Research Article

Etio-Prevalence of Environmental Bacterial Species Causing Subclinical Mastitis in a Cohort of Buffaloes at Khyber Pakhtunkhwa

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Abstract | Subclinical mastitis is multifactorial inflammation of mammary glands in dairy animals, resulting in changes in milk quality, milk production, and economic losses to dairy farmers. It mainly occurs due to non-contagious environmental bacterial species. In Pakistan, it is the major disease of different dairy animals including bovines. However, only a little information is available about bacterial profile of the disease. A cross-sectional study was conducted to find the Etio-prevalence of bacterial species causing subclinical mastitis in a cohort of buffaloes at Khyber Pakhtunkhwa. 120 quarter samples were collected from suspected buffaloes in selected areas of Peshawar, Charsadda, Mohmand Agency, and Dara Adam Khel. Initially, California Mastitis Test was performed for screening of positive samples. Afterward, the bacterial profile was confirmed through biochemical testing. The quarter wise prevalence of subclinical mastitis was 25%. Within this, contribution of Gram-negative bacteria was 68% and that of Gram-positive bacteria was 32%. Among 30 positive samples, percentage prevalence of different bacterial species was: *E.coli* (37%), *S. aureus* (23%), *Pseudomonas* (20%), *Streptococcus* (10%), *Proteus* (7%) and Salmonella (3%). The study reported high percentage of *E. coli* in cases of subclinical mastitis. It may be due to transfer of pathogen from cow to buffaloes and from the environment in herds of mixed farming. The study results may be helpful in developing the strategic policies against the control of disease.

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Introduction

Bovine mastitis is multi-etiological inflammation of parenchyma in udder, causing chemical, bacteriological and physical changes in mammary glands (Hussain et al., 2017). It causes economical losses in terms of poor animal health, low milk production, treatment cost, milk wastage, and unhygienic milk production (Salomäki, 2015) Clinical symptoms of the disease include swelling, hardness of udder, reddening of skin, rise in body temperature, change in milk color and reduction in milk quality and quantity. Epidemiologically, mastitis involves complex interaction among three major factors including the infectious agents, host and the environment. Host factors comprise of lactation stage, age, udder anatomy, immunity and pre mature birth (Radostitis et al., 2000). Two different forms of

the disease include Clinical mastitis (CM) that is characterized by change in milk color and clots in milk with swollen, heated and painful mammary glands and Subclinical mastitis (SCM) which is featured by the presence of different bacterial species without producing any clinical symptoms (Hasan et al., 2016).

On the basis of involvement of different bacterial species, the SCM may be contagious or environmental. Contagious mastitis occurs due to bacterial spread, residing on skin of teats and inside of udder, during milking from one animal to the other. Such bacterial species include gram positive commensals such as *Staphylococcus aureus, Streptococcus agalactiae* and other related species. About 55-60% of SCM occurs due to contagious bacteria (Hasan et al., 2016 #228) Environmental mastitis occurs in 45-50% cases due to genera like *Escherichia, Serratia*, *Enterobacter*, and *Klebsiella*. Species of these genera inhabit soil, digestive tract, animal manure, dirty bedding material and unclean housing environment. (Verma et al., 2018).

Coliform mastitis is a term used for mastitis caused by Gram-negative bacteria which are present on contaminated cattle teats and invade the mammary glands through teat sphincters. In mammary glands, such bacteria grow rapidly and release endotoxins that affect the immune system of affected animals. The clinical symptoms include high fever, rapid weight loss, reduced appetite, dehydration, diarrhea, poor milk quality and reduced production (Hogan and Smith, 2003).

In Pakistan, the average prevalence rate of mastitis, in different parts of the country, ranges b/w 20 – 60% (Hameed et al., 2012). The most prevalent pathogenic bacteria are *S. aureus, S. hyicus, S. epidermidis, S. capotus, Streptococcus dysglactiae, S. pyogenes and Corynebacterium bovis* (Oviedo-Boyso et al., 2007). Therefore, bacteriological diagnosis and epidemiological studies are critical to design effective control strategies for mastitis in the country.

