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Emerging public health challenges of *Listeria monocytogenes* isolated from different ecological niches in Egypt: Food, humans, animals and environment

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Abstract: Serious outbreaks of foodborne disease have been caused by *Listeria monocytogenes* found in retail delicatessens and the severity of disease is significant, with high hospitalization and mortality rates. Little is understood about the formidable public health threat of *L. monocytogenes*in all four niches, humans, animals, food and environment in Egypt. This study analyzed the presence of *L. monocytogenes* collected from the four environmental niches and bioinformatic analysis was implemented to analyze and compare the data. PCR was used to detect virulence genes encoded by pathogenicity island (LIPI-1). *prf*A amino acid substation that causes constitutive expression of virulence was common in 77.7% of isolates. BLAST analysis did not match other isolates in the NCBI database suggesting this may be a characteristic of the region associated with these isolates. A second group included the NH1 isolate originating in China, and BLAST analysis showed this *prf*A allele was shared with isolates from other global locations such as Europe and North America. Identification of possible links and transmission pathways between the four niches, helps to decrease the risk of disease in humans, to take more specific control measures in the context of disease prevention, to limit economic losses associated with food recalls and highlightens the need to treatment options.

Keywords: *L. monocytogenes;* Humans; Animals; Food; Antimicrobial and virulence genes; Bioinformatic analysis; *prfA* phylogenetic analysis

1. Introduction

Listeria monocytogenes is a facultative intracellular pathogen,which may be classified as asapronoses (or saprozoonoses) with its environmental reservoir [1]; where it may grow effectively outside of the host and to infect a relatively wide range of hosts (polyhostality) [1]. *L. monocytogenes*is present in natural ecosystems and is widely disseminated in different environmental niches [2]. It has been isolated from humans, and more than 50 species of wild and domestic animals, including mammals, birds, fish, crustaceans and ticks in addition to environmental sources such as animal silage, soil, plants, sewage, stream water and processing environment [3-6]. Although the genus *Listeria* includes many species, due to the pathogenicity of *L. monocytogenes* in human hosts and its capability to thrive in harsh environments, previous genome sequencing and research efforts were largely focused on this species [7-11]. Almost all cases of human listeriosis are related to *L. monocytogenes* and in the present decade, numerous serious outbreaks of foodborne listeriosis have been recorded causing severe symptoms, such as septicemia and meningitis, predominantly in the immunocompromised and elderly populations as well as in pregnant women, who may give birth to stillborn infants or severely infected newborns [4,9,12-21].

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The differences in the pathogenic potential of the *L. monocytogenes* strains, which are correlated with the flagellar antigen groups, have been demonstrated *in vitro* and *in vivo* and at least three distinct genetic lineages exist [22,23]: lineage I: strains associated with epidemic outbreaks of listeriosis (serotypes 1/2b, 3b, 4b, 3c); lineage II: strains isolated from sporadic cases of listeriosis (serotypes 1/2a, 1/2c, and 3a); and lineage III: strains rarely associated with cases of listeriosis (serotypes 4a and 4c). Based on somatic (O) and flagellar (H) antigens, 13 serotypes of *L. monocytogenes* have been recognized and identified alphanumerically: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c,4a, 4ab, 4b, 4c, 4d, 4e and 7 [22,23]. Over 95% of isolates in human and animal listeriosis in addition to foods belong to serotypes 1/2a, 1/2b and 4b [15,22-24]. Epidemiologically important *L. monocytogenes* clones were identified as being associated with listeriosis outbreaks in different countries and continents [11,25-29]. The molecular pathogenesis of *L. monocytogenes*is determined by multiple key virulence factors, such as internalins, haemolysin, phospholipases, actin polymerization protein and other minor virulence factors such as extracellular proteins (*iap*), antioxidant factors, metal ion uptake systems and stress response mediators [8]. The expression of these virulence factors is directly modulated by the regulator gene *prf*A [[8,30]. Loss of virulence-associated genes such as the *prf*A cluster during the evolution of *Listeria*, have played a critical role in the transition of *Listeria* species from facultative pathogen to saprotroph [7], suggesting that *Listeria* tends to evolve through loss of virulence rather than acquisition of virulence characteristics.

Analysis of domestic animal and farm environmental *L. monocytogenes* revealed that some strains associated with human infection circulate within the biosphere and the agroecosystems may contribute to the transmission of these pathogens to the food chain [9] posing a major health issue [9]. Although the role of the wild nature as an original source of listerial infection was suggested, the information about clone distribution among *L. monocytogenes* disseminated in natural ecosystems and their phylogenetic relationships with epidemiologically important clones although scarce but of increasing interest [25].

Foods are considered to be the major vehicle for human cases of listeriosis [9]. Of particular significance are ready-to-eat (RTE) foods, including processed foods that have been exposed to the processing environment prior to packaging. RTE foods are those that are normally eaten raw or handled, processed, mixed, cooked, or otherwise prepared into a form that is eaten without further control steps capable of inactivating the bacterium, such as cooking or held for extended periods at refrigeration or chill temperatures, which allow growth to high numbers although, prevalence of *L. monocytogenes* in the food environment may not necessarily be proportional with prevalence in the food product [9,31].

In order to improve understanding of the pathogen's ecology, the current study was aimed to investigate the occurrence of *L. monocytogenes* in retail food or clinical cases of infection to establish their potential virulence and antibiotic resistance in Egypt. This may allow to identify possible links and transmission pathways between these niches, the diversity in virulence, resistance, and other clinically

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relevant traits that make *L. monocytogenes* a formidable public health threat and, in further consequence, help to prevent the infection of humans and animals to take more specific control measures in the context of disease prevention and to limit economic losses associated with food recalls.

