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Asian Journal of Animal and Veterinary Advances

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OPEN ACCESS Asian Journal of Animal and Veterinary Advances

ISSN 1683-9919
DOI: 10.3923/ajava.2017.302.310

Research Article
Ecological Study on *Listeria monocytogenes* and the Extent of its Resistance to Different Disinfectants in Dairy Farm for Improving Animal Health

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Abstract

Background and Objective: Resistance of pathogenic bacteria to disinfection process represent a potential threat to the animal health and food industry in developing countries. This study designed to monitor the main source of *Listeria monocytogenes* (*L. monocytogenes*) in animal's environment and assess the resistance pattern of *Listeria monocytogenes* to different disinfectants used for improving animal health and product quality. **Materials and Methods:** Animal, human and environmental samples, (n = 80, 40, 200, respectively) include [milk, animal feces, human stool, air dust, water, biofilm, bulk tank milk swabs, feedstuff and soil] are collected from a dairy cattle farm. Samples were cultured for isolation and identification of *Listeria monocytogenes* using biochemical tests and PCR. Sensitivity test of *Listeria monocytogenes* to different disinfectants [benzalkonium chloride (BC), hydrogen peroxide (H_2O_2), Virkon®S, TH^{4+} and sodium hypochlorite] was evaluated using broth macrodilution method. Resistant bacteria to disinfectants were screened for the presence of resistant gene using PCR. Data was analyzed by Chi square test. **Results:** The highest percentage of *L. monocytogenes* was isolated from soil, milk equipment, biofilm and feedstuff (45.0, 42.5, 32.5 and 25.0%, respectively). *Listeria monocytogenes* was moderately resistant against benzalkonium chloride1000 mg L^{-1}, Virkon®S 2%, TH^{4+} 1%, H_2O_2 5% and sodium hypochlorite 500 mg L^{-1} (50, 40, 50, 50, 60, 40, 20, 30, 30 and 40%, respectively) at both 12 and 24 h of exposure times. Furthermore, resistance *qacED1* gene was found in *L. monocytogenes* isolates. **Conclusion:** *Listeria monocytogenes* was highly resistant against all tested disinfectants at both 4 and 6 h of exposure times. Animal feces, soil and milk equipment serve as the main reservoir of *L. monocytogenes* in a dairy farm. Regular monitoring of microbial sensitivity to disinfectants used and improving the disinfectants power have an essential role for controlling of *L. monocytogenes* resistant bacteria in dairy cattle farms.

Key words: *Listeria monocytogenes*, reservoir, biocides, *qacED1* gene, benzalkonium chloride

Received: August 27, 2017 Accepted: September 22, 2017 Published: October 15, 2017

Citation: Asmaa Nady Mohammed and Sahar Abdel Aleem Abdel Aziz, 2017. Ecological study on *Listeria monocytogenes* and the extent of its resistance to different disinfectants in dairy farm for improving animal health. Asian J. Anim. Vet. Adv., 12: 302-310.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Listeria monocytogenes is a relevant foodborne pathogen responsible for a potentially fatal listeriosis disease that results in meningitis, septicemia or abortion. It can affect humans, wild and domestic animals[1]. Although rare, the mortality rate of listeriosis is 25-30% worldwide[2]. Listeriosis outbreaks in Europe and United States are still being notified disease despite the efforts that have been made by food industries and sanitary authorities [3].

In the farms, Livestock animals can contribute to *L. monocytogenes* prevalence in three ways: Using of livestock manure as fertilizer, contamination of irrigation water through runoff from livestock facilities and contamination of produce fields by direct runoff from livestock from the adjacent facilities [4].

Although *L. monocytogenes* can be found in cattle environment include water, soil and feces: Human and animals are likely to be an important reservoir[5]. *Listeria monocytogenes* has the ability to form biofilms which can contribute to its ability to colonize food processing facilities. Moreover, it is also resistant to many detergents and disinfectants used. Therefore, it can survive in food processing environments and become persistent. Such persistence of *L. monocytogenes* has been shown at a larger scale and smaller facilities of different production sectors[6-8].