The aim of present study is to report the etio-prevalence of bacterial species which cause SCM in buffaloes in different areas of Khyber Pakhtunkhwa, Pakistan. Our results may prove beneficial in the selection of antimicrobial medicines for the treatment of SCM/coliform mastitis.

Materials and Methods

Study population

A cross-sectional study was conducted on a cohort of lactating buffaloes at various small dairy farms in Khyber Pakhtunkhwa and sampling sites were Peshawar, Charsadda, Mohmand Agency, and Dara Adam Khel (Figure 1). The present study was approved by Institutional Review Board (IRB) of advanced studies and performed at Kohat University. The samples were obtained from Mastitis Section, Veterinary Research Institute (VRI) Peshawar. The samples were processed for about four months from March – June, 2018. All the material and information were collected after consent approval from livestock's owners.

Collection of milk samples

Before taking samples, the udder and teats were cleaned with potassium permanganate solution and teats were dried with paper towel. Each teat opening was scrubbed with a separate cotton gauze that was soaked in 70% ethanol. After cleaning the teats, initial few streams of milk were discarded to avoid the contamination of bacteria in teat canals. Afterward, the milk samples were collected in sterilized tubes and about 5-10 ml milk was taken in each tube. Samples were kept in ice boxes until transported to the research center for further investigation.

Test for screening of SCM

Overall 120 quarter milk samples were obtained from apparently normal buffaloes suspected for SCM. Samples were collected randomly from the sampling sites and each sample was tested by California Mastitis Test (CMT) to measure the somatic cell count (SCC) and criteria for SCC according to NMC guidelines (Roy et al., 2009) Digital electric pH meter was used to measure pH of each milk sample to detect SCM as adopted by (Shahid et al., 2011). Based on tests results, thirty positive mastitis samples were subjected further for bacteriological examination to identify various bacterial species for determining their prevalence.

Identification of bacterial species

To isolate different bacterial species causing subclinical/coliform mastitis, 50µl of each CMT positive milk sample was added in 3-4ml of blood agar and MacConkey's agar. Each culture was then incubated for 48 h at 37°C and bacterial growth was recorded after 24 and 48 h of incubation. The cultures were maintained in glycerol stock solutions for

