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STUDY ON OCCURRENCE OF *LISTERIA MONOCYTOGENES* IN SOIL OF PUNJAB PROVINCE AND ITS ASSOCIATED RISK FACTORS

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ABSTRACT

Listeriosis, caused by *Listeria monocytogenes (Lm),* has zoonotic implications and has a wide host range. It has unique potential to cross blood brain barrier and placental barrier which results in encephalitis, meningitis and abortions. Initially only animals were considered as its victim but later on it emerged as an important food borne human pathogen and so far, it has been isolated from various food samples and environmental samples too. The current study is based upon the determination of genome-based distribution of *L. monocytogenes* (*hly* gene) in various soil samples collected from selected districts of Punjab province, Pakistan. The selection of the districts was based on historical information about simultaneous prevalence of the soil borne human and animal diseases... A total of 970 soil samples were processed independently by real-time PCR and an association between occurrences in soil was correlated to several categorical variables. The genome was detected in 17 samples (1.75%, 95 % CI: ±0.5), each originating from different location across districts under study. A greater number of positive soil samples for *Lm genome* were detected in district Attock followed by Sheikhupura, Faisalabad, Sargodha, Sahiwal and Lahore. The odds for occurrence of *Lm* were more at places near to water sources [2.85, CI:95% (0.9256, 8.831) p=0.05] and having more than 1000 animals per village [3.32, CI:95% (1.077, 10.27) p=0.02]. In conclusion, DNA of *Lm* is distributed in the soil of several districts of Punjab and fodder grown on such contaminated soil could be a potential risk factor to health of both animals and humans. Future studies are required to identify a causal relationship between presence of bacterium in soil to seroconversion in animal and human and prevalent serotypes across a wide geographical range across Pakistan.

Keywords: *Listeria monocytogenes*, Real time PCR *hly* gene, soil

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INTRODUCTION

Listeria monocytogenes is the etiological agent of listeriosis which is a highly infectious disease of zoonotic importance (Dhama *et al*. 2013). *L. monocytogenes* is widely distributed in nature across the world and has been isolated from water, vegetation, sediment, livestock, wild and domestic animals, feces of the animals and also from the surroundings of the animals (Locatelli *et al*. 2013)b. The infections of *L. monocytogenes* occur in sporadic or epidemic forms across the world and multiple outbreaks across the world are reported due to it (Malik *et al.* 2002). Listeria invades the host with the help of several unique virulence factors like Listeriolysin (LLO), ActA protein, phospholipases and metalloproteases. It has got the unique ability of crossing intestinal, blood brain and feto-placental barriers. (Camejo *et al*. 2011).In ruminants it can affect almost ten percent or less of the herd which results in 30 % or more morbidity rate. In ruminants this disease is characterized by septicaemic or encephalitic form. The encephalitic form of animal listeriosis includes nervous signs like circling, head pressing, excessive salivation, and unilateral facial paralysis. The septicaemic form is relatively rare and is mostly observed in the neonates. It is characterized by depression, loss of appetite, fever and death. Late term abortion in affected animals is observed around or after 12 weeks in sheep and 7 months in cattle (Nightingale *et al.* 2004). In addition, its direct contact with eyes in form of feed (silage) can also lead to eye infections and keratitis (Nightingale *et al.* 2005). Mastitis is rarely reported while gastro-intestinal infections are often seen in sheep (Clark *et al.* 2004). *Listeria monocytogenes* emerged as zoonotic pathogen in 20th century but still its transmission to humans is vague. Food processing environment is considered one of the potent source of contamination which can lead to transmission of *Listeria monocytogenes* in animals (Roberts and Wiedmann 2003).