For controlling of *L. monocytogenes*, it demands increasing knowledge of its habitats and contamination pathways for application of control strategies. Moreover, all the strains of *L. monocytogenes* aren't the same and they have differences in adaptation to environments, resistance to adverse conditions and virulence. Preventing contamination and growth of the pathogen control in foods represented an important step in inhibiting listeriosis. Despite in the food chain, elimination of *L. monocytogenes* is hardly possible but food contamination needs to be minimized and trying to prevention for reducing the incidence of listeriosis in humans[9].

Using of different disinfectants in destruction probability of *Listeria* species have been assessed in some scientific studies whereas the most frequently used disinfectants for *L. monocytogenes* are quaternary ammonium compounds (QAC), chlorine based and peroxygens[10,11]. The quaternary ammonium compounds (QAC) are the most widely useful in destroying *L. monocytogenes* while on other *Listeria* species have an adequate effect even in the presence of organic matter[12,13]. The main purpose of this study was to monitor the main source of contamination with *Listeria monocytogenes* at both levels of animals, their surrounding environment and human being then to assess the resistance patterns of *Listeria monocytogenes* against different disinfectants used and detect the resistant gene for improving product quality.

MATERIALS AND METHODS

Study area and animal husbandry: This study was conducted on a private dairy cattle farm located in Beni-Suef district (coordinates: 29°04'N31°05'E), Egypt during the study period from June, 2016 to May, 2017. The herd contained 150 lactating Friesian cows that produced an average of 15 kg of milk/day. Cows were milked two times/day (2 h/milking) in a parallel parlor equipped with 12 milking units. Milk passively flowed from the parlor to the milk house and subsequently enter into bulk tanks. Cows were kept into nine groups based on a stage of lactation and reproductive status in a partially sheltered yard on an earthy floor that bedded with straw materials.

Study design: Across-sectional study was carried out to determine the prevalence and monitor the main source of *Listeria monocytogenes* in animal's environment then assess the efficacy of different types of disinfectants against *L. monocytogenes* and detect the extent of its resistance to disinfectants used. A total number of (n = 320) animal, human and environmental samples including [milk, feces, human stool, air dust, water, biofilm, bulk tank milk swabs, feedstuff (silage) and soil samples] are collected from a dairy cattle farm. Collected samples were cultured for isolation and identification of *Listeria monocytogenes* using a molecular technique (PCR). The bactericidal efficacy of different disinfectants, benzalkonium chloride (BC) Quaternary ammonium compounds (QAC), hydrogen peroxide (H_2O_2), Virkon®S, TH^{4+} and sodium hypochlorite against *Listeria monocytogenes* at different concentrations and exposure times were evaluated using broth macrodilution method. Resistant bacteria to disinfectants used were screened for the presence of resistant gene by PCR.

Sampling collection: A total number of 80 animal samples (milk and fecal, n = 40 each) were collected directly under aseptic condition from apparently healthy dairy cattle using sterile containers, swabs that were kept and preserved on ice to be transferred to the lab of Animal Hygiene in the Faculty of Veterinary Medicine, Beni-Suef University. Meanwhile, 200 environmental samples including air dust, water, biofilm, bulk tank milk swabs, feedstuff and soil sample that were collected from different sites in farm particularly from the wetted area with high organic load twice/month

Table 1: Oligonucleotide primers sequence of target identified, virulence and resistant genes in *L. monocytogenes*