2. Results

The current study examines *L. monocytogenes* isolates from different retail food, or from animals or human clinical cases showing septicemia and abortion in the Greater Cairo Area selected based on the prevalence determined in previous national studies on milk samples [32-34]. *L. monocytogenes* were found in less than one percent (0.99%) or 20 of 2029 samples analyzed (including 1828 retail food samples, 136 veterinary, and 65 clinical samples). The results (Tables 1, S1 and S2) revealed that the prevalence of *L. monocytogenes* in the different samples were recorded in: Milk by-products, kariesh cheese (1/120); Chicken, broilers internal organs (3/120) and layers internal organs (3/120); Table eggs (1/100); Meat, meat by-products (hamburger 1/50); Ducks internal organs (1/60); Silage (3/90); Fish, frozen fish (1/100), fish filet (1/58), herring (1/66); Brain tissue, rabbit (1/30); fetal livers (goats 1/15); Septicemia (ewes 1/24 and women 1/65).

The 20 isolated *L. monocytogenes* along with seven additional isolates from previous surveillance of milk [32-34] were examined for differences in serotype, genotypic virulence, and phenotypic virulence, biofilm formation, and antibiotic sensitivity. Table 1 is a condensed version of all results (Tables S1 and S2) presenting phenotypic and genotypic differences between the isolates, with Figure 1 presenting a heatmap of the isolates to visualize these differences. Nine of the isolates were determined to be serotype 1 while 18 isolates were serotype 4. All 27 isolates demonstrated the presence of genetic elements harbored on the *Listeria* pathogenicity island (LIPI-1; *prfA, plcA, plcB, and actA*), although two isolates demonstrated the absence of listeriolysin O, encoded by the *hlyA* gene. All isolates showed the presence of the minor virulence factor gene, *iap*, encoding the extracellular protein p60. All isolates exhibited causation of keratoconjunctivitis (Anton's eye test), cytotoxicity of Vero cells, and successful infection and lethality toward mice and chick embryos indicating all were virulent isolates of *L. monocytogenes*. The flagellin encoding *flaA* gene, which contributes to effective invasion during infection,[8] was absent in only four of the 27 isolates. PCR screening of four key internalins encoded by *inlA, inlB, inlC,* and *inlJ* indicated the presence of all four genes in 12 isolates including the isolates from veterinary and clinical samples, while absent in two of the isolates. The frequency of the individual internalin genes *inlA, inlB, inlC,* and *inlJ* were 74.1% (20), 81.5% (22), 70.4% (19), and 66.7% (18), respectively. Notably, the isolates (4) from cow, she-camel, and buffalo milk, and the two isolates that caused septicemia were positive for the presence of all 11 genes examined.

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Biofilm formation was determined by glass test tube assay and microtiter plate method. The glass test tube which allows for qualitative analysis of attachment to glass surfaces and subsequent biofilm formation during disturbance showed moderate to strong biofilm formation by the isolates. The microtiter plate method for quantitative analysis of attachment and biofilm formation on plastic surfaces during static conditions displayed strong to very strong biofilm formation ranging from 0.13 to 0.56 of staining where the reference strains was 0.16.

The phenotypic antibiotic sensitivity results of the 27 *L. monocytogenes* isolates showed all were sensitive to all penicillins tested: ampicillin, amoxicillin/clavulanic, amoxicillin, penicillin G, cloxacillin and oxacillin; the 2nd generation fluroquinolones: ofloxacin, enrofloxacin, and ciprofloxacin; the aminoglycosides: amikacin, gentamicin, kanamycin, spiramycin; the glucopeptide, vancomycin; and rifamycin. All isolates were resistant to the fluroquinolones: flumequine and perfloxacin; the phenicol: chloramphenicol; the cephalosporins: cefotaxime and cephalothin; the lincosamides: lincomycin and clindamycin; and the polypeptide: bacitracin. The isolates showed 25.9% (7), 7.4% (2), 62.9% (17), 44.4% (12), and 7.4% (2) resistance to neomycin, streptomycin, tetracycline, sulphamethozole-trimethoprim, and erythromycin, respectively. The range of the multiple antibiotic resistance index (MAR_{index}) was from 0.28 to 0.43, with the greatest MAR_{index} attributed to the isolate from a case of human septicemia which showed resistance to 12 antibiotics including four of the five antibiotics showing variance within the isolates.

The *prfA* gene encodes a transcriptional activator of the determinants of pathogenicity in *L. monocytogenes*. The *prfA*gene was sequenced to determine possible differences in virulence and pathogenicity. There were seven nucleotide differences detected between codons 87 and 208 which caused five changes in the amino acid sequences compared to wild-type (Figure 2). Two of the changes were synonymous substitutions in the third position of codons for S127 and T170 in an isolate for cow milk and buffalo milk, respectively. The other five mutations were missense mutations. One isolate from cow milk showed a mutation of E101K and one isolate showed a mutation of G161D. The E101K and G161D mutations were not identified in the non-redundant protein database at NCBI by Blast nor the literature. Of the 21 isolates demonstrating the mutation of G145S, only one was absent of additional mutations. The G145S mutation was present with K130Iin four isolates, in one isolate which also harbored a G161D mutation, and in all 15 isolates with S184P mutations.

Examination of correlations between the phenotypic and genotypic characterizations of the 27 isolates of *L.* monocytogenes was performed (Figure 3). There was a significant positive correlation of the MAR_{index} with resistance to neomycin, sulphamethozole-trimethoprim, and erythromycin ($p<0.05$). The strongest correlation of the MAR_{index} was with the antibiotic combination of sulphamethozole-trimethoprim suggesting this resistance is most important in multi-antibiotic resistance of *L. monocytogenes.* The presence of the S184P mutation in the *pfrA* gene was positively and strongly correlated with the G145S

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mutation as expected based on initial analysis ($p<0.05$). There was a significant negative correlation of the S184P and K130I mutations in *pfrA* gene ($p<0.05$). Correlations of genes and phenotypes that are assumed to be independent of one-another included a strong negative correlation of streptomycin resistance with the flagellin encoding, *flaA*, gene and the *pfrA* gene with the G145S mutation ($p<0.05$). There was also a significant negative correlation between the internalin encoded by the *inlC* gene and the antibiotic combination of sulphamethozole-trimethoprim. PCA analysis (Figure 4) supports a number of these correlations, however, MANOVA analysis indicated no statistical differences in the levels of individual test and there was no strong differences in the test based on source or serotype ($p>0.56$).

prfA phylogenetic analysis

The 28 isolates clustered into 7 unique *prfA* alleles. The largest group included 14 isolates which included isolates each of the sample types, including the human isolate. BLAST analysis of this allele did not match other isolates in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), suggesting this may be a characteristic of the region associated with these isolates (Figure 5). The second largest group included the NH1 isolate originating in China, and BLAST analysis showed this allele was shared with isolates from other global locations such as Europe and North America (data not shown). No clear association of a single sample type and specific allele was apparent, with multiple alleles identified among most animal sources included.