In Pakistan very little work has been done on isolation of *L. monocytogenes*. Previously done work only encompasses *L. monocytogenes* count in ready to eat products (Hussain *et al*. 2015), raw milk (Chandio *et al.* 2007) and poultry meat (Mahmood *et al.* 2003). No study has ever been conducted in Pakistan to investigate the distribution of *L. monocytogenes* in the environmental

soil and also the effect of several risk factors on the presence of this bacterium. Therefore to establish baseline data about its epidemiology and distribution in the environment (particularly soil), an extensive study based on *hly* gene (as it is highly conserved in all *Lm* strains (Rodríguez-Lázaro *et al.* 2004)) detection through real time PCR was conductedin which highly disease prone districts of Punjab province were targeted.. This disease related information in both humans and animals were taken from health and livestock departments, government of the Punjab, respectively. In this study prevalence of *L. monocytogenes* was reported from areas with or without animal human interaction. Several risk factors including weather attributes, presence of domestic animals and distance from animal market, main road, and water bodies were also assessed for establishment of any association between samples positive for *L. monocytogenes*.

MATERIALS AND METHODS

Study design: This study was the continuation of the previously published work on prevalence of soil borne pathogens in Pakistan and is the part of the project entitled "Spatial ecology and epidemiology of soil-borne human and animal bacterial pathogens and their public health significance in Pakistan" (Shabbir *et al.* 2016). Briefly, Punjab province is the largest on the basis of population with around 110,012,442 individuals and second largest of Pakistan on basis of area with 205,334 square kilometers (GoP 2017). The majority of population (64%) of Punjab is associated with agriculture and livestock in rural areas (Pakistan bureau of Statistics 2010). In this study three staged sampling design was implied which involved primarily the selection of nine highly disease prone districts of Punjab (Directorates of Animal and Human Health, Punjab). During the second stage by assumption of 50% prevalence rate of Lm, 95 % confidence interval and 5 % level of significance a total of 4883 villages were targeted in selected districts. But for more precise results, randomly ten percent (n=485) villages were targeted representing each of the study district. During third stage, from each village based upon convenient sampling, five sites were selected. Four soil samples were taken from the places like livestock farm where close interaction between animal and human was observed, while the fifth soil sample was collected from a barren uncultivated land, away from that village and where there was possibly no human and animal interaction.

Sample collection: For the sampling purpose and as a standard protocol, upper 3 inches layer of ground soil was discarded and three aliquots of 500g of soil sample were collected in a clean labelled polythene zipper bag. During the whole process of soil sample collection, standard biosafety measures were adopted to avoid possible contact with any soil borne infectious agents of public health importance. A questionnaire (Fig.2) was also designed which was filled by asking specific questions from the farmers of the village. The questionnaire comprised of data relevant to village name, union council, number and type of animals (sheep/ cattle/ buffalo/ goat or any else) housed, common diseases encountered, vaccination history, distance from the nearest animal market or wetland, availability of any satisfactory veterinary facilities and its delivery system, etc. While performing interpretations of the results data, the above-mentioned information helped in establishing any possible associations between the risk factors and the prevalence of pathogens.

Molecular identification: The power soil DNA isolation Kit (MOBIO, West Carlsbad, CA, USA) was used for extraction of genomic DNA from the soil samples according to the manufacturer's protocol. Qualitative and quantitative analysis (ng/µL) of the extracted genome was carried out by using Nano drop (USA) at $A_{260/280}$ and $A_{260/230}$. Real time PCR assay (CFX 96, Bio-Rad) was used to determine the prevalence of *L. monocytogenes* in soil samples. Formerly reported primers (5′- CAT TAG TGG AAA GAT GGA ATG-3′, 5′- GTA AGC CAT TTC GTC ATC AT-3′) and probe (HEX-5′- TCA AGC TTA TCC AAA TGT AAG TGC AA-3′-BHQ) were adapted from the work of Le Monnier (Le Monnier *et al.* 2011). These oligonucleotides were able to amplify a 71 base pair fragment of *hly* gene (responsible for expression of listeriolysin O, a potent cytolysin used for getting entry into host cell by pore formation). The real time PCR reaction was carried out in a total reaction volume of 50 µL comprising of 500 nM of each primer (forward and reverse), 100 nM of probe, 25 µL master mix, 15 µL extracted DNA and remaining parts of nuclease free water. A total of 45 amplification cycles were performed involving following conditions: initial denaturation at 95°C for 10 min followed by denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 min. The real time assay was optimized and validated by using the positive controls (*Lm* culture, ATCC 19111).