Genes	Primers sequences	Amplified products	References
Species identified	*5'-CCT TTG ACC ACT CTG GAG ACA GAG C-3'*	553 bp	Abu Al-Soud and Rådström[18]
16S rRNA	*5'-AAG GAG GTG ATC CAA CCG CAC CTT C-3'*		
Virulence	*5'-GCA-TCT-GCA-TTC-AAT-AAA-GA-3'*	174 bp	Deneer and Boychuk[19]
hlyA	5'-TGT-CAC-TGC-ATC-TCC-GTG-GT-3'		
Resistant	*5'- TAA GCC CTA CACAAA TTG GGA GAT AT-3'*	362 bp	Chuanchuen *et al.*[23]
qacED1	*5'- GCC TCC GCA GCG ACT TCCACG-3'*		

throughout study period[14,15]. About 40 stool samples were collected from the targeted category, farm workers (livestock contact) after informed them about the risk and complication of *Listeria* infection, in sterile containers where thirteen from diarrheic one and the remaining samples were from apparent healthy. Data were collected from those individuals concerning, age, sex and the recorded signs (fever, anorexia, colic, diarrhea, vomiting) which are the most prominent signs for *Listeria* food poisoning in man. All samples were properly labeled, identified and immediately sent to the lab for further microbiological examination.

Isolation and identification of *L. monocytogenes*: Collected samples were enriched in *Listeria* enrichment broth for isolation of *L. monocytogenes* and incubated at 30°C for 48 h then 0.1 mL of the enrichment broth was inoculated onto *Listeria* selective agar base (Palcam agar, Biokar Diagnostics, Beauvais Cedex, France). The agar plates were incubated at 37°C for 48 h. Bluish gray colonies 0.5-1.5 mm in diameter were selected and sub-cultivated on tryptic soy agar supplemented with 0.6% (wt/vol) yeast extract (TSAY, Oxoid Ltd., Basingstoke, UK) for identification. Gram staining technique was applied and Gram-positive short rods with rounded ends were selected and the following biochemical tests were applied catalase, oxidase, TSI, methyl red and blood hemolysis tests according to Hitchins[16]. Based on the polymerase chain reaction (PCR) technique, DNA was isolated by dispersing an isolated colony in 100 mL 0.1 M Tris-HCL buffer (Sigma-Aldrich, St. Louis, MO, USA). A simple DNA extraction method including the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) as described by Walsh *et al.*[17]. Protein involved in the actin filament assembly, a PCR amplification of the hemolysin (*hlyA*) virulence gene and species identification gene *(16S rRNA*) were detected according to Abu Al-Soud and Radstrom[18], Deneer and Boychuk[19] (Table 1).

Disinfectants assay: Fifty strains of *L. monocytogenes* isolates were screened *in vitro* for susceptibilities to five different disinfectants at different concentrations and exposure times, benzalkonium chloride (BC) quaternary ammonium salt (QAC) group, Fluka Analytical, St. Louis, USA), TH^{4+} (quaternary ammonium+glutaraldehyde, SoGeVal, France), Virkon®S (potassium peroxymonosulfate, Antec international TD, UK), H_2O_2 (6th of October 3rd industrial area, Egypt) and sodium hypochlorite (10-15% available chlorine, 425044 Sigma-Aldrich) are approved for food industry[20,21].

***In vitro* efficacy of different disinfectants:** The antimicrobial activity of tested disinfectants was evaluated using broth macrodilution method according to Li *et al.*[22], with some modifications in ratio of disinfectants concentration to bacterial contact (3 mL: 1 mL) and different exposure times (4, 6, 12 and 24 h). Each disinfectant stock solution was serially diluted with tryptic soya broth (TSB) to obtain test solutions containing required concentrations. Disinfectants concentrations used: Benzalkonium chloride (BC) (100, 125, 250, 500 and 1000 mg L^{-1}) while, TH^{4+} (0.5 and 1%), Virkon®S (1 and 2%), H_2O_2 (3 and 5%) and sodium hypochlorite (250 and 500 mg L^{-1}) were prepared. Then aliquots of 1 mL from *L. monocytogenes* bacterial isolates (about 10^6 CFU mL^{-1}) were added to the tubes containing 3 mL of the prepared concentrations of disinfectant solutions separately and allowed to interact at different exposure times (4, 6, 12 and 24 h) then 1 mL of inocula was transferred to TSB containing tubes, incubated at 37°C for 24 h. Positive tubes are considered that exhibiting medium turbidity and formation of a surface of the thin skin or a precipitate in the bottom of the tubes compared to the negative control tube (1 mL bacterial suspensions). To confirm the presence or absence of *L. monocytogenes* bacteria, the suspension was inoculated in tryptic soya agar. The effectiveness of disinfectant confirmed the absence of bacterial growth on plates.

