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Original Article *Food Hygiene*

Evaluation of aqueous Ozone as a method to combat multidrug-resistant *Staphylococcus aureus* tainting cattle meat sold in Wasit marketplaces

Manal Hadi Ghaffoori Kanaan [1*], Sura Saad Abdullah[2]

[1]Agriculture Department/ Technical Institute of Suwaria, Middle Technical University, Baghdad, Iraq
[2]Al- Suwaria General Hospital / Wassit Health Office, Wassit, Iraq.

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Corresponding author: Manal Hadi
Iraq/manalhadi73@yahoo.com

ABSTRACT

Objective: To evaluate the effectiveness of ozonation treatment (1/2 ppm for up to 30 min at 3-7 centigrade) on the persistence of multidrug-resistance Staphylococcus aureus (MDR S. aureus) tainting cattle meat.
Design: Descriptive study
***Procedures*:** Nine testers of cattle's meat including imported (n = 3) and locally slaughtered (n = 6), which were stored at -18 centigrade and accepted positive for MDR S. aureus were subjected to aqueous ozone (O_3) as 1/2 ppm for 15 and 30 min.
Results: The results presented that after ozonation treatment (1/2 ppm at 3-7 centigrade), the whole testers which free from MDR S. aureus were 22.2% & 55.6% for 15 & 30 min, respectively. Additionally, the antimicrobial effectiveness of aqueous O_3 as 1/2 ppm on the decline of MDR S. aureus level (log_{10}CFU/ml) was measured against three contaminated testers of cattle's meat. The outcomes publicized that afterward handlings, the total decline of bacterial counts was 2-3 log_{10} (CFU/ml) after 30 min at 3-7 centigrade, this decline is extremely noteworthy from the opinion of public health. The effectiveness of aqueous O_3 (0.5 ppm/3-7 centigrade) with carcass drip to MDR S. aureus was assessed and the results displayed that 77.8% and 100% of the testers of aqueous O_3 were negative after ozonation treatments for 15 and 30 min, respectively.
Conclusion and clinical relevance: O_3 as 1/2 ppm is exceedingly active in dropping the amount of MDR S. aureus contaminated testers and this decline augmented as prolonged experience time to ozonation treatment. On the other hand, O_3 was highly effective in eradicating MDR S. aureus even in the presence of high levels of organic materials. These outcomes designated O_3 as a substitute promising approach to decline meat contagion with foodborne microorganisms for instance MDR *S. aureus*.
Keywords: Antimicrobial resistance, aqueous ozone, cattle's meat, Staphylococcus aureus, Wasit province.

1. INTRODUCTION

Meat can be tainted with a wide variety of pathogenic and spoilage microorganisms during the periods of slaughter, processing, and storage. In addition, it was considered one of the most important sources of foodborne pathogens [1]. Among these pathogens, S. aureus has mentioned as a celebrated opportunistic foodborne bacterium, which considered as a hazard due to its harmful effects on animal health and its ability to transmit from animals to humans and vice versa [2]. It is among the most prevalent causes of clinical infections globally and has garnered substantial public attention due to increasing mortality associated with MDR [3]. Multidrug-resistant S. aureus has been established in different meat-generating animals internationally [4]. The occurrence of livestock-associated MRSA in farmhouse animals is cumulative that lead to increase likelihood contamination of the food products [5]. Numerous researchers have perceived the universality of the isolation of MDR Staphylococci from meat testers [6-9]. This must be taken seriously, especially when the discovery of Waters et al. [3] documented that about 50% of grocery supplies with meat testers experienced in the United States are tainted by MDR S. aureus for not less than three classes of antimicrobials.