Data analysis: Results revealed about prevalence of *L. monocytogenes* by real time PCR (numerical variables) and risk factors (categorical variables) for each sample were compiled in a Microsoft Excel spreadsheet. Data were analyzed through chi square test (numeric variables) and odds ratio (categorical variables) by utilizing 95% confidence interval and 5% level of significance by SPSS version 20.0 (SPSS Inc., Chicago, IL USA).

RESULTS

The prevalence of *L. monocytogenes* in selected districts of Punjab: In Punjab province by targeting

highly disease prone districts inclusive of Lahore, Sheikhupura, Faisalabad, Sargodha, Attock, Chakwal, Sahiwal, Dera Ghazi Khan and Gujranwala, 970 soil samples were subjected to *hly* gene based real time PCR for detection of *Lm* which revealed presence of *Lm* DNA in 17 samples [1.75, 95% CI: (1.1-2.79)].

The origin of these positive soil samples was from six different districts including five (n=5) from Attock, four (n=4) from Sheikhupura, two (n=2) from Sahiwal, two (n=2) from Sargodha, three (n=3) from Faisalabad and one (n=1) from Lahore. The details of villages positive for genome of *Lm* soil villages are; Kot Chaji, Kalu Kalan, Ababakar, Malli Toalan, Ghora Maar from Attock; Chak 33, Chak 12, Chak 18, KalaKhatai from Sheikhupura; Chak Khana, Chak 15/NB from Sargodha; 78/5L, 174/9L from Sahiwal; Chak 374GB, Chak 41 GB, Chak 55 GB from Faisalabad and Wasti Amin Pura from Lahore. The peak distribution pattern of *Lm* genome was observed in district Attock (5.55%, CI95%, 2.4-12.36) followed by Sheikhupura (3.39%, CI 95%, 1.33-8.39), Faisalabad (2.02%, CI 95%, 0.69-5.79), Sahiwal (1.96%, CI 95%, 0.54-6.87), Sargodha (1.35%, CI 95%, 0.37-4.79) and Lahore (1.72%, CI 95%, 0.3-9.13). From districts of Chakwal, Gujranwala and DG Khan none of the soil samples were declared positive for presence of genome of *Lm* (Table 1). All the positive soil samples were plotted on map which showed their overall distribution in Punjab province (Figure 1).