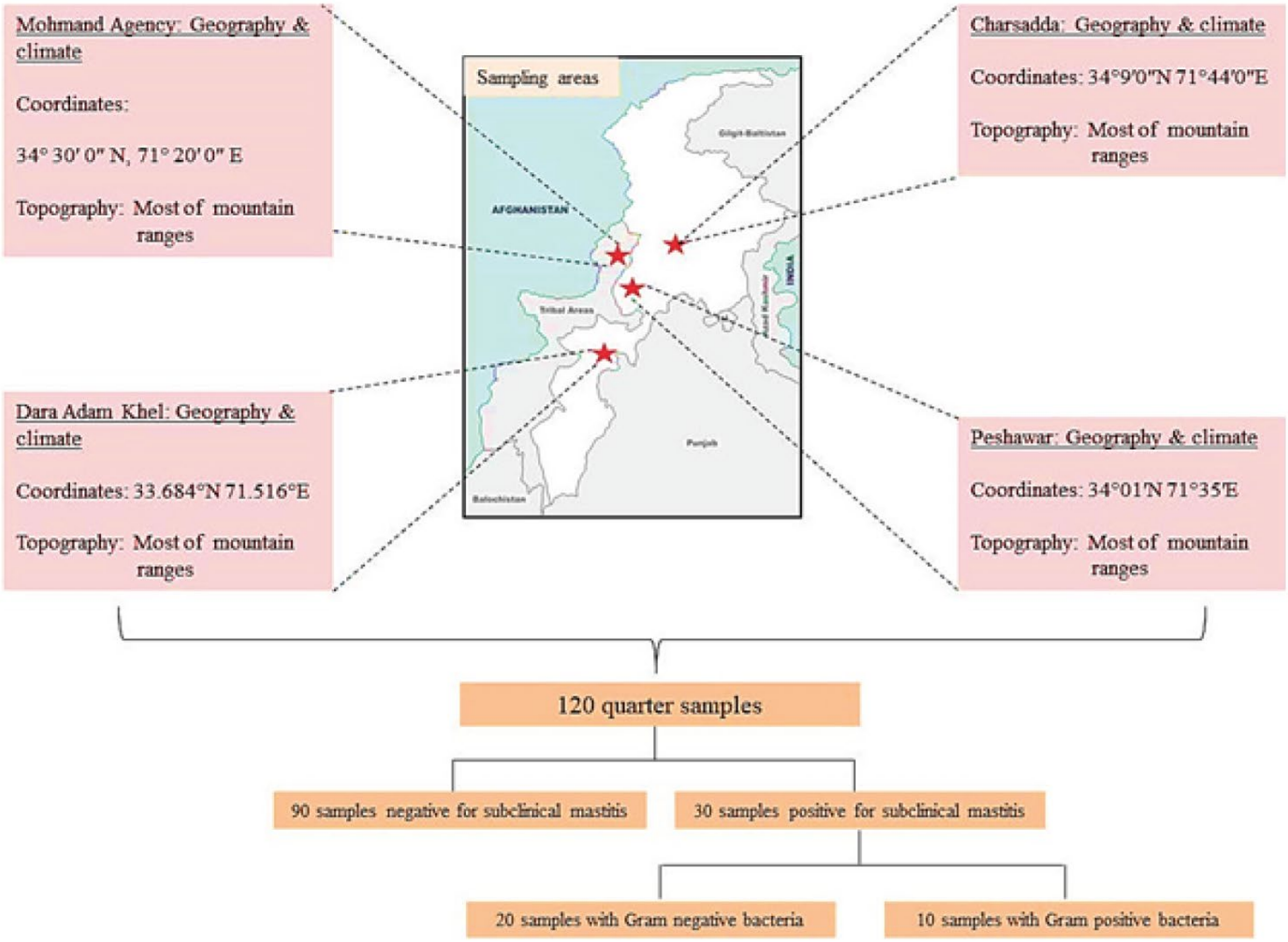

Figure 1: *Geographic coordinates of sampling sites and summary of study data.*

performing different biochemical tests like Gram's staining's, LF (Lactose Fermentation test), NLF (Non-Lactose Fermentation test), catalase, oxidase, TSI (triple sugar iron test) motility, H2S, and indole tests for isolation of Gram-negative bacteria whereas, coagulase and catalase tests were used for isolation of Gram-positive bacteria method adopted by Hameed et al., 2008.

Statistical analysis

Prevalence of SCM and its associated factors were calculated by determining the proportion of affected buffaloes out of all collected samples. Similarly, prevalence of each bacterial species was also determined. All the descriptive calculations were made by using Statistical Package for Social Studies (SPSS) software; Version 21. Moreover, the frequency tabulation and graphical presentation of trends of mastitis in Pakistan were also done by SPSS.

Results and Discussion

CMT Analysis

All 120 milk samples were tested by CMT and results revealed 25% quarter wise prevalence of subclinical mastitis in the studied population as 30 samples were positive for subclinical mastitis among 120 samples. CMT employed for screening of SCM also acted as an indicator of the Somatic Cell Count (SCC) in milk samples. Severe infection indicated higher values of SCC and negative CMT was considered for SCC ≤ 100,000 (Table 1).

Normally the pH of buffalos milk ranges between 6.6-6.9 but pH at 6.8 or more than 6.8 is indicative for SCM (Shahid et al., 2011). Following this pattern, the pH of CMT positive milk samples was checked and we found 21 samples positive for SCM among 30 CMT positive samples. Therefore, CMT is a better diagnostic test as compared to detection by pH meter. We categorized 30 SCM positive samples into + (8 trace positive), ++ (10 weak positive), and +++ (12 distinctive positive) by conjoining the results of CMT and pH values (Table 1).