To eliminate bacterial contaminants in meat numerous sanitizing interventions as well as treatments were developed via food manufacturing including washing by acid and salts [10], the use of chlorine dioxide [11], irradiation with gamma-rays [12], ultraviolet radiation and O_3 [13]. The use of chlorine is becoming more scrutinized because of toxicity issues and disinfection by-products that have proven harmful from the point of view of food and environmental safety [14]. Food researchers are seeking to discover a substitute agents used for cleanliness and sanitation characterized by their effectiveness to foodborne bacteria, inoffensive to human being and environment [15]. Recently, O_3 has been attracted the attention of food scientists as an alternative sanitizer [16], as of its influential disinfecting

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effectiveness, this gas can be practical use in the food manufacturing to eradicate bacteria plus to incapacitate viruses, fungi as well as their toxins [17]. On the other hand, there is no any detrimental deposits in/on products following ozonation, as it rapidly rots to O_2 [18]. Ozone usage has augmented due to its description as Generally Recognized as Safe (GRAS) by the Ministry of Food and Drug Safety (MFDS) in 1997 [19]. Ozone has been documented to eradicate foodborne microorganisms for instance Salmonella, Listeria monocytogenes and S. aureus [13]. Consequently, O_3 is measured to be greatest suitable method aimed at removing microbes of food protection concern [20].

In our country, cattle's meat is measured as the greatest widespread meat item in numerous populations and if we consider that when cooking meat, it is extremely important to disinfect the produce completely in order to eradicate foodborne pathogens such as S. aureus, so this study was conducted to assess the effects of O_3 treatment on the microbiological security of such products that have been retailed in the marketplaces of Wasit governorate.

2. MATERIALS AND METHODS

2.1. Ethical Approval

Meat samples were obtained from the markets, so there is no need for such approval.

2.2. Treating of samples

A total of nine samples including imported (n = 3) and locally slaughtered cattle's meat (n = 6), which were stored at -18 centigrade and accepted positive for MDR S. aureus using ordinary microbiological and biochemical exams and confirmed via Rapid ™ Staph Plus system (Remel, R8311009), latex agglutination kit Dry SPOT Staphytect Plus (Oxoid, DR0100M) and rapid latex agglutination assessment PBP2a kit (Oxoid, DR0900A) as pronounced earlier [21]. These samples distributed into two portions, the first one exposed to aqueous O_3 for 15 and 30 min using Aqua-6 O_3 originator (600 mg /h), while the second parts were stored at -18 centigrade for additional scrutiny.

2.3. Calculation of O3 concentration output

The concentration of O_3 generated by the Aqua-6 originator in water as ppm was done by CHE-Mets®-Kit as a way applied by [22]. In brief, A plastic tub was filled with water and covered with its lid, then aeration stone was implanted into the tub via a hole in the lid. Four experience times were taken (5,10,15 and 30 min). Next every experience, the water was altered, and the procedure was recurrent. To determine O_3 concentration in water five drops of A-7400 Activator were added into the empty sample cup then filled to the 25 ml mark with the aqueous O_3, then the CHE Met ampoule tip was placed into the cup and, the tip of the ampoule was broke. The ampoule was filled by the aqueous O_3, then upturned numerous times to mix comprehensively and dried by left for 1 min for color development. The ampoule was positioned among the color values till the top color was corresponding using High Range comparator [22]. The peak concentration among the experiences used was obtained at 15 and 30 min, which was 1/2 ppm in water (Figure 1).

2.4. The effect of aqueous O3 on MDR S. aureus

The first part of every sample was subjected to ozonation. In this experiment O_3 gas was inserted into the water using aeration stone (Diffuser) and disseminated it consistently all over the water. The Aqua-6 originator was fed with 1 L /min (600 mg/hr) of beaten air as a feed gas. The testers were defrosted at 4 centigrade for 18 h, then dipped into aqueous O_3 at 3-7 centigrade to allow dispersion of O_3 within the samples for two different experience times (15 and 30 min) in order to evaluate the effect of ozonation on MDR S. aureus (Figure 2).

A slice of (25 gm) of every treated tester was sliced and processed in a stomacher with 225 ml of buffered peptone water for 2 min, then 10 ml of the homogenate was mixed with 100 ml of Tryptone soy broth-yeast extract (TSB-YE), 10% NaCl and 1% sodium pyruvate. After incubation at 35 centigrade for 18 h, 20 µl of the culture was plated on Baird-Parker agar (Oxoid, CM1127) supplemented with egg yolk tellurite and incubated overnight at 37 centigrade as described previously [21].