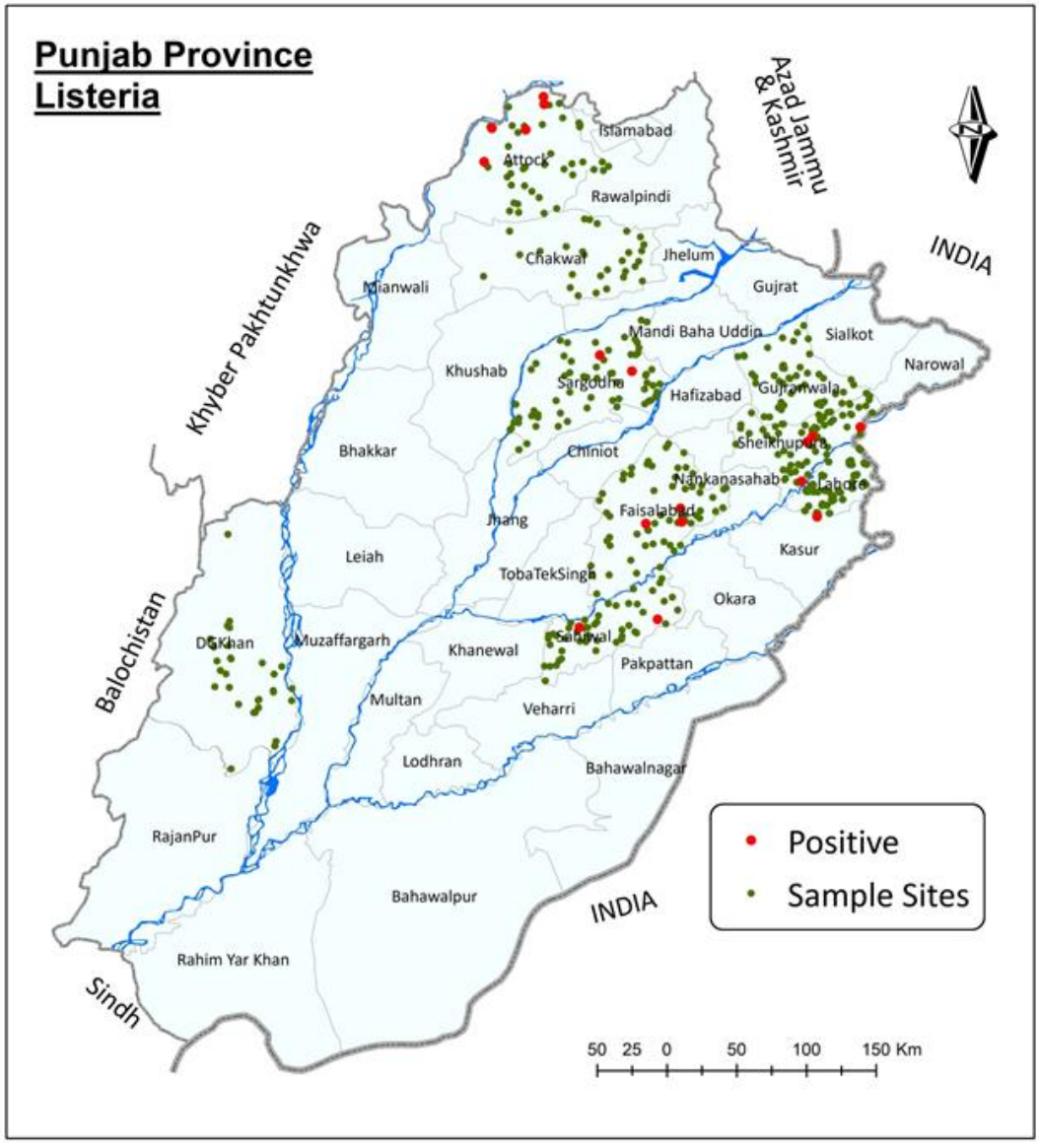

Fig. 1: The real time-based prevalence of *L. monocytogenes* across Punjab province, Pakistan

Table 1: Prevalence of *L. monocytogenes* in selected districts of Punjab Pakistan based upon Real time PCR detection.

District	Targeted Villages	Positive/total tested	Percentage	(95%CI)
Sheikhupura	59	4/118	3.39	(1.33-8.39)
Gujranwala	72	0/144	0.00	0.00
Faisalabad	74	3/148	2.02	(0.69-5.79)
Sargodha	74	2/148	1.35	(0.37-4.79)
Sahiwal	51	2/102	1.96	(0.54-6.87)
D.G. Khan	43	0/86	0.00	0.00
Chakwal	38	0/76	0.00	0.00
Attock	45	5/90	5.55	(2.4-12.36)
Lahore	29	1/58	1.72	(0.3-9.13)
Total	485	17/970	1.75	(1.1-2.79)

Correlation of risk factors with the sites positive for DNA of *L. monocytogenes:* The presence or absence of *Lm* DNA in relation to physical risk factors was evaluated by odds ratio test (OR). Categorical response (yes or no) was used to group all the physical risk factors to determine any correlation with the presence or absence of *Lm* DNA. For the estimation of precision of OR, 95% confidence interval was used. Upon evaluation of the data the presence of *Lm* DNA was positively correlated with distance from water sources [2.85(0.92-8.83), P=0.05] and number of animals [3.32(1.077-10.27)] P=0.02]. While the other parameters like distance from main road, distance from animal market and number of households in village were found not to be associated with the presence of *Lm* DNA (Table 2).

