter and sewage [3]. Also, it has been reported as being capable of attaching and developing biofilm on various surfaces, for example, stainless steel, polymers, rubber gaskets, plastic surfaces, polypropylene, and glass [4, 5].

Biofilm is a microbial consortia embedded in a self-production of exopolymer matrix composed mainly of exopolysaccharides (EPS). It is composed of microbial cells, enzymes, proteins, EPS and nucleic acids. The microbes living in these matrix benefit from nutrient and water supplies improved by lateral gene transference [6, 7] and protection against adverse environmental conditions such as a desiccation and chemicals, including detergents, disinfectants and antibiotics [8, 9]. Additionally, biofilm can function as a reservoir for pathogens and also as a source for disease outbreaks [10]. Additionally, it is a functional, three-dimensional consortia of microbial cells enveloped within extracellular polymers [11]. The microscopic examination of biofilm is made by structure using three dimensional (3-D) data such as Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) [12, 13].

The extracellular polymeric substances (EPS), which are excreted by microbes, are a complex gelatinous matrix of extracellular polymers containing exopolysaccharides, proteins, nucleic acids, lipids and humic substances. Most microorganisms in water distribution systems exist in biofilm on the inner surfaces of the pipelines [14]. The formation of biofilm is influenced by several factors such as concentration and quality of disinfectants, nutrients, water flow velocity, hydraulic conditions, temperature and pipe materials [15, 16]. Pipe materials can influence biofilm formation, especially in its early stages [17, 18]. In addition, Flemming *et al.* [19] estimated that only 5% of bacterial counts are present in the drinking water distribution network which can be detected in bulk water, which is commonly used for its quality control and the high percentage of bacterial densities (95%) adhere to the inner surface of pipe walls.

Many problems in drinking water pipes are due to the biofilm growth [20], and the persistence of pathogens [21]. Moreover, the biofilm growth can cause corrosion of the pipe, deterioration of water quality and organoleptic problems [22, 23]. The pipe material used in water distribution systems is another important factor that influences the proliferation of the distribution system of the biofilm. It has been
found that some pipes frequently experience some problems [24].

The most commonly used element in water disinfectant is chlorine, which is considered as a highly effective, highly soluble, more stable, easy to use, low cost and potent oxidizer. Moreover, it has been a residue to avoid regrowth of the microorganisms [25, 26].

*L. monocytogenes* biofilm was more resistant to antimicrobial agents and disinfectants, such as chlorine, ozone, hydrogen peroxide, and quaternary ammonium compounds [27-29]. Therefore, the main purpose of this research was to evaluate the effectiveness of different doses of chlorine to inactivate *L. monocytogenes* biofilm cells of six types of pipe materials, which are commonly used in the household at different biofilm ages.

**MATERIALS AND METHODS**

**Experimental Model Water Distribution System**

The present study was performed by using a laboratory-scale simulated distribution system. The designed system consisted of six different sets, of identical drinking water distribution pipes which are used up till now. The pipe materials were: polyvinyl chloride (PVC), Polypropylene (PP), polyethylene (PE), iron (Fe), copper (Cu) and rubber (R). The length of distribution pipe was one meter with an internal diameter of 3 cm. Tap water was pumped from the contact tank (30 liters of tap water) with a flow rate of 1.0 l/min into the simulated drinking water distribution pipe using a peristaltic pump. The culture of *Listeria monocytogenes* strain ATCC 25152 was used as inoculums, whereas the concentration of viable cells was (10^5 CFU/ml) of pumped water in the contact tank.

**Preparation of Bacterial Suspensions**

Strain of *L. monocytogenes* ATCC 25152 (planktonic cells) was cultured in a Brain-Heart Infusion Broth (OXOID, UK) and incubated at 37°C during 24h. After the incubation, the suspensions were harvested in the vortex. The initial counts of the inocula (6 log CFU/ml), which were used for biofilm formation, were determined by using the standard plate count method.

**Biofilm Sampling**

The biofilm samples that have been formed on the inner surface of the pipe material were collected after 10, 40 and 90 days. The biofilm samples were scraped with sterile cotton swabs, which were transferred to tubes containing 10 ml sterile water, and vortex agitator for 2 min. The detached biomass (biofilm suspensions) was diluted with a sterile saline solution for bacterial enumeration. The results were reported as the number of CFU/cm² [30].