Etio-prevalence of bacterial species

In order to uncover the etio-prevalence of bacterial profile in SCM, different biochemical tests were

Table 1: *Classification of SCM positive samples by combining the CMT findings with pH readings.*

Milk samples	Adjoining of CMT result with pH readings								
	Trace +ve < 300,000 SCC			Weak +ve SCC < 900,000			Distinctive +ve > 1 million SCC		
	6	6.5	6.7	6.8	6.9	7	7.2	7.3	7.5
1	-	-	+	-	-	-	-	-	-
2	-	+	-	-	-	-	-	-	-
3	-	-	-	-	-	-	+++	-	-
4	-	-	-	-	-	-	+++	-	-
5	-	-	-	-	-	-	-	+++	-
6	-	+	-	-	-	-	-	-	-
7	+	-	-	-	-	-	-	-	-
8	+	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	+++
10	-	-	-	++	-	-	-	-	-
11	-	-	-	-	-	-	+++	-	-
12	-	-	-	-	-	-	-	-	+++
13	-	-	-	-	++	-	-	-	-
14	-	-	-	-	-	++	-	-	-
15	-	-	-	++	-	-	-	-	-
16	-	+	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	+++
18	-	-	-	-	-	-	+++	-	-
19	-	-	+	-	-	-	-	-	-
20	-	-	-	-	++	-	-	-	-
21	-	-	-	-	-	++	-	-	-
22	-	-	-	-	-		+++	-	-
23	-	-	-	-	-	-	-	+++	-
24	-	-	-	-	-	++	-	-	-
25	-	-	-	-	++	-	-	-	-
26	-	-	-	-	-	-	+++	-	-
27	-	-	-	-	-	-	-	-	+++
28	-	-	-	++	-	-	-	-	-
29	-	-	+	-	-	-	-	-	-
30	-	-	-	-	-	++	-	-	-
Total	2	3	3	3	3	4	6	2	4
	8			10			12		

performed to identify particular groups and types of bacteria within each positive sample and then prevalence of each identified Genera was calculated as indicated in table 2, 3 and 4. Six different bacterial pathogens were identified among all 30 positive samples. These six General were *Escherichia*, *Pseudomonas*, *Proteus*, *Salmonella*, *Staphylococcus*, and *Streptococcus*. These General were grouped into Gram-negative bacteria and Gram positive bacteria and their prevalence rates, within SCM positive samples and out of all collected samples, are provided in Table 2. Among Gram negative bacteria four identified bacterial genera include *Escherichia* (55%; n=11/20), *Pseudomonas* (30%; n= 6/20), *Proteus* (10%; n= 2/20), and *Salmonella* (5%; n=1/20) as shown in Table 3. Whereas among Gram positive bacteria only two identified species were identified, *Staphylococcus* (70%; n=7/10), and *Streptococcus* (30%; n= 3/10) as presented in Table 4.

Table 2: *The prevalence of bacterial species causes subclinical/coliform mastitis in buffaloes.*

Bacterial species	Total No. of isolates in SCM positive samples	Prevalence (%) within 30 disease samples	Prevalence (%) within all 120 quarter samples
Gram Negative bacteria	20	67	***17***
E. Coli	11	37	9
Pseudomonas	6	20	5
Proteus	2	7	2
Salmonella	1	3	1
Gram Positive bacteria	10	33	***8***
Staphylococcus Aureus	7	23	6
Streptococcus	3	10	3

In Pakistan, *Bubalus bubalis* is considered to be the 'Black Gold' due to huge economic importance as they contribute61.8% of total milk produces annually (Ali et al., 2014). Subclinical mastitis reduces this number to about 40% by affecting the animal's health, compromising immunity and by deteriorating the quantity and quality of the milk (Sharif et al., 2007; Younus et al., 2018) . SCM also acts as a risk factor for zoonosis.

Despite its importance and prevalence as found in this study, only a few studies are available on epidemiology of subclinical/coliform mastitis in buffalos from KPK (Khan et al., 2017a; Khan et al., 2017b; Rafiullah et al., 2017) and only one study is available from Burewala, Punjab (Hameed et al., 2008).