Table 2: Risk factors association with prevalence of *L. monocytogenes* across Punjab, Pakistan.

Sr.#	Variable	Categories	Positive sites	Negative sites	P-value	Odds ratio
1	Animal human interaction	Yes	8	477	0.80	0.88
		No	9	476		(0.3394, 2.318)
2	Distance from animal market	More than 1 km	10	690	0.21	0.55
		Less than 1 Km	7	263		(0.2051, 1.445)
3	Distance from main road	More than 500m	9	588	0.06	0.33
		Less than 500m	8	365		(0.1242, 0.9233)
4	Distance from water source	Less than 500m	13	507	0.05	2.85
		More than 500m	4	446		(0.9256, 8.831)
5	Animal population	Less than 1000	13	471	0.02	3.32
		More than 1000	4	482		(1.077, 10.27)
6	No of house hold in village	More than 300	8	641	0.07	0.43
		Less than 300	9	312		(0.408, 3.345)

DISCUSSION

The prevalence of *L. monocytogenes* in soil of the Punjab province was 1.7 % and it was the first ever evidence of its presence in soil of Pakistan. The previous reports about *Listeria monocytogenes* in Pakistan were related to ready to eat food which estimated its burden up to 25% (Hussain *et al*. 2015) in it, 6% to 12% in milk (Chandio *et al.* 2007) , 4% in fresh fruits and vegetables (Vahidy *et al.* 1992), 2.5% in poultry meat and 12.5% in poultry meat by-products (Mahmood *et al.* 2003). Several reports across the world exhibit varying prevalence rates of *L. monocytogenes* in soil. In comparison to Pakistan the prevalence of *L. monocytogenes* in India (neighboring country) was bit higher (5%) in Varanasi (Soni *et al.* 2014) , 5% in Odisha (Sarangi and Panda 2012) whereas Malaysia reported 0% prevalence rate in its local soil (Tze 2013). Contrarily higher prevalence rate of 7.9% were observed in USA (Weller *et al.* 2015), in Canada 8.3 % (Dowe *et al.* 1997) and 17.2 % in Germany (Weis and Seeliger 1975). The observations made in the current study corroborates less percentage prevalence of *L. monocytogenes* in comparison to other countries .One of the foremost reason could be the varying climatic conditions and seasonal patterns prevailing across the world (MacGowan *et al.* 1994)).

Soil is fortified with bacterial DNA which is liberated actively from bacteria or autolytic changes

occurring after its death releases it into the environment (Palmen and Hellingwerf 1997). l. Bacterial DNA can persist in soil for varying periods of time depending upon the presence or absence of degradation enzymes, type and physical conditions of soil (DeSalle *et al.* 1992). In our study, *L. monocytogenes* was not detected in the soil samples of district of Chakwal, DG Khan and Gujranwala and one of the possible reason behind its absence might be the increased nucleases activity in the soil which destroyed the genome of *L. monocytogenes*. In addition the pervasiveness of too harsh and grim temperature conditions also leads to destruction of genomic DNA (Bauer *et al.* 2003). Soil constitutes of some genome extraction and PCR inhibitors like humic acid and heavy metals. These PCR inhibitors intervene the results and leads to decreased efficacy of diagnostic test (Wilson 1997). . According to the findings of Ranjard and Richaume (2001), soil is home to a number of entities which results in contamination of the desired genome during DNA extraction process. As a consequence of this contamination, the target genome gets diluted leading to lesser chances of its detection. Likewise, real time PCR in comparison to culture-based identification methods also depicts the reduced microbial load in soil samples (Locatelli *et al.* 2013) a.

