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Short Communication

Isolation and Identification of *Listeria monocytogenes* from Raw Vegetables and Meat Sold in Quetta, Pakistan

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AS, FA and TMA designed the experiments. AS, MN and HA performed the experiments. TMA, JR and RA analyzed the data. All authors contributed in Manuscript writing.

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ABSTRACT

Water and soil have been reported as the pivotal reservoirs for *Listeria monocytogenes* and its subsequent transmission to food chain, plant material and animals. In present study, 800 food samples consisting of beef and chicken meat, milk, salads and fresh vegetables were collected from retail markets of Quetta, Pakistan. Multiplex PCR was employed to identify the prevalent species and genes associated with virulence. Only 10 (1.25%) samples were found contaminated with *Listeria monocytogenes*. Milk samples were the most contaminated as 4 samples collected from retail shops or dairy farm were found positive for *Listeria*. While 2 samples of each beef, chicken meat and salad were contaminated with *Listeria*. The virulent genes *inl*A, *inl*B, *prf*A, *hly*A, *act*A, *plc*A and *iap* were detected in all 10 positive samples for *Listeria.* The positive samples inventories were analyzed and were found that water used to wash the meat and vegetables was contaminated with *Listeria.* This study concludes that better hygienic measures should be adopted to keep the food material germ free.

The disease listeriosis is caused by *Listeria monocytogenes* which affects the extreme age and immuno-compromised people more severally when compared to other population (Lomonaco *et al*., 2009). Around, 17% fatality rate has been documented because of *Listeria* infections globally which is highest among all food borne pathogens (Helwigh, 2006). *L. monocytogenes* is prevalent in environment and has ability to survive under very adverse conditions (Razavilar and Genigeorgis, 1998). Although undercooked and fast foods are the main source of infection but *L. monocytogenes* can contaminate any type of food including vegetable and meat, when hygiene measures are ignored (Büla *et al*., 1995). Listeriosis may lead to pre-term birth, septic shock and death (Helwigh, 2006). To cause such fatal diseases, *L. monocytogenes* is equipped with several virulent factors. The contamination of food with virulent strains of *L. monocytogenes,* have severe implications on human health (Jensen *et al*., 2008). Among virulence factors of *L. monocytogenes* internalins (Inl) have been reported as the key factor (Wieczorek *et al*., 2012). For example, InlA is responsible for adhesion and invasion into host cells while InlC helps the *L. monocytogenes* during cell to cell spread. Similarly, InlJ helps the bacteria to cross the cell barriers (Shen *et al*., 2013). Moreover, *L. monocytogenes* has the capability to secrete toxin known as listeriolysin S, which helps to evade the phagocytosis of bacteria. These pathogenic factors along with several other virulent factors play an important role to cause fatal infection (Shen *et al*., 2013).

To treat listeriosis penicillin/ampicillin and gentamicin are drugs of choice but several studies have reported the antibiotic resistance to these drugs (Wieczorek *et al*., 2012). Moreover, *L. monocytogenes* isolated from food samples have shown antibiotic resistant to multiple drugs which may be because of irrational use of antibiotics (Shen *et al*., 2013).

Outbreaks of listeriosis originated from consumption of contaminated food have been documented around the globe (Feng *et al*., 2011). Almost all types of food items including meat and vegetables have been reported to be involved in outbreaks of *L. monocytogenes* (Yücel *et al*., 2005). To control and minimize the infections caused by *L. monocytogenes*, the very first step is to identify the source and reservoir of bacteria (Franciosa *et al*., 1998). To achieve this goal, determining the prevalence and level of contamination of food items with *L. monocytogenes*

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Table I. Nucleotide sequence of the Oligonucleotide primers used for the detection of *Listeria monocytogenes* and their virulence associated genes.

