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Retrospective Study on the Association of Risk Factors of Johne's Disease along with Physiological Biomarker in Large Ruminants of Punjab, Pakistan

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Authors' Contribution
MA conducted the research. AZD conceptualized and supervised the study. MI and AAA served as members of supervisory committee. MU, MZI and QA wrote and proofread the manuscript. MH, AA and GM helped in laboratory work.

Key words
***Johne's disease*, MAP, ELISA, *Risk factors*, Hematology**

ABSTRACT

The present study elucidated sero-prevalence of *Mycobacterium avium* sub sp. *paratuberculosis* (MAP) in cattle and buffalo. Diarrhea is a major risk factor for MAP, so 1100 blood samples were collected from diarrheic animals or having history of some diarrheic episodes from four districts of Punjab i.e. Kasur (n=300), Rawalpindi (n=230), Lahore (n=230) and Bahawalpur (n=340). These samples were tested using ELISA and overall seroprevalence found was 39.64%. The major host-associated and non-associated risk factors of MAP were also considered. It was found that positive percentage of MAP was 2.20 times more in buffaloes than in cattle; 21.19 times more in females than in males; 1.04 times more in adult animals having age >6 years; and 1.20 times more in dry animals compared to milking animals. Furthermore, body condition score (BCS), type of housing, and condition of drinking water were also found to have significant ($p<0.05$) effect on the occurrence of MAP in cattle and buffalo. Moreover, hematological profile of MAP-positive animals showed significantly ($p<0.05$) lower values of RBCs, Hb, and PCV; whereas, levels of MCHC showed non-significant ($p>0.05$) rise in MAP-positive animals as compared to control animals. Thus, findings of the current study depict alarming situation of Johne's disease in Punjab, and help in understanding its associated risk factors and its effects on the health of cattle and buffalos.

INTRODUCTION

Dairy sector is one of the most vibrant segments of livestock Pakistan. It contributes almost 60.5 percent in agriculture sector which in turn has a share of 18.5 percent in Gross Domestic Product (GDP) of Pakistan (Economic Survey of Pakistan, 2018-19). One of the many problems dairy sector has been facing is Johne's disease also known as paratuberculosis. It is infectious enteritis of ruminants that cause huge economic losses to dairy farmers due to the loss of milk production and culling of diseased animals (Abbas *et al*., 2011). The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* which is non-motile, non-spore farming acid fast bacilli and facultative intracellular anaerobic in nature (Arsenault *et al*., 2014). The disease was first time reported in subcontinent in Lahore in 1912 (Twort *et al*., 1912). Its sero-prevalence in cattle and buffalo has been recorded 29% in Northern India (Singh *et al*., 2008), 33% in teaser bulls and 20% in breeding bulls in Pakistan (Abbas *et al*., 2011). The current position regarding the zoonotic potential of MAP is that there is some association between the causative agent of Johne's disease and human Crohn's disease (Liverani *et al*., 2014).

Diagnosis of MAP can be done by cultures, histological examinations and polymerase chain reaction (PCR). Histological examinations of the tissue are also rapid methods but currently serum ELISA and milk ELISA are good screening tests that are inexpensive, efficient and sensitive diagnostic methods (Ansar-Lari et al., 2013). It has also been reported that hematological profile of affected animals varies in this disease (Lybeck *et al*., 2011).

Detection and diagnosis of MAP is difficult due to long incubation period i.e. 4 months to 15 years (Nielsen

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et al., 2008). The long incubation period of Johne's disease makes its diagnosis through clinical signs at earlier stages impossible. Its cases are usually not reported and treated as general diarrheic cases. Consequently, it is feared that culling of MAP-positive animals is not done and Johne's disease has been becoming prevalent in Pakistan. What makes the situation even more uncertain, and worrisome, is the negligence and unawareness among farmer community public health workers and even veterinarians regarding MAP positive cases in Pakistan.

Keeping in view the aforementioned background, this study was designed to check seroprevalence of MAP using ELISA in cattle and buffalo of four districts of Punjab. The main risk factors of the disease were also taken into account. Moreover, hematological profile of the animals was also checked to determine the effects on the health of MAP-positive animals. It is anticipated that results of current study would be helpful to understand current status of MAP in Punjab, Pakistan and to plan future strategy for controlling this disease in dairy animals. Moreover, this study will also help in imparting knowledge of MAP in farmer community, public health workers, and veterinarians.

MATERIALS AND METHODS

Study population

The study was performed in cattle and buffalos of four districts of Punjab i.e. Kasur (n=300), Rawalpindi (n=230), Lahore (n=230) and Bahawalpur (n=340). These cattle and buffalo were either diarrheic or had a history of diarrheic episode in the past. Data about host associated risk factors including species (cattle and buffalo), gender (male and female), age (>6 years and <6 years), physiological parameters (milking and dry animals), BCS (1, 2, 3); and non-host associated risk factors mainly vaccination status (vaccinated and non-vaccinated), housing (open and confined housing) and water condition (clean water and Dirty water) were also considered and related data was collected in a questionnaire. Although vaccine is not available in Pakistan but it can be imported so its data was also considered.