3. Discussion

The assessment of the actual prevalence of listeriosis and an investigation of the environmental distribution and ecology of different strains are important steps towards finding a possible link between environmental reservoirs of *L. monocytogenes* and human infection. The review published by Walland et al. [35] provided an overview of listeriosis in animals and discusses our insufficient understanding of reservoirs and possible cycling of *L. monocytogenes* between animal and human hosts. This insufficient understanding is caused by gaps in our knowledge of the role of genetic subtypes in *L. monocytogenes* ecology and virulence, and the risk factors, *in vivo* diagnostics, and pathogenesis of listeriosis in animals. The ecology of *L. monocytogenes* strains in the reservoir of agricultural and animals environments is not known in Egypt.

The overall low prevalence of *L. monocytogenes* of less than one percent detected within this study is lower than previous studies, and may reflect increased awareness and vigilance [3,9,11,36-41]. A recent study by Leong et al. [42] of small food businesses in Ireland showed a similar decrease over the course of a three-year study. Leong et al. [42] found a comparable food and environmental occurrence of *L. monocytogenes* at 4.2% and 3.8%, respectively. The highest occurrence was within meat at 7.5% and the

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lowest in seafood at 1.8%. A similar trend was observed in the current study with occurrence of 2.4% in meat and 1.3% in seafood. The occurrence of *L. monocytogenes* in raw meat, milk, and cheese in this study was lower than that found in other countries [4,11,29,39-45]. The global *L. monocytogenes* population diversity in the implicated food vehicles could be attributed to several factors: i) Improvements in detection methodologies, ii) packing facility, iii) increases in populations of lactic acid bacteria, yeasts and molds that might have an impact on the microenvironment supporting growth of *L. monocytogenes*, iv) storage times and temperatures, v) consumer handling, vi) lack of adequate sanitation, vii) inadequate temperature control, viii) glove/hand issues, ix) environmental (hygienic conditions of the farms, hygienic conditions of the slaughter houses, rodents, workers, the slicer, trash handling and cleanup operations) contamination and subsequent cross contamination to other products, x) transport, xi) in the processing facilities, and xii) during handling at the outlets. In the cheese industry, where ripening and storage are critical stages of modernisation of the process, several additional parameters must be taken into consideration, including [40]: i) ripening duration, ii) storage temperature, iii) level of initial contamination, iv) postprocessing contamination, v) physico-chemical conditions of the cheese process, and vi) packaging.

Globally, the prevalence rates in fish shows great variability [3,44,46-50]: The prevalence of *L. monocytogenes* in fish, presumably from contaminated waters or during manipulation and processing with contaminated environment and/or equipment [48,51]. The *L. monocytogenes* isolated from our frozen fish samples indicates that *L. monocytogenes* can survive in frozen food products and that contaminated products can be a reservoir involved in human listeriosis outbreaks [9,20,52-54].

The presence of the genes of LPI-1, including *prfA, plcA, plcB,* and *actA* detected in all isolates highlights the potential for virulence by the isolates, which was further confirmed by infection and mortality of mice, chick embryos, and Vero cells [55]. The lack of detection of the listerolysin O encoding *hlyA* gene of LIPI-1 required for intracellular pathogenesis in two of the 27 isolates may suggest decrease sequence homology indicating evolutionary plasticity of the *hlyA* gene [56]. Similar lack of detection of this gene was observed by Al-Nabulsi et al. [57] and Ndahi et al. [58]. The presence of LIPI-1 in all the isolates indicates a potential health risk with the associated contaminated food products.

Attachment and formation of biofilm allows the colonization of surfaces including food processing environments [59]. The presence of LIPI-1 genes holy and *prf*A is required for efficient biofilm formation by *L. monocytogenes,* [60] however the current study was unable to detect a correlation between the strength of attachment and biofilm formation and detection of the *hlyA* gene or amino acid substitutions in *prfA*. The disruption of the *flaA* gene causes immotile cells and increased biofilm formation [61]. There was also no positive correlation with the absence of the flagellin encoding *flaA* gene and biofilm formation. The isolates lacking the *flaA* gene did not show an increase in biofilm formation, however, several genes play a role in biofilm formation and were not screened [61]. Likewise the presence of strong biofilm formation in two

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isolates might be due to disruption or overexpression of genes with a role in biofilm formation [61]. Further exploration of differences between environmental and clinical isolates in relation to attachment and biofilm formation will lead to greater understanding of the reservoirs of *L. monocytogenes*.