The 25% prevalence of SCM as found in this study is concordant with the results of previously reported studies in Faisalabad as they also found 25% prevalence (Ashfaq and Muhammad, 2008). Similarly, 21% prevalence was found in Lahore in 2011 (Mustafa et

Table 3: *Results of biochemical tests and characteristic features different Gram negative bacterial isolates.*

Bacterial isolates	Total No. of identified samples	Biochemical tests								Percent prevalence (%)
		LF	NLF	Catalase	Oxidase	TSI	Motility	H2S	Indole	
E. Coli	11	+, pink	-	+	-	+, A/A, +gas	+	-	+	55
Pseudomonas	6	-	+, colorless	+	+	-	-	-	-	30
Proteus	2	-	+, colorless	+	-	-	+	+	+	10
Salmonella	1	-	+, colorless	+	-	+, A/A	+	+	-	5
Total	20									100

Table 4: *The summary and prevalence of bacterial isolates within the group of Gram's positive bacteria.*

Bacterial isolates	Total No. of identified samples	Biochemical tests		Percent prevalence (%)
		Catalase	Coagulase	
Staph Aureus	7	+	+	70
Streptococcus	3	β Beta hemolytic	-	30
Total	10			100

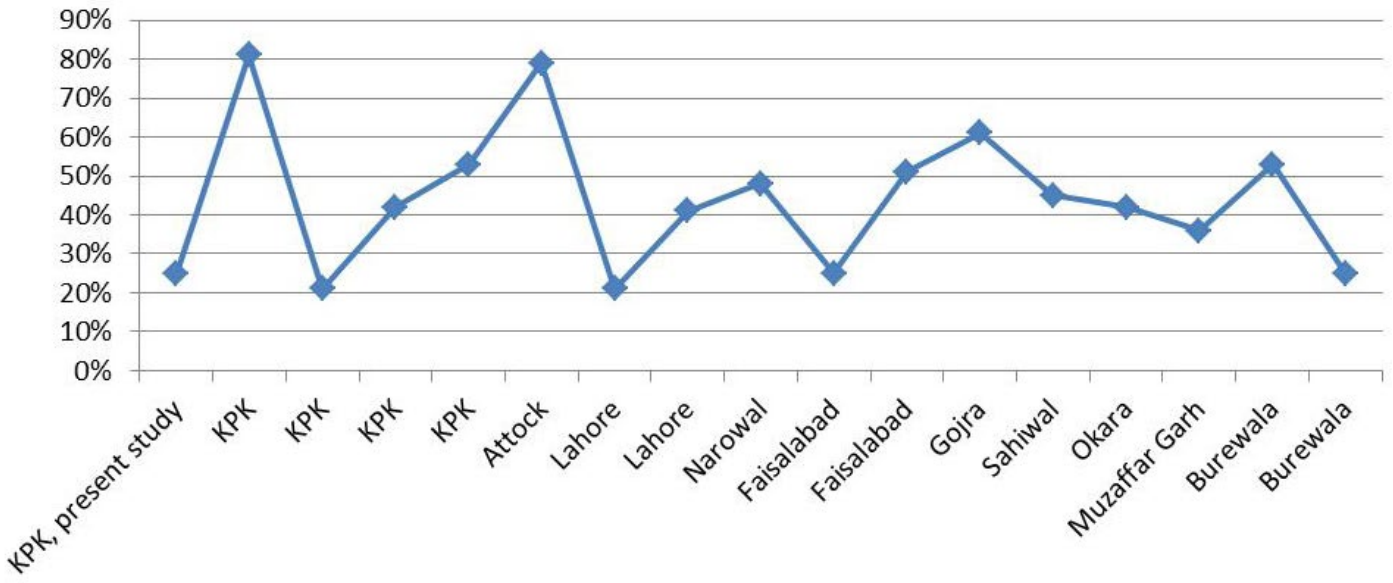

Figure 2: *Based on previous studies, the changing trends of disease prevalence in buffaloes from different parts of Pakistan.*

Table 5: *Comparison of current study with the previous studies in terms of percentage prevalence of identified bacterial species.*

References	Study area	Total collected samples	Total positive samples for mastitis	Over all prevalence (%)	Percent prevalence of bacteriological agents in positive samples for mastitis (%)					
					Gram negative bacteria				Gram positive bacteria	
					E. Coli	Pseudomonas sps	Proteus	Salmonella	S. Aureus	Streptococcus sps
Current study	KPK	120	30	25	37	20	5	3	23	10
Rafiullah et al., 2017	KPK	787	626	79	59	0	16	0	5	0
Ali et al., 2011	Narowal	150	72	48	16	16	0	5	32	9
	Lahore	150	61	41	15	15	0	7	22	13
	Okara	150	63	42	18	6	0	8	21	16
	Sahiwal	150	68	45	12	13	0	7	32	11
Hameed et al., 2008	Burewala	30	19	63	15	0	0	0	53	23
Ashfaq and Muhammad, 2008	Faisalabad	56	14	25	1.9	0	0	0	48	21