**Quantification of Biofilm Bacterial Cells**

The enumeration of total cells for biofilm samples was performed by the pour plate technique and by using Plate Count Agar medium (OXOID, UK). The replicate plates for biofilm samples, taken from six different pipe materials and three different ages were analyzed for each dilution within the appropriate count between 30 and 300 CFU, which were selected for enumeration [31].

**Electron Microscopy (EM) Examination**

Six biofilm samples of *L. monocytogenes* were developed on six different pipe materials after 90 days. They were examined by transmission electron microscopy (TEM) model JEM 2100- HRTEM. As for the investigation of the total samples by the negative contrast method, biofilm cells suspensions were applied to a Formvar coated grid stabilized by carbon. The samples were stained with 1% water solution of the ammonium molybdate (Sigma, United States) for 30 Sec [32].

**Determination of Exopolysacharides (EPS) at 90 Days Old**

The extraction of EPS from biofilm samples was carried out by using cation exchange resin method according to Michalowski et al. [33]. The polysaccharide content of crude EPS was determined by using phenol-sulfuric acid method and by the following the protocol described by Dubois et al. [34].

**Effect of Different Chlorine Concentration on Planktonic Cells and Different Ages of *L. monocytogenes* Biofilm**

Six biofilm samples, which were removed from different pipe materials (PVC, PP, PE, I, Cu and R) in different ages (10, 40 and 90 days old), were exposed to eight different chlorine doses (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/l) for 10 min. The biofilm cells and planktonic cells were subcultured into 100 ml Tryptic Soya broth (OXOID, UK) and incubated at 37°C for 24 h and then centrifuged at 3000 rpm for 20 min. The pellets were washed three times using sterilized distilled water and then the pellets were suspended in 10 ml sterile distilled water.

**Determination of Residual Chlorine**

Nine flasks (250 ml conical flask with quick fit stopper) containing 100 mL sterilized distilled water were inoculated with 0.5 ml of bacterial biofilm suspension, which was formed on six pipe materials at different ages and then it was inoculated by different chlorine doses and uninoculated chlorine water flask was used as a control. The determination of residual chlorine was carried out by DPD method according to [31].

**Statistical Analysis**

The relationship between chlorine doses, residual chlorine and log counts of *L. monocytogenes* biofilm cells was carried out by using regression coefficient analysis (R²). All the data was transformed in decimal logarithms and it was processed by SPSS version 14.0, by the computer application.

**RESULTS**

The results of this study explained the characteristics of biofilm formation by *L. monocytogenes*. In addition, the relationship between the effect of chlorine doses and its residual on log reduction of *L. monocytogenes* on different pipe materials during different ages were also explained.
Characteristics of Biofilm

The kinetics of the biofilm formation of *L. monocytogenes* and the biofilm on different pipe materials were explained by using the transmission electron photomicrograph (Fig. 1). The photomicrograph indicated that the cells were embedded in a polymer matrix and exopolysaccharides. Also, it was produced from the biofilm cells in different shapes. From Fig. (1), it was found that the pipe materials were affected by the quantity and shape of the exopolysaccharides produced from biofilm cells.

When comparing the amount of exopolysaccharides that was produced by the cells of biofilm formation on different types of pipes in both TEM and determination of EPS results, it is clear that, *L. monocytogenes* biofilm grown on copper pipe (203.7 μg/cm²) produced lower amount of exopolysaccharides than the others types of pipes Fig. (1-E). Also, the results of exopolysaccharides amount of PVC, PP, PE, iron, Cu, Rubber at 90 day-old was 384.2, 390.6, 375.4, 411.5, 203.7 and 289.3 μg/cm², respectively.