Of the nine second parts three samples (local cattle1st, local cattle 2nd and imported cattle1st) were taken to assess the effectiveness of aqueous O_3 on the decline of MDR S. aureus count (log_{10}/ml) after conducts for 15 and 30 min at 3-7 centigrade. First, these samples were processed as described previously to detect the initial bacterial count, then subjected to ozonation for two experience times and possessed after each experience. Miles and Mizra technique [23], was adopted to detect the influence of ozonation on MDR S. aureus through estimated the sum of colony forming units (CFU) in a bacterial broth in which a series of decimal ten dilution of enrichment broths were diluted with sterile BPW tubes (1ml broth / 9ml BPW), dilutions were made to at least 10^{-8} , then 5 × 20 µl of each dilution were dropped onto surface of the Baird-Parker agar and allowed to spread and dry naturally before inversion and incubation at 37 centigrade for 24 h. The colonies were counted in the drops where the highest number of full-size discrete colonies were seen (usually drops containing between 10-20 colonies were counted). The microbial load log_{10} titers were adjusted by the low of Miles and Mizra [23].

CFU per ml = Average number of colonies for a dilution × 50 × dilution factor [23].

2.5. Detection the effectiveness of aqueous O3 with meat drip against MDR S. aureus

One ml of aqueous O_3 for all samples was injected to 5 ml of (double strength) Trypton Soya Broth (Oxoid, CM0129) with 0.6% Yeast Extract (TSB-YE) and incubated at 35 centigrade for 18 h, then 20 µL of the culture was plated

onto Baird-Parker agar (Oxoid, CM1127) improved with egg yolk tellurite then incubated overnight at 37 centigrade.

2.6. Statistics

Analysis of data were implemented by MedCalc Software bvba version 18 (BE,USA). Two samples Chi-square (χ2) between proportions was implemented to evaluate significance between proportions with a 5% significant level https://www.medcalc.org/.

3. RESULTS

In this study the effectiveness of aqueous O_3 against MDR S. aureus contaminating cattle's meat was inspected and the obtainable results are set in Table 1. The results showed that after treatment with aqueous O_3 (1/2 ppm/15 min), two testers (22.2%) were free (no growth on agar surface) besides seven testers (77.8%) were positive (more than 100 colonies /plate). While, when experience time was delayed to 30 min, five samples (55.6%) were negative, and four samples (44.4%) were positive (less than 40 colonies / plate). Statistically there is no significant effect ($p > 0.05$) for the experience times (15 and 30 min) on the effectiveness of aqueous O_3 at the same concentration (1/2 ppm) against MDR S. aureus tainting cattle's meat ($\chi^2 = 1.995$, $p = 0.158$).

Table 1. Effect of aqueous ozone (0.5 ppm) for 15 and 30 min on MDR *S. aureus* tainting cattle's meat.

Source cattle's meat	Total No.+ve for MDR	Total No.-ve for MDR *S. aureus* after ozone treatment (%)	
		15 minutes	30 minutes
Local	6	1 (16.7%)	3 (50%)
Imported	3	1 (33.3%)	2 (66.7%)
Total	9	2	5
Effectiveness (-ve)		2/9 (22.2%)	5/9 (55.6%)

Table 2. Antibacterial effectiveness of aqueous ozone (0.5 ppm) for 15 and 30 min on the reduction of MDR S. aureus count (log10 / ml) for three positive samples.

Sample's code	Log_{10}/ml count before ozone treatment	Log_{10} / ml count after ozone treatment				
		15 min.	Log_{10} Decre-ased	30 min.	Log_{10} Decre-ased	Total log_{10} Decre-ased
Local cattle 1st	1.2×10^5	1.1×10^3	2	1.1×10^2	1	3
Local cattle 2nd	1.4×10^6	1.4×10^5	1	1.3×10^4	1	2
Imported cattle 1st	1.7×10^4	1.7×10^3	1	1.6×10^2	1	2

Table 3. The effectiveness of aqueous ozone (0.5 ppm) contained meat drip against MDR *S. aureus* after treatments of cattle's meat for 15 and 30 min.