Detection of *qacED1* resistance gene in *L. monocytogenes*: All *L. monocytogenes* isolates were cultured on peptone water (at 37°C for 24 h) for DNA extraction. Two hundred µL of bacterial suspension were added to 10 µL of proteinase K and 200 µL of lysis buffer. All components were incubated for 10 min at 56°C then 200 µL of 100% ethanol was added to the lysate. The sample was washed then centrifuged

following the manufacturer's recommendations. Nucleic acid was eluted with 100 µL of elution buffer provided in the kit. The PCR primers targeting the *qacED1* gene was applied on resistant *L. monocytogenes* isolates that showed resistance to benzalkonium chloride (BC) quaternary ammonium compound (QAC) according to Chuanchuen *et al*.[23] (Table 1).

Statistical analysis: All data were recorded using the Microsoft excel spreadsheet then prepared for analysis. The prevalence of *L. Monocytogenes*, frequent distribution of bacteria in human stool, animals samples, their surrounding environment and *in vitro* sensitivity of *L. Monocytogenes* to different disinfectants used were calculated by the use of non-parametric tests (Chi-square test) using statistical package for social sciences (SPSS) software (version 22.0 for Windows, SPSS Inc., Chicago, IL) and probability level is $p<0.001$.

RESULTS

The prevalence rate of *L. monocytogenes* bacteria isolated from dairy cattle farm based on bacterial finding was 28.1% whereas *L. monocytogenes* prevalence in cattle's environment was significantly higher 30.0% compared to prevalence rate in both animals and human samples (26.3 and 22.5%, respectively) at $X^2 = 69.0$, $p<0.001$ is shown in (Table 2). Concerning, frequent distribution of *L. monocytogenes* bacteria isolated from different examined samples (Table 3) clarified that 90 positive bacterial isolates out of 320 examined samples were recovered (60, 21 and 9 from the environment, animal and human, respectively). The highest percentage of *L. monocytogenes* was isolated from environmental samples, soil, milk equipment (bulk milk tank swabs), biofilm and feedstuff (45.0, 42.5, 32.5 and 25.0%, respectively) at $X^2 = 57$, $p<0.001$ followed by animal fecal, milk and human stool samples (30.0, 22.5, 22.5%, respectively) while, both water and air dust samples recorded the lowest percentage (6.7 and 0.0%, respectively).

Referring to *in vitro* antimicrobial efficacy of different disinfectants against *L. monocytogenes* isolates from different samples as shown in (Table 4). It has been revealed that *L. monocytogenes* isolates exhibited significantly high resistant profile at $p<0.001$ to all disinfectants, sodium hypochlorite, hydrogen peroxide (H_2O_2), Virkon®S, TH^{4+}, benzalkonium chloride at the different tested concentrations after 4 and 6 h of exposure time. Meanwhile, sensitivity of *L. monocytogenes* to benzalkonium chloride (1000 mg L^{-1}), Virkon®S (2%), TH^{4+} (1%), H_2O_2 (5%) and sodium hypochlorite (500 mg L^{-1}) was gradually increase (50, 60, 50, 50 and 40, 60, 80, 70, 70 and 60%, respectively) at both 12 and 24 h of exposure times compared to other concentrations used at the same contact times. On contrast, the susceptibility of

Table 2: Prevalence rate of *L. monocytogenes* in examined dairy farm during the study period

Examined farm	Total examined (No.)	Positive (No.)	Prevalence of *L. monocytogenes* (%)
Animal samples	80	21	26.30
Environmental samples	200	60	30.00
Stool samples	40	9	22.50
Total	320	90	28.10

$X^2 = 69.0$, $p<0.001$

Table 3: Frequent distribution of *L. monocytogenes* isolated from different examined samples during the study period