![Fig. (1). Transmission electron photomicrograph of biofilm formation of *L. monocytogenes* on different pipe materials (A) PVC, (B) PP, (C) PE, (D) I, (E) Cu and (F) R.](image)

![Fig. (2). The relationship between the chlorine doses, residual chlorine and log reduction of *L. monocytogenes* planktonic cells.](image)
Effect of Different Chlorine Doses and its Residual on *L. monocytogenes* Planktonic Cells

In this study *L. monocytogenes* ATCC 25152 was used as a reference strain (planktonic cells). The initial count of planktonic cells of *L. monocytogenes* (1.6x10^6 CFU/ml) was exposed to eight chlorine doses (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/l). The obtained results showed that the most effective chlorine dose was 3.0 mg/l, which led to complete inactivation (100% removal) (Fig. 2).

Biofilm Cells Formed on Different Pipe Materials in Different Ages

The six samples of *L. monocytogenes* biofilm cells were scraped from six different pipeline materials (PVC, PP, PE, I, Cu and R) in three different ages (10, 40, and 90 days-old) and were exposed to the same eight chlorine doses (Figs. 3-8).

As for the *L. monocytogenes* biofilm cells were collected from PVC pipeline in different ages (10, 40, and 90 days-old), it is clear that, the initial log counts of 10 days-old biofilm cells were 6.6 CFU/cm². In this age, 3.0 mg/l of chlorine dose was able to complete the removal of cells (Fig. 3A), while the initial logs count of 40 and 90 days-old of biofilm cells were 6.6 and 6.3 CFU/cm², respectively. The log reduction was reached to 4.7 and 3.9 CFU/cm², respectively at the obtained ages (Figs. 3B, C).

In the case of PP pipeline, the initial count of biofilm formation of *L. monocytogenes* at 10 days-old was 6.5 CFU/cm² (Fig. 4A). Results demonstrated that, 3 mg/l of chlorine dose was able to do a complete removal of the initial log counts. While 40 and 90 days-old biofilm cells (the initial count 6.6 and 6.5 CFU/cm²) the log reduction was 4.71 and 4.16 CFU/cm², respectively (Fig. 4B, C).

By regarding the biofilm cells of *L. monocytogenes* growing on the PE pipe, the initial log counts of different ages were, respectively, of 6.5, 6.3 and 6.7 CFU/cm² in 10, 40 and 90 days-old. The results indicated that, 3 mg/l of chlorine dose was able to reduce the biofilm cells (4.21 and 5.50 log) for 90 and 40 days-old (Fig. 5B, C) Whereas, the biofilm cells of 10 days-old *L. monocytogenes* were completely removed (6.5 log CFU/cm²) (Fig. 5A).

As shown in Fig. (6), it can be explained that the biofilm formation of *L. monocytogenes* on the iron pipes revealed more resistant to different chlorine doses in all ages. On the other hand, the high level from chlorine dose (3 mg/l) was not able to cause a complete inhibition of biofilm cells, not only for 90 days-old biofilm (older biofilm), but also for 10 days-old biofilm (younger biofilm). In addition, the initial log count of the obtained ages was, respectively, 6.5, 6.7 and 6.8 CFU/cm² at 10, 40 and 90 days-old.

In case of biofilm formation of *L. monocytogenes* on Cu pipe materials, the results revealed that 10 days-old biofilm cells were completely removed (6.3 log reduction) at 2.2 mg/l of chlorine doses. While 40 and 90 days-old of biofilm cells were completely inactivated at 3 mg/l. Whereas, the initial log count was 6.3, 6.5 and 6.1 CFU/cm² in different ages, respectively (Fig. 7).
Fig. (4). The relationship between the chlorine doses, residual chlorine and log reduction of *L. monocytogenes* biofilm cells on PP pipes (A) 10 days-old, (B) 40 days-old and (C) 90 days-old.

Fig. (5). The relationship between the chlorine doses, residual chlorine and the log reduction of *L. monocytogenes* biofilm cells on PE pipes (A) 10 days-old, (B) 40 days-old and (C) 90 days-old.
Fig. (6). The relationship between the chlorine doses, residual chlorine and the log reduction of *L. monocytogenes* biofilm cells on I pipes (A) 10 days-old, (B) 40 days-old and (C) 90 days-old.