Source cattle's meat	No. of samples tested	Effectiveness of aqueous ozone (0.5 ppm)	
		15 min.	30 min.
Local	6	5 (83.3%)	6 (50%)
Imported	3	2 (66.7%)	3 (66.7%)
Total	9	7	9
		7/9 (77.8%)	9/9 (100%)

The effectiveness of aqueous O_3 (1/2 ppm) on the decline of MDR S. aureus count (log_{10}/ml) was experienced using two experience times (15 and 30 min) and the acquired data is given in Table 2. Our outcomes indicated that the bacterial counts before treatments were (1.2×10^5) , (1.4×10^6) and (1.7×10^4) for local cattle1st , local cattle 2nd and imported cattle1st, respectively. Whereas after ozonation for 15 min these counts were declined to reach (1.1×10^3) , (1.4×10^5) and (1.7×10^3) for local cattle1st , local cattle 2nd and imported cattle1st, respectively. And this decline was further increased when experience time was prolonged to 30 min to reach (1.1×10^2), (1.3×10^4) & (1.6×10^2) for local cattle1st , local cattle 2nd and imported cattle1st , respectively.

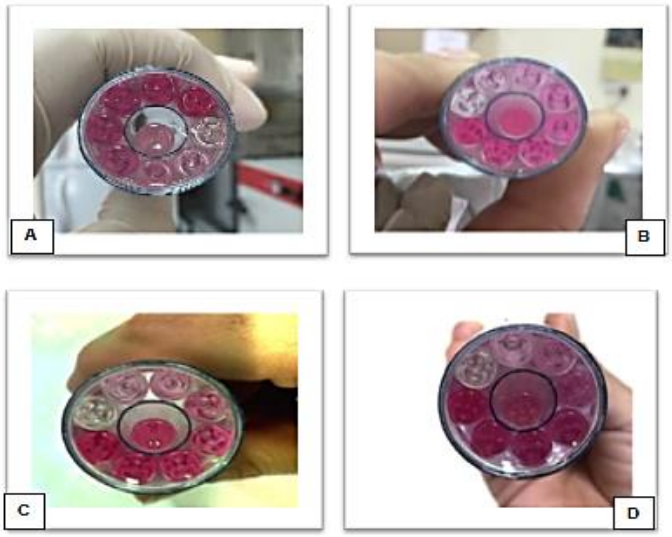
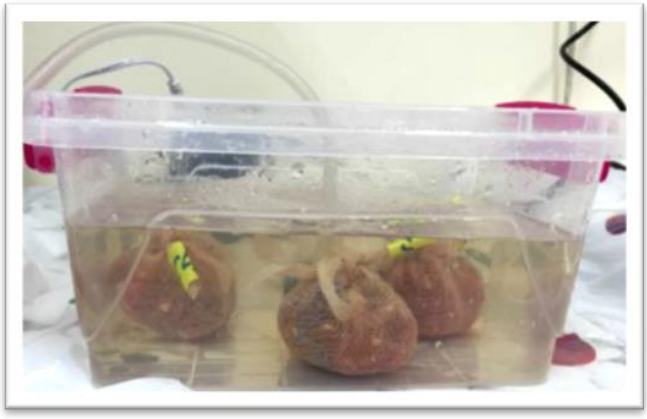


Figure 1. A, B, C, and D: ozone concentration output (ppm/in water) of the ozone originator at diverse experience times: (A) : for 5 min; (B) : for 10 min ; (C) : for 15 min, and (D) : for 30 min.



The effectiveness of aqueous O_3 (0.5 ppm) contained meat drip against MDR S. aureus was evaluated and the results displayed that 77.8% and 100% of the testers of aqueous O_3 were free (no growth on agar surface) after treatments of cattle's meat for 15 and 30 min, respectively (Table 3). Statistically there is no significant effect ($p > 0.05$) for the experience times (15 and 30 min) on the effectiveness of aqueous O_3 containing organic materials at the same concentration (1/2 ppm) against MDR S. aureus ($\chi^2 = 2.123$, $p = 0.145$).