Soil of the six districts Faisalabad, Sheikhupura, Attock, Sahiwal, Lahore and Sargodha were positive for DNA of *L. monocytogenes*. Soil inhabits a variety of microbes but they considerably outnumber in surface soil especially encompassing macro pores (Bundt *et al.* 2001). The courses formed by earthworms, plant roots and soil inhabitants are termed as macro pores (Fierer *et al.* 2007). Majority of the positive soil samples were less than 500 meter away from water sources like ponds, canals, drains or rivers etc. High moisture content extends the survival time of *L. monocytogenes* (McLaughlin *et al.* 2011, Strawn *et al.* 2013).When the moisture levels are sustained at 7 % and 17 % it can survive for 180 days in clay type soil and 300 days in fertile type soil (Welshimer 1960). Soil of the rainy areas are rich in moisture content and it has shown positive association with the presence of *L. monocytogenes* (Weller *et al.* 2015).

On evaluation of risk factors, positive association (OR $\geq$ 1) for prevalence of genome o*f L. monocytogenes* was observed between following two variables ie. distance from water sources and animal population. High density of animals in an area and high population of humans built a strong interaction between them. Other variables like distance from animal market and main road were negatively associated with the presence of *L. monocytogenes*. The greater number of positive sites were adjacent or near to water sources, which gets contaminated as a consequence of entry of *L. monocytogenes*. The manure or runoff water from livestock barn leads to contamination of water and soil (Soupir *et al.* 2006). The infected animals might be the active source of its spread by secreting it in their feces and the runoff water from such contaminated farms disseminates it all around. In a study by Colburn *et al.* (1990), 62% of freshwater sources which were encountered by livestock were declared positive for *L. monocytogenes* and contrarily water sources not encountered by livestock showed prevalence of 5.9% (Arvanitidou *et al.* 1997).Furthermore water used for cultivation of crops must be investigated as source of spread of *L. monocytogenes* because sometimes sewage water also gets mixed with water used for irrigation. A study in France depicts almost 60% prevalence of *L. monocytogenes* in wastewater plant and on transmission of this water to rivers or other watering bodies it resulted in its further spread (Paillard *et al*. 2005).

Conclusion: In conclusion the genome of *L. monocytogenes* is present in soil of Punjab, Pakistan and its distribution are correlated to distance from water sources and density of animal population the existence of zoonotic *L. monocytogenes* in soil is a potential threat to health of both humans and animals. We are in dire need of extended studies to determine its molecular characterization, extensive surveillance across the country and its transmission patterns in humans and animals

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SAMPLING PROFORMA

Soil Pak #:……Dated:……Time: …..……

1): District:…… Tehsil:……

Union Council:…… Village:……...……

a): Geographical Co-ordinates: Long…….……Lat……..

b): Farmer's Name:……c) Cell……

d): Address:…….

2): Environmental Variables: Wind Flow:……Temperature……Humidity:

3): Animal Type……..Sex…….Age……

Water source…….Distance from main road……

4): Animal Housed in the Farm:……..……

Cattle	<5	5-10	>10
Buffalo	<5	5-10	>10
Sheep	<5	5-10	>10
Goat	<5	5-10	>10
Dog/Cat	<5	5-10	>10
Horse/Donkey	<5	5-10	>10

5): Nearest Animal Market:……..…….

6):	Common Diseases:	HS	FMD	PPR	ET	Anthrax Mastitis
7):	Vaccination:	HS	FMDV	PPRV	ETV	Anthrax Pox
8):	Poultry Vaccination:		NDV		AIV	Others
9):	Fertilizer Used: Yes / No		Organic / Inorganic			Both

Proforma filled by:……Signature:……..……