RC= Residual Chlorine  
LC= Log Count Reduction

Fig. (7). The relationship between the chlorine doses, residual chlorine and log reduction of *L. monocytogenes* biofilm cells on Cu pipes (A) 10 days-old, (B) 40 days-old and (C) 90 days-old.

RC= Residual Chlorine  
LC= Log Count Reduction
Additionally, when the biofilm cells of *L. monocytogenes* were grown on the inner surface of R materials, the initial log count of three different ages was 6.4, 6.6 and 6.6 CFU/cm², respectively. The results of biofilm cells, which scraped after 10 days, were completely reduced, when exposed to 3 mg/l of chlorine dose. On the other hand, the log reduction after 90 and 40 days-old of biofilm cells were, 4.0, and 4.6 CFU/cm² (Fig. 8).

Finally, from the obtained results (Figs. 2-8), it is clear that the most effective chlorine dose was 3.0 mg/l. Also, the residual free chlorine dose ranged between 0.55 - 1.5 mg/l.

**Statistical Analysis**

The statistical analysis using regression coefficient (R²) expresses the relationship between residual chlorine and log reduction count of biofilm cells in different ages (Table 1).

**DISCUSSION**

*L. monocytogenes* is widely spread throughout the land and water environments. It is often isolated from samples of different sources such as soil, feces, water, decaying plant material, vegetables, and silage [35]. A high proportion of bacteria *L. monocytogenes* was observed in the treated household and industrial sewage. The contaminated sewage plays an essential role in the transmission of *Listeria* in the water environment. It leads to their presence in rivers, lakes, sea and groundwater. The transmissions of *L. monocytogenes* through water can cause a dangerous disease, called listeriosis, for human beings and animals [36]. The literature data determined the frequency of *L. monocytogenes* in water samples reaches up to 62% [37]. It is also notable that these bacteria, owing to a high resistance to unfavorable external conditions, are able to survive in the environment for a long time [38]. *L. monocytogenes* was able to produce biofilm on the surfaces and it was rapidly adaptable to changing environmental conditions [39]. Due to this fact, the presence of *L. monocytogenes* bacilli in water ecosystems may be a cause of the sporadic or epidemic listeriosis incidence, which poses a serious hazard for the health of people and animal [40].

In this study by using TEM, it is clear that the biofilm cells, which grow in different pipe materials, are embedded in a polymer matrix and in exopolysaccharides. Moreover, exopolysaccharides were produced from the biofilm cells in different shapes. In addition, *L. monocytogenes* biofilm grows on copper pipe produced lower amounts of exopolysaccharides than the others. Therefore, EPS plays a vital role in the build-up of biofilm [41, 42]. In comparison to many other biofilm studies, they showed cells surrounded in a heavy, slimy polymeric matrix layers [43, 44].

There are many factors that affect the formation of biofilm such as the type, the doses of disinfectant, the nature and concentration of organic and inorganic compounds in the water, type of pipe composition and water temperature. Momba et al. [45] demonstrated that, a variety of disinfection processes such as ozonation, chlorination, monochloramination, hydrogen peroxide, and UV irradiation are used to prevent the formation of biofilm on the surface of pipeline composition in water distribution networks. The efficiency

![Fig. (8).](image-url)
organic carbon levels and consistently maintained chlorine control and biological treatment of water to reduce assimilable based pipes decreases the efficiency of residual chlorine in as to ensure microbiologically potable water [25].

...can be destroyed or irreversibly inactivate by chlorination, so microbes, which have passed through the treatment stage, development of biofilm in the water distribution system. The considered as the most common approach to prevent the de...

...the development of biofilm production by-products to health are very small, in comparison tant residual. The WHO mentioned that the risks from disinfection 

...LeChevallier for the eradication of bacterial biofilm [50, 51]. Additionally, iron pipes corrosion that influences the efficiency of chlorine...of these biocides in the prevention of biofilm production varies because of their different abilities to penetrate the biofilm. Also, they reported that the pipe material plays a very crucial role in the maintenance of a high water quality because it can supply the bacteria in the water with nutrients through the leaching of organic compounds.