	Bacteriological findings		
Samples	Examined (No.)	Positive (No.)	Distribution of *L. monocytogenes* (%)
Fecal	40	12	30.0
Milk	40	9	22.5
Biofilm	40	13	32.5
Bulk tank milk swabs	40	17	42.5
Air dust	20	0	0.0
Water trough	30	2	6.7
Feedstuff (silage)	30	10	25.0
Bedding	40	18	45.0
Stool	40	9	22.5
Total	320	90	28.1

$X^2 = 57.0$, $p<0.001$

Table 4: *In vitro* sensitivity of *L. monocytogenes* isolates against different types of disinfectants

		Sensitivity (%) of *L. monocytogenes* isolates at different exposure time (h)							
		4 h		6 h		12 h		24 h	
Bacterial isolates disinfectants tested	Conc. (mg L^{-1})	S	R	S	R	S	R	S	R
Benzalkonium chloride	125	0.0	100	0	100	0	100	10	90
	250	0.0	100	10	90	20	80	30	70
	500	0.0	100	20	80	30	70	50	50
	1000	0.0	100	40	60	50	50	60	40
Virkon® S	1.00%	0.0	100	10	80	40	60	50	50
	2.00%	10	90	30	70	60	40	80	20
TH^{4+}	0.50%	0.0	100	20	80	50	50	60	40
	1.00%	20	80	30	70	50	50	70	30
H_2O_2	3.00%	10	90	30	70	40	60	60	40
	5.00%	20	80	30	70	50	50	70	30
Sodium hypochlorite	250	0.0	100	10	90	30	70	50	50
	500	0.0	100	10	90	40	60	60	40
p-value		0.321		0.073		0.001		0.001	

S: Sensitive (absence of bacterial growth), R: Resistant (bacterial growth)

L. monocytogenes to sodium hypochlorite at tested concentrations (250 and 500 mg L^{-1}) was not exceed 10% at both 4 and 6 h of exposure times.

Concerning, polymerase chain reaction for detection of *L. monocytogenes* virulence genes (Fig. 1). It has been found that the hemolysin (*hlyA*) gene was the most prevalent genes found in *L. monocytogenes* isolates from different examined samples. Based on PCR for detection of antimicrobial resistance gene (Fig. 2). Results revealed that *qacED1* gene responsible for resistance to quaternary ammonium compound (QAC) was found in three representative resistant strains of *L. monocytogenes*.

DISCUSSION

Cross-resistance between disinfectants used and antibiotics in livestock farms may become a challenge to the food processing industries and public health in the future. In the present study, the prevalence rate of *L. monocytogenes* was higher in cattle's environment compared to its prevalence in cattle and human stool. Furthermore, animal feces, soil and milk equipment (bulk milk tank swabs) serve as the main reservoir of *L. monocytogenes* in a dairy farm. Although soil can be a natural reservoir of *L. monocytogenes* in livestock farm, it can be transferred to human through food chain throughout the application of animal's manure to agriculture land. *Listeria monocytogenes* had the ability to form biofilms on stainless steel surfaces of milk equipment that initially might be caused by fecal and/or environmental contamination and subsequently enhance their survival and spread. These results can be attributed to improper cleaning and irregular disinfection of milk equipment besides frequent accumulation of animal manure on an earthy floor of the yard. Moreover, animal feces harbors the highest percentage of *L. monocytogenes* compared to human stool samples. Manyi-Loh *et al.*[24], highlighted that microbial agents in animal manure make it a potential source of environmental pollution and humans infections. However, Shiwakoti[4] showed that in the soil of livestock farms, the prevalence of *L. monocytogenes* (29.7%) is greater than that of all other soil types. This can be explained by in livestock animals, the prevalence of *L. monocytogenes* was relatively high due to the accumulation of animal manure in yards from the animal directly.