An important element of the LIPI-1 virulence determinant is the *prfA* gene. The *pfrA* gene encodes the key regulator (the PfrA protein) involved in the activation of pathogenicity determinants in *L. monocytogenes*. The high representation of the G145S substitution in PfrA suggests the majority of isolates would have putative constitutive over expression of virulence factors [62]. However, this over expression in virulence can lead to decreased fitness outside the host and decrease environmental survival [63]. Additional mutations of K130I, G161D, or S184P were present in PrfA in isolates with the G145S mutation. Substitution of K130 with glutamine (K130Q) caused complete loss of PrfA activity [64], however the isoleucine (K130I) substitution may not have disrupted the putative functional pocket in which it is found or the G145S substitution causing constitutive activity was not abolished by K130I. The detection of the S184P mutation within 14 of the 21 isolates with the G145S mutation is interesting as the S184 forms direct hydrogen bonds with the nucleotides within the major groove of the DNA binding site [65]. An alanine substitution of S184 (S184A) decreases DNA binding and virulence in mouse model infections [65], however the S184P mutation did not lead to changes in infection of mice or chick embryos in the current study. The G161D mutation was in one isolate and is most likely does not influence the DNA binding domain of PrfA, however, G161 is conserved with the G180 of *srv*, a PrfA-like Group A *Streptococcus* regulator of virulence, within the putative DNA-binding domain [66]. The detected negative correlations between the G145S mutation and streptomycin resistance was driven by two isolates from silage that were streptomycin resistant and had no detected mutations within the PrfA protein, suggesting that this was an artifact of low isolate number. The understanding of the benefit and cost of these mutations of PrfA on the regulation of LIPI-1 mediated virulence in the host and environment will inform models of potential risk of environmental strains of *L. monocytogenes.*

In addition to the LIPI-1, the internalin family of proteins are necessary for attachment and host cell invasion in non-phagocytic cells. Internalins are associated with or anchored to the cell wall, or may be secreted and together mediate diverse functions in virulence. Currently only the surface proteins InlA and InlB are linked with internalization *per se* [67]. InlA is necessary for the recognition and invasion of epithelial cells and is essential in invasion of enterocytes and to crossing the placental barrier, while InlB is important for liver and spleen colonization but does not appear necessary for crossing the intestinal epithelium [67]. The small secreted internalin InlC may contribute to InlA-mediated internalization and InlJ contributes to virulence after intravenous infections, however, InlC and InlJare not known to affect internalization, intracellular proliferation, nor cell-to-cell spread [67]. The isolates showed difference in carriage of the internalins encoded by the *inlA, inlB, inlC,* and *inlJ* genes. There were 12 isolates with all

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four of these internalins, while 7 isolates lacked InlA but did carry InlB. There were no detected differences in the virulence of the isolates based on infection and mortality of mice, chick embryo, and Vero cells suggesting that the presence of other determinants present could facilitate an invasive pathology or the isolates contained a sequence variant of InlA or InlB not detected in the assay. Interestingly there was a significant negative correlation of the detection of InlC and phenotypic resistance to the antibiotic combination of sulphamethozole-trimethoprim suggesting that strains with InlC may currently have decreased incidences of mechanisms yielding resistance to suplfamethozole-trimethoprim. However, greater number of isolates and identification of the specific mechanisms of antibiotic resistance will be needed to further support this potential correlation. The absence of genes encoding the InlC or InlJ in only four of the isolates suggests a *L. monocytogenes* population with potentially diverse markers associated with infectivity. *L. monocytogenes* isolates from imported frozen fish lacked the four main internalin genes (*inlA, inlB, inlC*, and *inlJ*) in contrast to the previous studies where internalin genes were present in almost all previously examined *L. monocytogenes* isolates from all 4 niches –humans, animals, food and environment [47-49,68-76].

L. monocytogenes exhibiting resistance to antibiotics commonly used for the treatment of listeriosis including ampicillin and other penicillins, and gentamicin resulting in treatment failure is of major concern [77]. While antibiotic resistance of *L. monocytogenes* isolates from food, the environment, and human have been reported during the present decade [50], differences between the phenotypic antibiotic resistance of isolates from different ecological niches appear to vary by antimicrobial usage in humans and animals of different geographical location [3,47,51,78]. The isolates in this study showed sensitivity to antibiotics within the penicillin class, 2nd generation fluoroquinolones, glucopeptide class, and rifampicin while showing complete resistance to phenicols, polypeptides, and lincosamides. The isolates also showed the characteristic intrinsic resistance to cephalosporins and 1st generation fluoroquinolones [79]. The isolates were variable in their resistance to the macrolide, erythromycin, the aminoglycosides, neomycin and streptomycin, tetracycline, and the combination folic acid pathway inhibitors sulphamethozole-trimethoprim indicating acquired resistance from mutation or horizontal gene transfer. The prevalence of 44% (12/27) of isolates showing sulphamethozole-trimethoprim resistance is concerning as there are very few reports of this resistance. Importantly, this is a treatment option utilized in cases of penicillin resistance and so presents a significant risk for human treatment of listeriosis. Bertsch et al. [80] detected the presence of the trimethoprim resistance genes *drfA, drfD,* and *drfG* within isolates demonstrating resistance to trimethoprim. Horizontal gene transfer of tetracycline resistance has been identified previously in *L. monocytogenes* in relation to *tet*(M) gene on a Tn*916* family conjugative transposons [80] and plasmid [81] and *tet*(S) bearing conjugative plasmid [82]. Plasmid mediated resistance to erythromycin and streptomycin has also been documented in *L. monocytogenes* [82,83]. The MAR_{index} indicates the relative level of

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resistance to antibiotics. The $MAR_{index}$was driven by the antibiotic resistance which was variable in the isolates. The highest MAR_{index} was identified in the isolate from clinical septicemia with the variable resistance to neomycin, tetracycline, erythromycin, and sulphamethozole-trimethoprim. The majority of higher MAR_{index} were associated with isolates from chickens (layers or broilers), silage, and frozen fish which was driven by the presence of resistance to tetracycline and sulphamethozole-trimethoprim. *L. monocytogenes* strains resistant to one or more antibiotics have been recovered from environmental, food and from sporadic cases of human listeriosis [3,22,23,47,50-52,78]. Importantly, the common-use drug treatment combination of ampicillin-gentamicin was not disrupted in any of the isolates indicating high potential success of treatment. However the high resistance to sulphamethozole-trimethoprim is concerning.