4. DISCUSSION

Ozone is the perfect solution for beef processing industry, it has been used for plant cleaning, disinfecting, and sanitizing due to its extraordinary oxidation possessions that conveys microbicides properties [24]. The outcomes (Table 1) showed that 22.2% and 55.6% of samples were negative after treatment with aqueous O_3 for 15 and 30 min, respectively. Moreover, the results (Table 2) also revealed that a total reduction as 2-3 $\log_{10}$/ml in the levels of MDR S. aureus was achieved through ozonation treatment of meat for 30 min.

Eradication of microorganisms via O_3 can be linked to its highly un stability which lead to its rapidly decompose into free radicals, so the effectiveness of O_3 is attributed to the oxidizing influence of these free radicals, its capability to diffuse over biotic membranes, then destroying pathogens by attacking and oxidizing the cell walls of the organism and continue to break molecular chain down until it's nothing but carbon dioxide and water [25, 26].

The outcomes of this experiment directed that the sum of surviving microbial cells symbolized by CFU from testers after ozonation treatment were less than from untreated samples. As well as the number decreased with increasing the experience time at the same concentration (1/2 ppm), reasonable clarification possibly due to increasing the experience time lead to longer the contact time of ozone with microorganisms, leading to lower inactivation rate. Besides, O_3 treatments were done at 3-7 centigrade, and in general the antimicrobial activity of O_3 decreased with increasing temperatures. So, when a temperature of aqueous medium decreases this will lead to increasing O_3 solubility and stability, enhancing its availability in the medium and consequently efficacy rises [26].

The effectiveness of O_3 to advance the microbial quality of meat had formerly been well-known by Cárdenas et al. [17] who found that gaseous O_3 as 154×10^{-6} kg m^{-3} at 0-4 centigrade for 3 h reduced total aerobic mesophilic heterotrophic microorganisms and Escherichia coli on beef testers by 0.5 $\log_{10}$ and 0.6 - 1.0 $\log_{10}$ cycles, respectively. Reagan et al. [27] established that aqueous O_3 as 0.3 to 2.3 ppm reduced aerobic plate counts by 1.30 $\log_{10}$ CFU/ cm^2 and concluded that ozonation treatment can be used as suitable intervention for removing physical and bacterial contamination from beef carcasses. Another study conducted by Novak and Yuan [28], to determine the effect of aqueous O_3 treatment as 3 ppm for 5 min on the survival of three-strain cocktails of Clostridium perfringens, Escherichia coli O157:H7 (E. coli O157:H7), and Listeria monocytogenes on beef surfaces, they found that reductions following treatment were 1.28, 0.85, and 1.09 $\log_{10}$ CFU/g, respectively. Castillo et al. [29] found that water wash followed by a spray of O_3 solution as 95 mg /L for 30 s at 80 lb/in^2 reduced E. coli O157:H7 and Salmonella Typhimurium by 2.0 - 3.6 and 1.9 - 3.6 $\log_{10}$ CFU/cm^2, respectively. Novak and Yuan [30] concluded that O_3 treatment of beef surfaces enhanced the effectiveness of cooking temperatures against enterotoxin-producing strains of Clostridium perfringens in which the vegetative cells on beef surfaces were reduced from 5.59 ± 0.17 $\log_{10}$ CFU/g to 4.09 ± 0.72 $\log_{10}$ CFU/g and 3.50 ± 0.90 $\log_{10}$ CFU/g after combined treatments of aqueous O_3 as 5 ppm and subsequently heating at 45 and 55 centigrade, respectively. Cho et al. [31] found that O_3 as 10×10^{-6} kg O3 h^{-1} at 4°C for 1 day reduced the original concentrations of E. coli O157:H7 in the inoculated ground beef samples by 0.53 $\log_{10}$ CFU/g and concluded that O_3 continuously eradicated E. coli O157:H7 on the surface of the beef testers and it can be an antibacterial substance for meat produces in a refrigerator.