Listeria monocytogenes has been isolated from domestic, livestock and wild animals in both infections and latent states, in animal feces and their surrounding

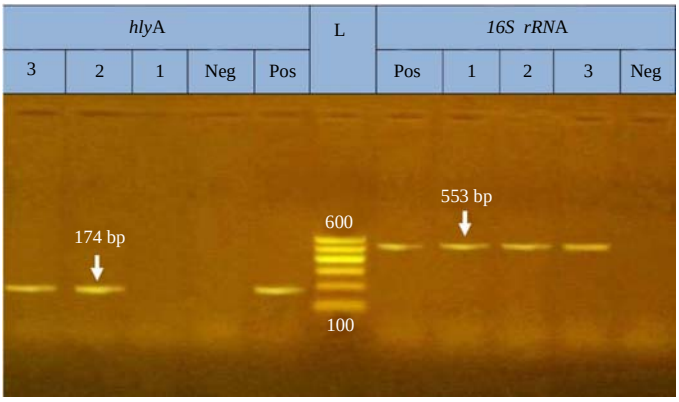

Fig. 1: PCR amplification of the 174 bp fragment of *hlyA* virulence gene (Lane 2-3) and *16S rRNA* (Lane 1-3) from *L. monocytogenes*, Pos (control positive), Neg: (control negative) L: DNA Ladder

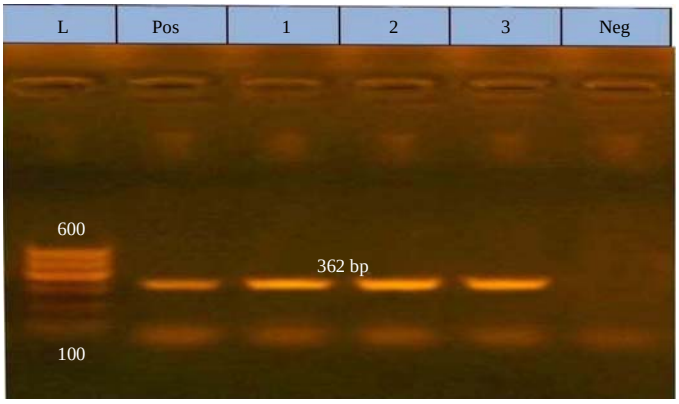

Fig. 2: PCR amplification of the 362 bp fragment of *qacED1* gene from *L. monocytogenes* (Lane 1-3), Pos (control positive), Neg: (control negative) L: DNA Ladder

environment[25]. Our findings were in harmony with those found by Jiang *et al.*[26], who revealed that 35.4% of cattle in a herd were shedding *L. monocytogenes* in feces. As well, in the milk system, the presence of *L. monocytogenes* was caused by fecal contamination[27]. *Listeria monocytogenes* has the ability to form biofilms and resist to antibacterial agents that cause bacterium stresses[28,29]. On the other hand, *Listeria monocytogenes* was isolated in the least percentage in water and air dust in this study. In animal's environment, *L. monocytogenes* is easily detected and able to persist for long periods in soil, animal feces, water and feed[30].

Concerning, the susceptibility of *L. monocytogenes* isolates against different disinfectants tested *in vitro* revealed that *L. monocytogenes* was significantly high resistant to all tested disinfectants after 4 and 6 h of exposure time. Meanwhile, it exhibited gradual increase in its sensitivity to benzalkonium chloride (1000 mg L^{-1}), Virkon®S (2%), TH^{4+} (1%), H_2O_2 (5%) and sodium hypochlorite (500 mg L^{-1}) after 24 h of exposure time. These results indicated that increasing the exposure time and disinfectants concentration led to increase the sensitivity of *L. monocytogenes* against disinfectants used. On the other hand, the highest percentage of bacteria was resistant to different disinfectants so that cross-resistance between disinfectants and antibiotics was possible. This explained by a stable higher resistance of *L. monocytogenes* due to exposure of bacteria to a sub-lethal concentration of disinfectant[31]. Therefore, the high resistance of bacteria may occur through adaptation, acquisition of genetic resistance elements, biofilm formation or stress responses[32,33]. In practice exposure of *L. monocytogenes* to quaternary ammonium compounds (QAC) led to adaptation but higher concentrations of BC was not possible to adapt a resistant strain of *L. monocytogenes*[34].