4. Materials and Methods

This work was performed in accordance with the recommendations in the updated Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NRC, 2010). All procedures were approved prior to experimentation by the Cairo University Ethical Committee in compliance with the United Kingdom (UK) Animals (scientific procedures) Act of 1986.

4.1. Food and clinical samples

Prevalence studies were carried out in the Greater Cairo Area (GCA) which includes the cities in the Cairo, Giza and in the Qalyubia Governorates, with a total population estimated at 20,500,000 (as of 2012); area: 1,709 km^2; density: 10,400/km^2 to detect the presence of *Listeria monocytogenes* in retail food and from animals or human clinical cases showing septicemia and abortion. A total of 2039 samples were collected from different environmental niches and tested for the presence of *L. monocytogenes* following International Organization for Standards 11290-1 (NF EN ISO 11290-1, 1996) during the year 2016: Pasteurized milk (n=77); Milk by-products, kariesh cheese (n= 120) and yogurt (n= 70); Chicken, chicken filet (n=100), broilers internal organs (n=120) and layers internal organs (n=120); Table eggs (n=100); Meat, retail meat (n=100) and meat by-products (hamburger n= 50 and basturma n= 50); Duck internal organs (n=60); Silage (n=90); Frozen seafood, fish (n=100), fish filet (n=58), herring (n=66) and shrimp (n=50); Brain tissue, cattle (n=25), sheep (n=20) and rabbits (n=30); Abortion, uterine discharge (cows, n=20 and ewes n=10) and fetal livers (cows n=15 and goats n=15); Septicemia (cows n=18, buffaloes n=14, ewes n=24, goats n=20 and women n=65). Samples consisting of 100 g from different retail stores were kept on ice during transportation and analysis was initiated within 4 h.

4.2. Isolation, identification, and serotyping of L. monocytogenes

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Isolation of *Listeria* was performed as in Osman et al. [32,34]. Briefly, subsamples of 25 g or 25 mL were aseptically transferred into sterile stomacher closure bags containing 500 mL of half-strength Fraser enrichment broth with CCFA supplement (pre-enrichment broth) and homogenized for 1 min. Samples in pre-treatment broth were incubated at 30°C for 48 h. The pre-enrichment culture was diluted 1:100 into 10 mL of full strength Fraser enrichment broth with CCFA supplement (enrichment broth) and incubated at 35°C for 48 h. A loopful of the enrichment culture was streaked onto PALCAM *Listeria* agar plates and incubated at 37°C for 24 to 48 h for presumptive isolation and differentiation for *Listeria* species. Colonies were transferred onto tryptic soy yeast extract agar (TSYEA) and incubated at 30°C for 24 h. Strains were identified using the API *Listeria* system and the Oxoid Microbact *Listeria* 12L system. As previously described [32,34], isolates were examined for morphological and biochemical characteristics by Gram stain, tumbling motility at 20-25°C, catalase test, Methyl Red (MR) and Voges-Prosakuer tests, hemolysis determined by 5% sheep blood agar, carbohydrate utilization, CAMP test, and phosphatidylinositol-specific phospholipase C (PI-PLC) assay. The enrichment and isolation resulted in 20 identified *L. monocytogenes.* In addition, seven previously isolated *L. monocytogenes* from raw milk samples collected from ewes, goats, buffaloes, and cows were included in this study [32,34]. Serotype was assayed following manufacturer's instructions of commercially available antisera against serovars 1 and 4 (Behringwerke AG).

4.3. Antibiogram profile

The 27 isolated *L. monocytogenes* were tested for their resistance to 28 antibiotics belonging to 11 drug classes by the Kirby-Bauer disk diffusion antibiotic sensitivity testing and interpreted based on CLSI standards [84] or manufacture's' instructions. The classes of antibiotics and individual antibiotics were: Penicillins: Ampicillin (25 µg), Penicillin G (10 IU), Amoxicillin/clavulanic acid (10 µg), Cloxacillin (5 µg), Oxacillin (1 µg), Amoxicillin (25 µg); Fluorquinolones: Ofloxacin (10 µg), Enrofloxacin (10 µg), Ciprofloxacin (5 µg), Flumequine (30 µg), Pefloxacin (30 µg); Aminoglycosides: Amikacin (30 µg), Gentamicin (10 µg), Kanamycin (30 µg), Neomycin (10 µg), Streptomycin (10 µg); Cephalosporins: Cefotaxime (30 µg), Cephalothin (30 µg); Lincosamides: Lincomycin (2 µg), Clindamycin (2 µg); Phenicol: Chloramphenicol (30 µg); Tetracycline: Tetracycline (30 µg); Glycopeptide: Vancomycin (30 µg); Rifamycin: Rifampicin (5 µg); Macrolide: Erythromycin (15 µg), Spiramycin (100 µg); Polypeptides: Bacitracin (10 units); Folic acid metabolism inhibitors: Trimethoprim-sulfamathoxazole 1:19 (25 µg).

4.4. Pathogenicity and biofilm formation

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The pathogenicity of the 27 *L. monocytogenes* isolates was accessed, as previously implemented by Osman et al. [32,34], by: Anton's eye test for purulent keratoconjunctivitis, the infection and mortality of experimental mice and chick embryos, cytotoxicity of Vero cells and biofilm formation by Christensen's tube method and microtiter plate assays.