Sample collection and processing

Based on selection criteria, 10 ml of blood samples were extracted from animals and equally poured in plain and EDTA mixed vacutainers for ELISA and hematology, respectively. These samples were transported in ice packed cooler to Medicine Laboratory, University of Veterinary and Animal Sciences, Lahore for further processing. All the samples were centrifuged for 5 min at 3000 RPM for collection of serum which was stored in Eppendorf tubes in deep freezer for further analysis.

Serological diagnosis

Serological diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* was done by commercially available ELISA (enzyme linked immuno sorbent assay) kit containing MAP (*Mycobacterium avium* subsp. *paratuberculosis*) antigen coated on it (IDEXX MAP ELISA Kit) according to manufacturer's guidelines.

Hematological profiling

The hematological profile mainly red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of all the animals was also determined in University Diagnostic Laboratory (UDL), University of Veterinary and Animal Science, Lahore.

Statistical analysis

The data was analyzed by applying Pearson Chi Square test, simple 't' test and one way ANOVA using Statistical Package of Social Sciences (SPSS) 2015 version. The values were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Out of 1100 blood samples collected from selected animals 436 were found positive through ELISA. The positive percentage were 31.33%, 42.61%, 54.35, and 35% in district Kasur, Rawalpindi, Lahore, and Bahawalpur, respectively as shown in Table I. So, Jhone's positive animals were found maximum in district Lahore which were 2.21 times greater in number than those found in district Bahawalpur (Table I). A similar study conducted on seroprevalence of Johne's disease in India showed 29% positive cases by indirect ELISA (Gupta *et al*., 2012). Age and stage of disease are the main factors in case of ELISA because ELISA easily detects antibodies in heavy shedders and in later stages of disease. It is important to highlight that MAP has a comparatively long latent period, and the antibody levels against MAP only can be detected at the end of the latent period by the ELISA test (Collins, 2011).

When different risk factors were taken into account then contrasting results were found. From host associated risk factors, buffalos were found to have higher positive percentage (46.61%) of MAP as compared to cattle (28.44%) as shown in Table II. The positive percentage in both genders showed that disease was 21.19 times higher in females (42.79%) compared to males 3.41% (Table II). This may be due to extra burden of milk production in females. In a similar study it was found that Johne's disease does persist in males as sero-prevalence 20.0% in breeding bull and 33.3% in teaser bull (Abbas *et al*., 2011). Whereas, no such contrasting difference was found in two age groups (Table II).

The link between physiological parameter and disease prevalence was also considered in this study. The prevalence of Johne's disease was found 1.20 times higher in milking animals (43.45%) than dry animals (39.06%). Similarly, BCS of animals was found significantly associated with MAP. Animals having BCS 1 had higher prevalence (66.82%) of MAP followed by those having BCS 2 (23.93%) (Table III). Whereas, animals belonged to BCS-3 category did not have any MAP-positive case. So, risk of MAP decreases with the increase in BCS score. The same finding was asserted by Ott and other in another study (Ott *et al*., 1997).

Table I. District wise positive percentage and statistical analysis of MAP in large ruminants.

Parameter/ Districts	Total (n)	Positive (%)	Od ratio (OR)	Chi Square value X^2	p value	Confidence interval (CI 95%)
Kasur	300	31.33	0.8474	33.35	<0.0001	CI: 0.2635 to0.3679
Rawalpindi	230	42.61	1.379			CI: 0.3639 to 0.4907
Lahore	230	54.35	2.211			CI: 0.4789 to 0.6066
Bahawalpur	340	35.00				CI: 0.3012 to 0.4021

Table II. Statistical analysis of host associated risk factors of MAP in large ruminants.

Parameters	Total (n)	Positive (%)	Od ratio (OR)	Chi square value X^2	P Value	Confidence interval (CI 95%)
Species						
Buffalo	678	46.61	2.20	35.91	0.0001	CI: 0.4288 to 0.5037
Cattle	422	28.44				CI: 0.2434 to 0.3292
Gender						
Female	1012	42.79	21.19	52.47	<0.0001	CI: 0.3977 to 0.4586
Male	88	3.41				CI: 0.0117 to 0.0955
Age						
<6 years	912	35.80	1.04	0.06	0.80	CI: 0.3667 to 0.4302
>6 years	188	38.83				CI: 0.3215 to 0.4595
Body condition score (BCS)						
1	434	66.82	227.2	233.70	<0.0001	CI: 0.6226 to 0.7109
2	610	23.93	35.6			CI: 0.2072 to 0.2748
3	56	0.00				CI: 0 to 0.0642
Physiological parameter						
Dry animals	145	43.45	1.20	1.01	0.31	CI: 0.3565 to 0.5158
Milking animals	955	39.06				CI: 0.3601 to 0.4219

Table III. Relationship of non-host associated risk factors with MAP.