Target genes	Sequence of the primers (5'-3')	Expected amplicon size (bp)	References
hly	**F:**CATTAGTGGAAAGATGGAATG **R:**GTATCCTCCAGAGTGATCGA	730	Blais and Phillippe, 1995.
*plc*A	**F:**CTGCTTGAGCGTTCATGTCTCATCCCCC **R:**CATGGGTTTCACTCTCCTTCTAC	1484	Notermans *et al*., 1991
*prf*A	**F:**CTGTTGGAGCTCTTCTTGGGTGAAGCAATCG **R:**AGCAACCTCGGTACCATATACTAACTC	1060	Notermans *et al*., 1991
*inl*A	**F:**GGCTGGGCATAACCAAATTA **R:**CTTTTGTTGGTGCCGTAGGT	629	Montero *et al*., 2015
*inl*B	**F:**CCTAAACCTCCGACCAAACA **R:**CCATTTCGCGCTTCTCTATC	293	
*inl*J	**F:**TGTAACCCCGCTTACACAGTT **R:**AGCGGCTTGGCAGTCTAATA	238	Liu *et al*., 2007
iap	**F:**ACAAGCTGCACCTGTTGCAG **R:**TGACAGCGTGTGTAGTAGCA	131	Furrer *et al.,* 1991
*inl*C	**F:**AATTCCCACAGGACACAACC **R:**CGGGAATGCAATTTTTCACTA	517	Liu *et al*., 2007
*act*A	**F:**CGCCGCGGAAATTAAAAAAAGA **R:**ACGAAGGAACCGGGCTGCTAG	839	Suarez *et al*., 2001

is of utmost importance. In developed world studies have been conducted in routine to check the prevalence of *L. monocytogenes* in environment and food items but data is lacking from underdeveloped countries especially from Pakistan. This pilot study was designed to evaluate the contamination level and prevalent strains of *L. monocytogenes* in vegetables and meat sold in Quetta, Pakistan.

Materials and methods

From January to December, 800 food samples were collected randomly from street vendors and retailers in different areas of Quetta, Pakistan. The food items included poultry meat (n=200), beef meat (n=200), vegetables/salad (n=200) and fresh milk (n=200). The collected samples were transported to laboratory in sealed cold box for further processing.

The collected food samples were analyzed to determine the presence of *L. monocytogenes*. The enrichment, isolation and characterization were performed as been described earlier in detail (Chen *et al*., 2015).

Multiplex PCR was employed to detect the *Listeria* (Doumith *et al*., 2004) isolated from different food samples by targeting the virulent genes by specific primers. The details of primers used are given in Table I.

Results and discussion

To ensure food safety, detection of pathogenic bacteria should be a fundamental objective. The application of molecular techniques has facilitated the identification and characterization of major virulence-associated genes in *L. monocytogenes*. *L. monocytogenes* is made of multiple strains showing varied virulence potential. To implement effective control and prevention measures against *L. monocytogenes* infections, it is necessary to identify virulent, disease-causing strains from avirulent, non-pathogenic strain that are relatively harmless.

In current study, 800 samples were collected and screened for *L. monocytogenes*. Overall, the prevalence of *L. monocytogenes* was 1.25% (10/800). Out of 10, *L. monocytogenes* isolates, 1% (1/100) was found in fresh chicken, 1% (1/100) in processed chicken, 2% (2/100) in minced beef, 1% (1/100) in milk collected from dairy farm, 3% (3/100) in milk obtained from retail shops and 2% (2/100) in fresh salad whereas no *L. monocytogenes* isolate was found in diced beef samples (0/100) and fresh vegetables (0/100) (Table II).

The ten isolates of L. *monocytogenes* isolated from different food sources were analyzed for the presence of virulence associated genes employing the multiplex PCR (Supplementary Fig. 1 and 2).

All the ten *Listeria* isolates (100%) were positive for *hly*A, *plc*A, *prf*A, *act*A, *iap, inl*A, and *inl*B genes while 70% of the isolates were positive for *inl*C gene and only 50% for *inl*J gene (Table III).

The overall prevalence of *L. monocytogenes* contamination in food samples observed in the present investigation was 1.25% (10/800). However, results of this study indicate lower prevalence as compared to other studies conducted by Nayak *et al*. (2015) in India (9%), Braga *et al*. (2017) in Uruguay (11.2%), Ismaiel *et al*. (2014) in Egypt (13.3%), Chen *et al*. (2015) in China (22%) and by Montero *et al*. (2013) in Chile (25%) in foods. Likewise, investigating the raw meat samples in

China Wu *et al*. (2015) found a higher prevalence of *L. monocytogenes* isolates (23.7%). In Iran Pournajaf *et al*. (2016) isolated *L. monocytogenes* in 5.2% of the processed meat samples and 7.4% of dairy products. Difference in prevalence may be due to the types of analyzed food during the investigation in this study.

Table II. The prevalence of *Listeria monocytogenes* isolates in different food items in Quetta.

Food items	No. of samples analyzed	Samples contaminated with *Listeria* (n/%)
Fresh chicken	100	1 (1%)
Processed chicken	100	1(1%)
Beef diced	100	0(0%)
Beef minced	100	2(2%)
Milk dairy farm	100	1(1%)
Milk retail shops	100	3(3%)
Fresh vegetables	100	0(0%)
Fresh salad	100	2(2%)
Total	800	10 (1.25%)

Table III. Occurrence of virulence-associated genes present in *L. monocytogenes* isolated from various foods.