4.5. Molecular confirmation of L. monocytogenes and detection of virulence genes

The 27 isolates, 20 isolates from recent sampling and the 7 resuscitated from frozen glycerol stocks (40% glycerol stored at -70°C), freshly grown on TSYEA were used for DNA extraction. DNA extraction and PCR confirmation of species were performed as previously described [33]. Colonies of isolates were boiled in 400 µL of TE (10 mMTris-HCl, 1 mM EDTA, pH 8.0) for 10 min and centrifuged at 14,000 rpm for 10 min. The supernatant was immediately used for PCR reactions. The genus was confirmed by PCR as previously described utilizing primers (Table S3) specific for the 16S rRNA gene of *Listeria.* PCR reaction were performed in 50 µL containing 2 µL of DNA extract, 1.5 mM $MgCl_2$, 250 µM dNTPs, 1x Ampli PCR buffer, 0.5 µM of each primer, and 1.25 U of AmpliTaq DNA polymerase and filled with molecular grade water to volume. The reaction was overlaid with mineral oil and tubes were placed in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The conditions for amplification were initially denatured for 94°C for 4 min followed by 25 cycles of 1 min at 94°C, 60°C for 1 min, 72°C for 1 min; ending with final extension for 5 min at 72°C. *L. monocytogenes* strain (ATCC 19115) and an *E. coli* strain (ATCC 25922) were included as positive and negative controls, respectively. Intragenic regions of 11 virulence genes (*prfA, hlyA, inlA, inlB, inlC, inlJ, plcA, plcB, Iap, actA,* and *flaA*) were examined by PCR. The primers are included in Table S3. The *L. monocytogenes* virulence genes form the LIPI-1 pathogenicity island (*plcA, plcB, prfA,* and *actA*), the genes encoding internalin proteins (*inlA, inlB, inlC* and *inlJ*), an adhesion protein (*lap),* and a flagellin protein (*flaA*). Bacterial isolates and reference strains were subjected to PCR assay. The PCR conditions were optimized to 1 mM of each primer, 0.65 UTaq DNA Polymerase, 0.2 mMdNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, and 2 µL of template in 25µl final volume. Amplification was performed in a thermal cycler with an initial denaturation step of 95°C for 5 min, followed by 35 amplification cycles of 15 sec at 95ºC (denaturation), 30 sec at 55°C (annealing), and 90 sec at 72°C (primer extension) followed by a final extension step of 72°C for 10 min. The PCR products were determined by separation by electrophoresis in 1.5% agarose and TAE (Tris-acetate EDTA) buffer. Gels were visualized with a UV transilluminator after staining with ethidium bromide.

Partial *prfA* gene PCR products were purified with GeneJET PCR Purification Kit, sequenced using BigDye Terminator V3.1 Cycle Sequencing kit with forward and reverse primers following manufacturer's instructions, and resulting reaction were detected by ABI 3730 xl DNA analyzer. The sequences were trimmed at the 5' and 3' end to remove quality less than a confidence of 30 and were aligned to full length

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prfA and merged into a single sequence representing the PCR product. The sequences were aligned using Mega7 [85] software and the Muscle alignment [86] with default settings. Single nucleotide changes and the subsequent amino acid change, if applicable, were visualized in Mega7. The gene sequences of *prfA* gene fragments determined in this study were deposited in the GenBank database under accession numbers: KP271933, KP271934, KP271935, KP271936, KP271937, KP271938, KP271939, KP271940, KP271941, KP271942, KP271943, KP271944, KP271945, KP271946, KP271947, KP271948, KX906905, KX906906, KX906907, KX906908, KX906909, KX906910, KX906911, KX906912, KX906913, KX906914 and KX906915.

4.6. Statistical Analysis

Analysis was performed only on factors that showed differences. Antibiotic resistance phenotypic profiles, gene presence, and biochemical results were converted into numerical coding. Sensitivity to an antibiotic was represented as 0 and resistance was represented as 1. Presence or absence of a specific gene (eg. *hlyA*) was represented as 1 and 0, respectively. Statistical analysis was performed using the open statistical program R [87]. Heatmap representations with dendrograms and partitions were plotted using the function 'heatmap.3' in the 'GMD' package [88]. Correlations for variables were calculated using the 'cor' function and 'cor.test' function to determine significance. Significant correlations were visualized utilizing the 'corrplot' function from the 'corrplot' R package [89]. False Discovery Rate function was used to correct *p*-values for multiple comparisons [90]. The R packages 'FactoMineR' [91] and 'factoextra' [92] were used to perform and visualize Principal component analysis (PCA). Multivariant statistical analysis was performed using functions in the R package 'vegan' [93]. Binomial similarity matrices were calculated for the isolate profiles using 'vegdist' function as input for permutational multivariate ANOVA (MANOVA) analyses using the 'adonis' function.

4.7. Phylogenetic analysis of the prfA gene

Partial *prfA* gene PCR products were purified with GeneJET PCR Purification Kit, sequenced using BigDye Terminator V3.1 Cycle Sequencing kit with forward and reverse primers following manufacturer's instructions, and resulting reaction were detected by ABI 3730 xl DNA analyzer. The sequences were trimmed at the 5' and 3' end to remove quality less than a confidence of 30 and were aligned to full length *prfA* and merged into a single sequence representing the PCR product. The sequences were aligned using Mega7 [85] software and the Muscle alignment [86] with default settings. Single nucleotide changes and the subsequent amino acid change, if applicable, were visualized in Mega7.

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436 The *prfA* nucleotide sequence of each isolate was trimmed, in frame, to 360 bp in length. A
437 translation alignment was performed using Geneious software [94], followed by phylogenetic NeighborNet
438 analysis using SplitsTree software [95,96].
439

440 ## 5. Conclusion

441 While additional isolates would be preferable in a study such as this, the low incidence of isolation is an
442 indication of good practices in food preparation that can be further improved. Additional isolates may have
443 strengthen the detected significant correlations while also identify additional correlations. In the future, it
444 will be essential to carry out systematic characterization studies of *L. monocytogenes* subtypes in all 4
445 niches –humans, animals, food and environment – in order to improve understanding of the pathogen's
446 ecology. and high resolution population genomic analysis of both human and animal strains will allow
447 tracing evolutionary origins and to predict how often *L. monocytogenes* host switches have occurred. These
448 studies will be important to predict if animals represent a reservoir for the emergence of new virulent strains
449 affecting humans, which can facilitate assessment of their potential public health significance, and
450 conversely if human to animal host switches may represent a significant threat to food security to help to
451 mitigate infection of humans and animals through specific control measures in the context of disease
452 prevention and to limit economic losses associated with food recalls, and animal health. Furthermore, the
453 molecular characterization of the host-pathogen interactions which are central to host adaptation could lead
454 to the identification of novel therapeutic targets for the control of bacterial infections.
455

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Author contributions

KMO and AS supervised the research work, designed the experimental work, literature search, data collection and prepared the manuscript. AO was responsible for the sample collection and carried out the experiments. ADK conducted the bioinformatics analysis and interpretation. EMF carried out the phylogenetic analysis of *prfA* gene sequences among isolates in this study. KMO, AS, ADK and EMF were involved in the preparation of the manuscript. All authors read and approved the final manuscript.