The usage of chlorine as a disinfectant was to prevent and control the biofilm formation. It was indicated that, the residual chlorine, after the disinfection of L. monocytogenes biofilm by 3.0 mg/l chlorine dose, ranged between: 0.55-1.5 mg/l with a contact time of 10 min although the EPA demonstrated that the maximum residual chlorine level was 4.0 mg/l [46]. On the other hand, the effectiveness of disinfectant residual depends on the concentration, the contact time and the presence of microorganisms [47].

...There are various investigations showing that, the adequate disinfectant residuals can control the biofilm accumulation [48]. Momba [49] found a large increase of biofilm microorganisms on test coupons in the absence of a disinfectant residual. The WHO mentioned that the risks from disinfection by-products to health are very small, in comparison with insufficient disinfection. Therefore, the development of safe and effective alternative disinfection methods is highly desirable. The chemical disinfection, especially chlorine, is considered as the most common approach to prevent the development of biofilm in the water distribution system. The microbes, which have passed through the treatment stage, can be destroyed or irreversibly inactivate by chlorination, so as to ensure microbiologically potable water [25].

...The results of this study showed that the most effective chlorine dose was 3.0 mg/l, which led to a complete inactivation of planktonic cells and biofilm cells, which were collected from different pipe materials in 10 days-old, except in the case of used iron pipeline. Also, the biofilm formations of L. monocytogenes on iron pipes in all ages were more resistant to different chlorine doses. This may be due to the iron pipes corrosion that influences the efficiency of chlorine for the eradication of bacterial biofilm [50, 51]. Additionally, LeChevallier et al. [52] found that the corrosion of metal based pipes decreases the efficiency of residual chlorine in the plumbing system. In spite of an adequate corrosion control and biological treatment of water to reduce assimilable organic carbon levels and consistently maintained chlorine residuals; the development of biofilm on the inner surface of iron pipe rapidly occurred and more diverse microorganisms occurred than on plastic polyvinyl chloride (PVC) pipes [50, 53].

...In this study, in the case of biofilm formation of L. monocytogenes on copper pipes, all biofilm ages (10, 40 and 90 days-old) were completely inactivated by using 3.0 mg/l of chlorine dose. This may be due to the release of copper residuals that have antibacterial properties expressed by the damage of cell membranes and nucleic acid structure [54]. So, the copper plumbing material is widely used. This may be due to the easy fittings with different pipe varieties [55, 56]. Metal-based materials can form the corrosion products on pipe surfaces and release metals into water as a result of chemical or biological reactions [57, 58]. Moreover, Lehtola et al. [59] found that, the biofilm formation in copper pipes was slower than in polyethylene (PE) pipes, and that copper ions led to the decrease of the count of microorganisms in water while plastic pipes such as PE, which have been used recently, are more economic pipes than traditional metal plumbing materials. But, the most disadvantage of PE is the release of biodegradable organic compounds and phosphorus, which can enhance microbial regrowth and biofilm accumulation.

...When comparing the ages of different pipe materials, the results showed that, after 40 days-old, it can be more resistant to the dose of chlorine. It could be revealed that, the presence of EPS, which coated biofilm cells, protected the cells from any disinfectant or antimicrobial agents. The EPS matrix is prevented by the penetration of antimicrobial agents from reaching the microorganisms within the biofilm by the diffusion limitation and/or the chemical interaction with the extracellular proteins and polysaccharides [6, 60, 61] whereas the biofilm EPS plays a vital role to protect the biofilm cells towards antimicrobial agents [62]. Biofilm cells are more resistant than planktonic populations as they include chlorine and antibiotics [63, 64]. Moreover, chlorine is proved to be less effective on older L. monocytogenes biofilm [65].

**CONCLUSION**

It could be concluded that, L. monocytogenes biofilm was more resistant to chlorine when compared to planktonic
cells. Older biofilm is more difficult to remove than younger biofilm and planktonic cells. Chlorine is less effective on older *L. monocytogenes* biofilm. As for the residual chlorine, 1.0 mg/l was found in older *L. monocytogenes* biofilm occurrence on all tested plumbing materials, except the copper pipe, that was found 1.1 mg/l.

**CONFLICT OF INTEREST**

The authors confirm that this article content has no conflict of interest.

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**REFERENCES**


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