The effectiveness of O_3 as an intervention against S. aureus and MDR S. aureus was previously established by Kammer [32] who found that the killing effect of O_3 is highly dependent on the relative humidity when examine the effect of O_3 treatment on S. aureus by which 92% and 0% of S. aureus were survived after exposure to gaseous O_3 as 0.3 ppm for 1 h with relative humidity of 15-25 and 85-95, respectively. Also, concluded that O_3 is a potent decontaminating agent and has a good potential for use as an agent for disinfection and sterilization. Another study conducted by de Boer et al. [33] who using O_3 as an intervention against methicillin resistant S. aureus (MRSA) contaminated home environment of a colonized hospital employee to eradicate these bacteria from a carrier with eczema and they pronounced O_3 in gaseous phase as an efficacious intervention to eradicate MRSA from a widely contaminated home environment. Burgassi et al. [34]

evaluated the bactericidal effect of different concentrations of O_3 when used as a gas, or dissolved in saline on S. aureus, MRSA and Pseudomonas aeroginosa that suspended in their culture media, and they found that no viable bacteria of (S. aureus and MRSA) were noticed after O_3 exposure as 5 to 320 mg/ L for 15 min at 20 centigrade. Song et al. [35] in their experiment measured the efficacy and care of current O_3 on the cure of skin contagion with MRSA, and they stated that ozonated oil can fumigate up to 98% of S. aureus and MRSA in 5 and 15 min, respectively. Also, they recorded that aqueous O_3 (1 mg/L) can sterilize 100% of S. aureus and MRSA in 1 min. Most recently, Kanaan [22] reported that ozonation treatment as (0.5 ppm) for 45 min of chicken meat resulted in 2-4 log reductions of MRSA.

The results showed that 77.8% and 100% of the samples of aqueous O_3 were free (no growth on agar surface) after treatments of cattle's meat for 15 and 30 min, respectively (Table 3). The results of this study showed that aqueous O_3 were successfully killed MDR S. aureus in the presence of meat drip due to powerful oxidizing power of ozone lead to highly ozone's reactivity that make it much more efficient and effective for sterilization of all form of bacteria and viruses in potable water, another advantage provided adequate microbiological controls are implemented, is that the aqueous O_3 that has been used for disinfection can potentially be re-used for the initial cleaning stages, either directly or after re ozonation to attain the required quality.

In principle, the outcomes of this experiment agreed with the outcomes obtained by Strasser et al. [36] who found considerable reduction of total bacterial count and complete removal of pathogens when eviscerated chickens treated with aqueous O_3 as 4-12 ppm at 4 centigrade for 30 min and concluded that properly filtered chiller water can be safely recycled, saving water and energy. The results of this study were also agreed with Graham et al. [37] who concluded that chiller overflow water using for pre-washing of chicken carcasses with O_3 as 4 - 8 ppm could be reconditioned after filtration, as well as they demonstrated that O_3 treatment and filtration of chiller water led to elimination of pathogens including Campylobacter, Clostridium perfringens, and Staphylococcus. They also stated that pilot chiller bath water at 4°C maintained at 2 - 4 ppm ozone remained clear and microbial counts were equivalent to a commercial three - stage chlorinated chiller. Additionally, Kanaan [38] found that that ozonated water as 0.5ppm were 100% negative (no bacterial growth on agar surface) for Campylobacter jejuni after treatments of chicken carcasses for 30 and 60 min at 4 centigrade in the presence of residual protein, fat, and blood. Also, she concluded that the effectiveness of O_3 did not affect by the presence of organic materials.

Conclusion

Based on the defined conditions, the attainable data of our study concluded that O_3 is exceedingly effective against MDR S. aureus positive samples and this effectiveness increase with increasing the experience time to ozonation treatment at the same concentration, these findings are greatly significant from the public health perspective. As well as the effectiveness of aqueous O_3 (1/2 ppm) did not affect by the presence of meat drip and was extremely efficient in excluding MDR S. aureus after treatments of meat for 15 and 30 min. These outcomes suggested the plausible usage of aqueous O_3 as a promising alternative involvement to disinfect meat products in order to eliminate common foodborne bacteria such as S. aureus either at abattoirs or prior cooking at home and restaurants.

Authors' Contributions

The laboratory work involved in this study, organize, writing and revising the manuscript was attained by MHGK. Analysis of data and interpretation of the results was attained by SSA. All researchers have read and approved the final version of the manuscript.

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Conflict of Interest

The researchers pronounce they do not have any conflict of interest.

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