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Additional Information

Competing Interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

Not applicable.

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Table 1. Virulence and antibiotic resistance profiles that showed variability among the *L. monocytogenes* isolates in this study

	Virulence genotype						Biofilm formation		PrfA mutations					
Source of isolated *L. monocytogenes*	***hlyA***	***flaA***	***inlA***	***inlB***	***inlC***	***inl j***	**CT**	**MPA (O.D.)**	**E101K**	**K130I**	**G145S**	**G161D**	**S184P**	**Accession Numbers**
Cow milk	+	+	+	+	+	+	Strong	0.12	+	-	+	-	+	KP271933
Cow milk	+	+	+	+	+	+	Strong	0.12	-	-	+	-	+	KP271934
Buffalo milk	+	+	+	+	+	+	Moderate	0.15	-	-	+	+	-	KP271935
She-camel milk	+	+	+	+	+	+	Strong	0.56	-	-	+	-	+	KP271936
Ewe milk	+	+	+	+	+	ND	Strong	0.11	-	+	+	-	-	KP271937
Goat milk	+	+	+	+	+	ND	Moderate	0.2	-	+	+	-	-	KP271938
Goat milk	+	+	+	+	+	+	Strong	0.2	-	-	-	-	-	KP271939
Kariesh cheese	+	+	ND	+	+	+	Strong	0.16	-	-	-	-	-	KP271940
Hamburger	+	+	ND	+	+	+	Strong	0.56	-	-	+	-	+	KP271941
Broilers	+	ND	ND	ND	ND	+	Strong	0.12	-	-	+	-	+	KP271942
Broilers	ND	+	ND	ND	ND	ND	Strong	0.11	-	-	-	-	-	KP271943
Broilers	+	+	+	+	ND	+	Strong	0.11	-	-	-	-	-	KP271944
Layers	+	+	+	+	ND	+	Moderate	0.2	-	+	+	-	-	KP271945
Layers	+	+	+	+	ND	ND	Strong	0.1	-	+	+	-	-	KP271946
Layers	+	+	ND	ND	ND	ND	Strong	0.12	-	-	+	-	+	KP271947
Table eggs	+	+	+	+	+	ND	Moderate	0.15	-	-	+	-	+	KP271948
Duck	+	+	+	+	+	+	Moderate	0.15	-	-	+	-	+	KX906914
Silage	+	+	+	+	+	+	Strong	0.1	-	-	+	-	+	KX906909
Silage	+	ND	+	+	+	+	Strong	0.1	-	-	-	-	-	KX906910
Silage	+	ND	+	+	+	+	Strong	0.1	-	-	-	-	-	KX906911
Goat fetal liver	ND	ND	+	+	+	+	Strong	0.12	-	-	+	-	+	KX906913
Ewe blood (Septicemia)	+	+	+	+	+	+	Strong	0.12	-	-	+	-	+	KX906912
Woman blood (Septicemia)	+	+	+	+	+	+	Strong	0.12	-	-	+	-	+	KX906908
Frozen fish	+	+	ND	ND	ND	+	Strong	0.1	-	-	+	-	+	KX906905
Frozen fish	+	+	+	+	ND	ND	Strong	0.12	-	-	+	-	+	KX906906
Herring	+	+	+	+	+	ND	Moderate	0.15	-	-	+	-	-	KX906907
Rabbit (brain)	+	+	ND	ND	+	ND	Strong	0.1	-	-	+	-	+	KX906915

ND represents not detected

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Figure 1.pdf

Figure 1.Heatmap of Individual isolates showing hierarchical clustering of isolates and factors. Binary factors (such as antibiotics or genes) indicating presence as green (relative response 1) or absence as red (relative response 0). Clustering is based on Wald-like test (D_2) and for binary data.

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Figure 2.pdf

Figure 2. Protein alignment of PrfA protein from *L. monocytogenes*isolates. Alignment was made to PrfA from *Listeria monocytogenes*strain NH1 (Genebank: GCA_002969195.1). Differences to NH1 are highlighted and can be found in Table 1.

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Figure 3.pdf

Figure 3. Correlation matrix of Virulence and Antibiotic Resistance profiles that were different among the recovered *Listeria monocytogenes*. Only correlations that were significant ($p<0.05$) are represented in the matrix.