Source	No. of isolates	Virulence-associated genes								
		hlyA	plcA	prfA	actA	iap	inlA	inlB	inlC	inlJ
Food	100%	+	+	+	+	+	+	+	-	-
	70%	-	-	-	-	-	-	-	+	-
	50%	-	-	-	-	-	-	-	-	+

In this study the highest prevalence of *L. monocytogenes* contamination was observed in milk samples (3%) obtained from retail shops followed by fresh salad (2%) and minced beef samples (2%). These findings highlight the potential role of these food types in the spread of *L. monocytogenes* and alert the health personnel about proper handling and cooking (Cossart *et al*., 2011). Wu *et al*. (2015) observed higher contamination frequency (5.7%) in vegetables in China. In another study, Cordano and Jacquet (2009) analyzed frozen and fresh vegetables from 2000 to 2005, reporting a *L. monocytogenes* prevalence of 26 and 10%, respectively.

L. monocytogenes comprises multiple strains that exhibit varying virulence potential. Although the differences in the virulence potential is not completely due to the presence of these genes, but these virulence makers play an important role in the pathogenicity of *L. monocytogenes*. Thus, detection of these markers may represent a quick approach for preliminary discrimination of potentially virulent strains from avirulent strains of *L. monocytogenes*. It has been suggested that diagnosis of pathogenic *Listeria* spp. and listerial infections should ideally be based on virulence markers (Notermans *et al*., 1991). Moreover, the importance of PCR has been investigated for detection of *L. monocytogenes* from foods (Gouws and Liedemann, 2005).

In the present study a multiplex PCR was standardized for the simultaneous detection of *inl*A gene and the *hly*A, *plc*A, *prf*A, *act*A, *iap*, *inl*B, *inl*C and *inl*J gene for the virulence determination of the pathogen.

Genotypic characterization of *L. monocytogenes* isolates from the different food samples in this study revealed the presence of *inl*A gene in all isolates (100%); these results are similar to those obtained by Liu *et al*. (2007) in USA and Almeida *et al*. (2000) in Brazil. Study conducted by Jung *et al*. (2003) showed that the *inl*A gene is species-specific, thereby suggesting their species-wide sequence conservation. Multiple virulence factors, including phosphatidylinositol phospholipase C (*plc*A), haemolysin (*hly*A), actin polymerization protein (*act*A), invasive associated protein (*iap*), and internalin A (*inl*A), are necessary for the pathogenesis of *L. monocytogenes*. Therefore, detection of just one virulence factor by PCR is not always sufficient to identify *L. monocytogenes* strains. In addition, it is plausible that spontaneous mutations enable the removal of one or more virulence determinants in some *L. monocytogenes* strains. Thus, simultaneous detection of multiple virulent genes in a single assay is desirable as it reduces the time and labor involved and will be useful for large-scale investigations for detection of pathogenic strains of *Listeria* (Rawool *et al*., 2007). According to this study, the prevalence of *inl*C and *inl*J virulence genes was 70% and 50% respectively which is comparable with the findings by Pounajaf *et al*. (2016) reporting the prevalence of inlC and inlJ genes in 64% and 57% of clinical samples and 84% and 78% of food specimens, respectively. These findings implicate a role of the virulence genes *inl*A, *inl*C and *inl*J in the pathogenesis of *L. monocytogenes* isolates.

In this study the multiplex PCR detected *hly*A gene in 100% of *L. monocytogenes* isolates. Similar findings were reported by Jallewar *et al*. (2007). However, the result of this study is not in agreement with the result of Usman *et al*. (2016), who reported the carriage of *hly*A gene by 25%. It may be possible that some *L. monocytogenes* strains may lack one or more virulence determinants because of spontaneous mutations (Cooray *et al*., 1994).

Conclusion

The overall prevalence of *L. monocytogenes* in food was found to be 1.25%. *L. monocytogenes* carried the virulence genes including *hly*A, *plc*A, *prf*A, *act*A, *iap*,

*inl*A, *inl*B, *inl*C and *inl*J genes indicating the pathogenic potential of the *Listeria* isolates. The highest prevalence in milk, fresh salad, minced beef samples in this study highlight the potential that these foods may play an important role in the spread of foodborne pathogen and alert to health personnel about the need to proper handling and cooking in individuals.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190402110434

Statement of conflict of interest

The author declare no conflict of interest.

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