al., 2011), and KPK in 2015 (Khan et al., 2015). In Pakistan most of the studies conducted on mastitis only showed the prevalence rates without identifying the specific bacterial agents relevant to specific types of mastitis in buffaloes (Bachaya et al., 2005; Bachaya et al., 2011; Mustafa et al., 2013; Mustafa et al., 2011; Shahid et al., 2011; Sharif et al., 2009) In present study, thi are was tried to be addressed and trends of prevalence of mastitis in different areas of Pakistan is given in Figure 2. It was concluded that environmental variations greatly influence the prevalence rate of mastitis in different parts of the country (Khan et al., 2015). The current study was conducted to find the etiological prevalence of associated bacterial species causing SCM in buffalos and the statistical analysis revealed an overall prevalence of *E coli* (37%), *Staphylococcus aureus* (23%), *Pseuodomonas* (20%), *Streptococcus* (10%), *Proteus* (7%) and *Salmonella* (3%) i n milk of infected affected buffaloes and these findings were concordant to other studies as shown in table 5 (Ali et al., 2011; Ashfaq and Muhammad, 2008; Hameed et al., 2008; Rafiullah et al., 2017).

The present study reported highest prevalence of *E. coli* (37%) in Pakistan out of identified bacterial isolates however, the result is concordant with international study as prevalence of *E. coli* is reported as 44% in Egypt. Moreover, in the same study it was calculated that ratio of weak positive results in buffaloes was about 49% high as compared to strong positive results (Ahmed et al., 2018) as calculated in the present study. But in cows the ratio of strong positive results was high. One of the risk factor for increased prevalence of *E. coli* is poor hygiene measures (Navaneethan et al., 2017). Moreover, our results are also comparable with studies in Bangladesh (Haque et al., 2018; Islam et al., 2016). *E. coli* is associated with cow's environment. But in current studies high prevalence of *E. coli* in buffaloes indicated t hat it has equal chances to cause mastitis in buffaloes due to its environmental transmission in herds of mixed cattle (Memon et al., 2012). In more explanatory sense, the SCM, due to its undetected nature, has tendency to remain in the body for long time in chronic form (Memon et al., 2012). indicates. *E. coli* usually causes chronic mastitis but recent studies have shown that its tendency to cause acute infection is increasing possibly due to the involvement of novel disease causing factors that enhance the survival of bacterial strains (Herry et al., 2017). Cattle are natural reservoir for Shiga Toxin producing *E. coli* (STEC) stains which cause interspecies infections (Persad and Lejeune, 2015). In the present study the type of *E. coli* strain is not clear. So, for this further PCR based identification is required. In the present study, contributing risk factors for *E. coli* mastitis were mixed farming, poor hygiene feed, environmental variations and lack of proper sanitation measurements.

Finally, culturing of bacteria is standard method to confirm SCM in bovines (Sudhan and Sharma, 2010) but Dasohari et al. (2017) discussed that culturing technique is costly, time consuming, and requires sophisticated laboratory setting. Additionally, it may give false positive results where the infection is non-random (Dasohari et al., 2017). Therefore, in current study, the glycerol stock cultures were used to isolate and identify bacteria.

Conclusions and Recommendations

Multifactorial nature of the mastitis and the development of resistance in bacteria against different antibiotics make the therapeutic measures useless in controlling the disease. There is poor understanding of the risk factors that govern the prevalence of pathogens in the disease animals. Due to this, such kinds of studies are indispensable for effective treatment to control and prevent the countrywide spread of disease and also to increase the farms productivity. Collectively, mastitis can be monitored by utilization of preventive and hygienic measures.

Author's Contribution

LU and AK have equal contribution to this research work. Additionally, LU analysed the data and wrote the paper. NA, FA and MS have completed the sampling and performed the CMT test. LU, AK and MS have performed the biochemical tests. TAK and NB has designed the study and funded for the research work.

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