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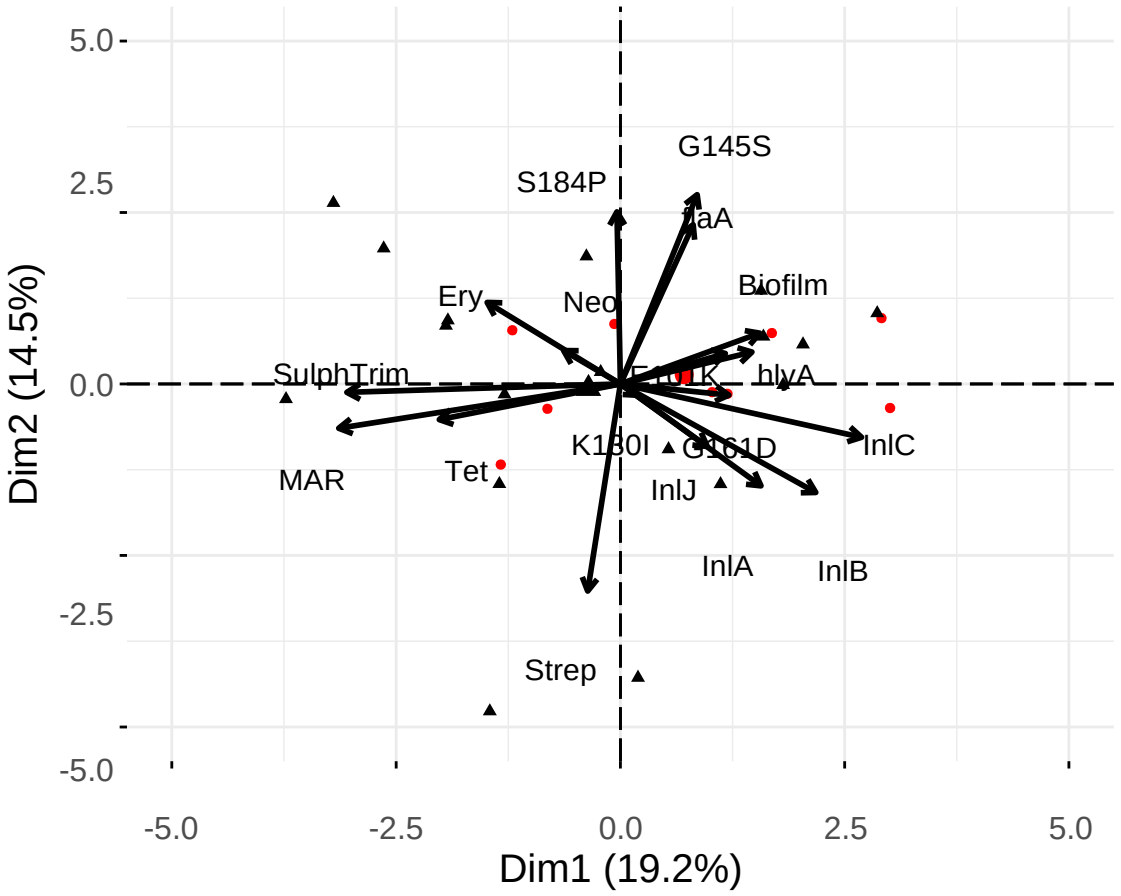

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Figure 4. Principle component analysis of factors and relationship with serotype and individual isolates.

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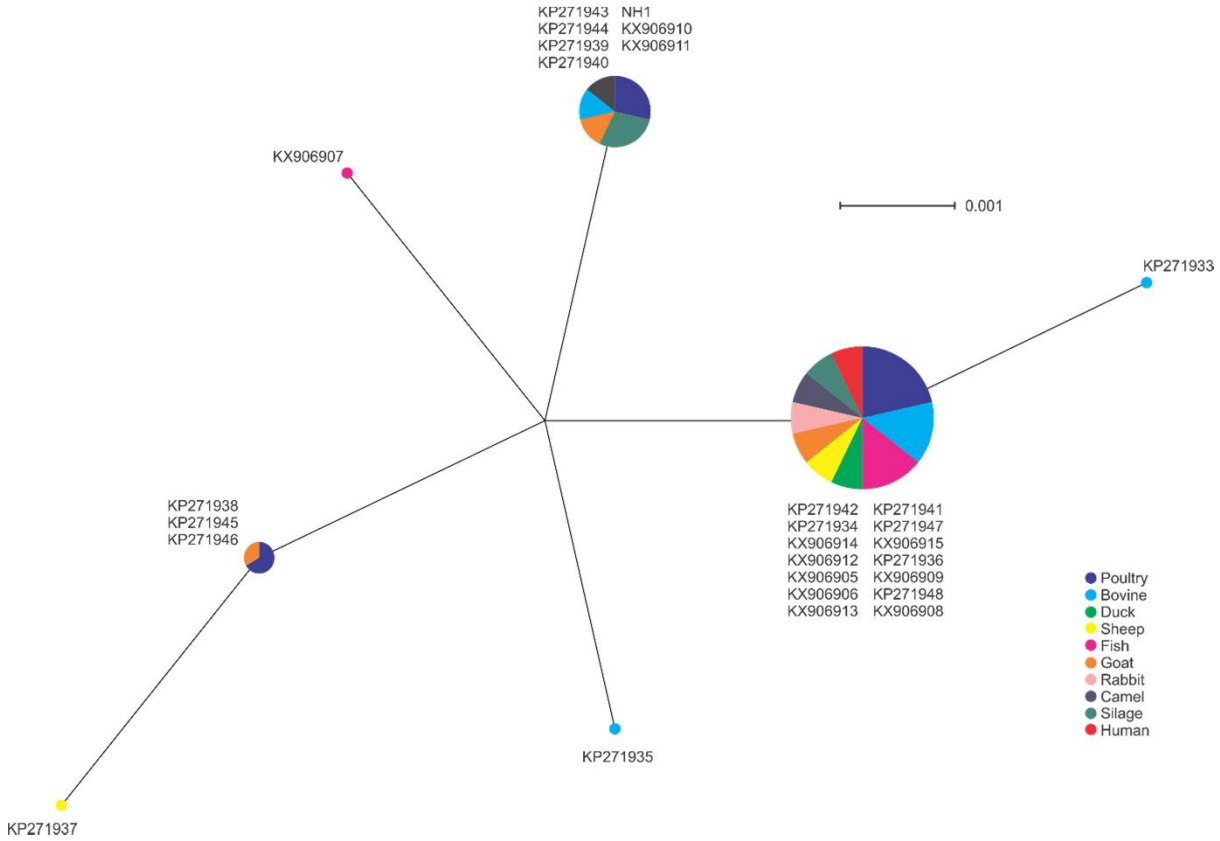

Figure 5. Phylogenetic analysis of *prfA* gene sequences among isolates in this study. Node size indicates proportion of isolates sharing a specific genotype.