Parameters	Total (n)	Positive (%)	Od ratio (OR)	Chi square value X^2	p value	Confidence interval (CI 95%)
Vaccination status						
Non-vaccinated	1035	42.13	95.38	45.36	<0.0001	CI: 0.3915 to 0.4516
Vaccinated	65	0.00				CI: 0 to 0.0558
Type of housing						
Confined	631	50.40	3.02	71.61	<0.0001	CI: 0.465 to 0.5428
Open	469	25.16				CI: 0.2145 to 0.2928
Water condition						
Dirty	947	40.13	1.16	0.68	0.41	CI: 0.3705 to 0.4328
Clean	153	36.60				CI: 0.2938 to 0.4448

This study also showed that the animals living in confined housing (50.40%) was 3.02 times more prone to MAP than those in open housing (25.16%) as described in Table III. So, in developed countries the higher prevalence rate of Johne's disease may be due to commercial farming and confined environment. Besides, the results of vaccination status were the most significant ($p<0.05$). Non-vaccinated animals showed 42.13% MAP-positive results by ELISA while no MAP-positive animal was found from 65 vaccinated animals targeted in this study (Table III). This finding confirmed that vaccination is the best way to control Johne's disease, however more studies are required for further confirmation. Source of drinking water also played a significant role in the occurrence of Jhone's disease. Animals drinking dirty water had 1.16 times higher prevalence of MAP (40.13%) than those living on clean water (36.6%) (Table III). This fact has been strongly proven in another study where animals drinking well water showed higher prevalence (26.83%) then tap water (16.67%) (Sun *et al*., 2015). Hematology parameters of MAP-positive animals and control-negative animals also showed significant differences (Table IV). Hematological profile of MAP-positive animals showed significantly ($p<0.05$) lower level of RBCs, Hb, and PCV; and non-significant ($p>0.05$) rise MCHC in MAP-positive animals when compared to those of control animals. Furthermore, MCV and MCH showed non-significant ($p>0.05$) change (Table IV). The same trend of hematology parameters was observed when these were compared to control negative animals of different districts using multiple comparison test (Tuckey's test) (Table V). These results coincide with a study conducted in Egypt by Abdelaal *et al*. (2019).

Table IV. Statistical analysis of hematology in control negative and MAP-positive animals.

Parameters	Groups		p-value ($p<0.05$)
	Control negative animals	MAP-positive animals	
RBCs ($10^6/\mu L$)	7.48 ± 1.00^a	4.16 ± 0.077^b	< 0.0001
Hb	9.09 ± 1.14^a	7.05 ± 0.084^b	< 0.0001
PCV	38.6 ± 5.05^a	21.85 ± 0.25^b	< 0.0001
MCV	51.4±7.77	52.11±0.60	0.646
MCH	14.21±2.28	14.76±0.28	0.392
MCHC	27.4±6.48	30.23±3.58	0.697

Within a row, values with different superscripts ^{a,b} variable significantly ($p<0.05$) from each other.

CONCLUSIONS

It is concluded from the study that animals having diarrhea or history of diarrheic episode had greater chances (39.64%) of having Johne's disease. Besides, different host associated (species, gender, age, physiological parameters, and BCS) and non-associated (vaccination status, housing, and drinking water) risk factors were significantly ($p<0.05$) related to MAP. Furthermore, hematological profile confirmed that MAP significantly ($p<0.05$) undermines animal's health. Based on these findings, it is suggested that MAP screening should also be made an integral part of time-to-time screening of diarrheic animals.

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Table V. One Way ANOVA results of hematology parameters of MAP-positive and control negative animals in different districts

Parameters	Groups					p-value ($p<0.05$)
	Control	Kasur	Rawalpindi	Lahore	Bahawalpur	
RBCs($10^6/\mu L$)	7.48 ± 1.00^a	4.12 ± 0.64^b	4.08 ± 0.62^b	4.13 ± 0.63^b	4.29 ± 0.54^b	< 0.0001
Hb	9.09 ± 1.14^a	7.12 ± 0.65^b	7.01 ± 0.60^b	7.05 ± 0.66^b	7.01 ± 0.75^b	< 0.0001
PCV	38.6 ± 5.05^a	21.91 ± 2.25^b	22.04 ± 1.80^b	21.43 ± 1.66^b	22.02 ± 2.07^b	< 0.0001
MCV	51.4±7.77	51.92±4.74	51.38±5.02	52.87±4.72	52.27±4.32	0.938
MCH	14.21±2.28	14.62±2.00	14.74±2.34	14.03±2.22	15.64±2.04	0.303
MCHC	27.4±6.48	27.2±6.26	25.87±5.46	26.67±5.50	27.87±6.47	0.408

Within a row, values with different superscripts; ^{a,b} variable significantly ($p<0.05$) from each other.

Statement of conflict of interest

The authors have declared no conflict of interest.

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