In the present study, despite some disinfectants used on a dairy farm there is an irregular removal of organic matter which led to reducing the effectiveness of the disinfectants and/or its effect was not obvious and at the same time gave a chance to foodborne pathogenic bacteria to become high resistance to disinfectants used. Morente *et al.*[35], concluded that bacterial resistance to biocides is becoming an emerging threat in the food chain. So that to remove organic compounds, cleaning the surface prior to disinfection is necessary. Otherwise, the disinfection will be useless. Thevenot *et al.*[36], revealed that using of disinfectants in the presence of organic matter resulting in less disinfectant coming into contact with the microorganism. Furthermore, industrial facilities and contamination of meat with animal feces might be occurred due to hygienic failing during animal slaughtering[3].

With regard to *qacED1* gene responsible for resistance to quaternary ammonium compound (QAC) was detected in *L. monocytogenes* isolates. About 40% of isolates were resistance to benzalkonium chloride (BC) at a concentration of 1000 mg L^{-1} after 24 h of exposure time. Langsrud *et al.*[37], pointed out that during using antimicrobial agents or disinfectants, the disinfectant resistance *qac* genes was closely linked to the antibiotic genes could lead to co-selection. Meanwhile, Tamburro *et al.*[38], found that among the tested (BC) resistant strains of *Listeria monocytogenes*, *lde* gene presented the highest expression level.

Control of *L. monocytogenes* should be started at farm level to mitigate its source in animal's environment that contribute to the overall reduction of *L. monocytogenes* in the food chain. Animal manure should be treated appropriately to kill *L. monocytogenes* and other potential pathogens before it is used as fertilizer. Under laboratory conditions, *L. monocytogenes* survived for 43 days in manure-amended soil and in fecal waste applied to land for 128 days[39]. To overcoming foodborne infections, cleaning is an essential key element as well as the use of disinfectants and bactericidal agents together, could potentially reduce the incidence of these infections[40]. Furthermore, in animal manure reducing the level of bacterial pathogen need to store it in heaps for a certain period in the animal housing before use as a fertilizer[24]. *Listeria monocytogenes* has the ability to survive in adverse conditions and might not be completely inactivated[41]. To reduce the microbial load of *L. monocytogenes* in fresh produce the application of antimicrobial agents should be used[42]. Proper cleaning and alternation between two disinfectants are commonly used to avoid buildup of resistant strains in environments. However, in practice, microorganisms resistant to one of the disinfectants may become resistant to the other which led to cross-resistance between disinfectants[43].

CONCLUSION

Based on the study results, it can be concluded that cattle feces, soil and milk equipment serve as the main reservoir of *L. monocytogenes* in dairy farm. *Listeria monocytogenes* bacteria exhibited highly resistant profile to different disinfectants used, sodium hypochlorite, hydrogen peroxide (H_2O_2), Virkon®S, TH^{4+}, benzalkonium chloride at the different tested concentrations after 4 and 6 h of exposure times whereas *qacED1* gene responsible for resistance to quaternary ammonium compounds (QAC) was detected in *L. monocytogenes* isolates. Regular testing the sensitivity of *L. monocytogenes* to disinfectants used besides improving the disinfectant power are represent an essential key elements for controlling of *L. monocytogenes* resistant bacteria.

SIGNIFICANCE STATEMENTS

This study highlights on resistant bacteria surviving disinfection process represent a potential threat to animal health and food industry. Tracking the main source of *L. monocytogenes* in animal's environment and assess *L. monocytogenes* sensitivity to different types of disinfectants used are essential elements for efficient control. Furthermore, this study stated that control of *L. monocytogenes* should be started at farm level to mitigate its source.

ACKNOWLEDGMENT

The authors would like to acknowledge Projects Funding and Granting Unit in Beni-Suef University, Egypt, for funding the project (the 6th stage of competitive projects, grant number 3) and supporting the research.

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