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Clinicopathological alterations in subclinical ketosis in Chilika buffaloes, detected by patient-side urine test and influence of risk factors on prevalence

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ABSTRACT

The present study was carried out by recruiting 526 lactating Chilika buffaloes, kept in 29 herds, belonging to small and marginal farmers in 16 villages of three Chilika Lake-adjoining districts-Puri, Khordha and Ganjam, in the state of Odisha, India during the period from December 2019 to June 2020 to assess the prevalence of subclinical ketosis in their native tract, and to weigh the influence of risk factors, namely parity, lactation stage, and milk yield. All the buffaloes were screened by Rothera's test on urine samples and Ross test on milk samples, that diagnosed subclinical ketosis in 41 animals, constituting 7.79% of the total tested buffaloes. Blood, milk and urine samples were collected from 20 lactating buffaloes, consisting of 10 apparently healthy buffaloes, and another ten with subclinical ketosis and analyzed for serum biochemical parameters, milk fat and solid not fat (SNF). The urine samples were tested using multi-diagnostic urinalysis strip. The highest prevalence of subclinical ketosis was observed in 4th parity (15.38%), followed by 3rd parity (11.20%), and in buffaloes yielding ≥ 3 kg of milk/ day (17.07%). The subclinical ketosis was more common during peak production, and was associated with significant decline in milk production, hepatic dysfunction and had sever hypoglycaemia, along with elevated mean serum triglycerides (mg/dl), activity of alanine aminotransferase (ALT), Aspartate Aminotransferase (AST). Serum calcium, magnesium and phosphorous levelS were significantly lower in buffaloes with subclinical ketosis than their apparently healthy counterparts.

Keywords: Chilika buffaloes, Ketosis, Liver function, Milk, Rothera's test

Ketosis is a vital metabolic disorder in dairy cattle, caused by negative energy balance due to disturbances in carbohydrate and fat metabolism. It is characterized by increased concentration of ketone bodies, namely acetone, acetoacetate and β-hydroxy butyrate in the body fluids and tissue, associated with a concurrent decrease in blood glucose levels (Taylor, 1994; Dann *et al.* 2005). This phenomenon prevails, at least in part, from gradually decreasing feed intake, and higher rate of milk synthesis during transition period (Grant and Albright, 1995). The negative energy balance mobilizes available fat from adipose tissue to serve as the main source of energy. The transition from carbohydrate to fat metabolism enhances ketones in blood, called hyperketonaemia, resulting in clinical disease or impaired milk production (Herdt, 2000).

Ketosis in dairy animals may appear as a primary disease or in association with other pathological conditions (secondary ketosis) that occur mainly during winter and spring seasons. Negative energy balance leading to hypoglycaemia and ketonemia are the primary cause of the disease as maintenance of adequate concentration of glucose in the blood is critical in high yielders during first few weeks of parturition for the regulation of energy metabolism (Foster, 1988). A significant increase in energy requirements during late pregnancy and early lactation makes dairy cows highly susceptible to negative energy balance (Turk *et al.* 2008). The metabolic adaptation to negative energy balance (NEB) requires interactions of metabolic fuels and its failure may occur in various tissues like liver, adipose tissue and others (Herdt, 2000). Augmented production of reactive oxygen species (ROS) and development of oxidative stress occurs as a result of intensified oxidation of non-esterified fatty acids (NEFA) in the liver (Miller *et al.* 1993).

Considerable economic losses take place due to decreased milk yield, failure of the dairy animals to return to the normal potential even after recovery, cost of treatment, decreased market value of animal because of sever wasting and occasionally from death and disposal of animal. Ketosis mostly occurs in secondary form in India, as a result of unhygienic farm conditions, and the cases of pure primary ketosis are comparatively less (Lean *et al.* 1994).

The clinical ketosis, as determined by ketonemia, are

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more likely to have serum biochemical derangement and higher oxidative stress indices compared to subclinical ketosis and apparently healthy buffaloes (Mohamed *et al*. 2010). Primary subclinical ketosis is most commonly seen in Murrah buffaloes within the first two months of parturition in the third lactation impacting production seriously, with characteristics clinical signs without variation in vital parameters (Kumar *et al.* 2015). Recently, the seasonal effects on milk yield and blood metabolites have been established in dairy cows fed under a high ambient temperature (Thammacharoen *et al.* 2021). Very limited studies have been conducted on the clinical and therapeutic aspect of ketosis in buffaloes, despite the fact that ketosis significantly affects the productivity (Anantwar and Singh. 1993; Ali and Teli, 2007; Ghanem and El-Deeb, 2010). Chilika buffalo (water buffalo) is a unique breed of buffalo, which spends the whole night grazing on weeds in Chilika water, and they usually return to their master's house next day morning. This breed has an exceptional competence of converting the saline biomass of the lake into the most precious milk and dung. The present study determined the prevalence of subclinical ketosis, its risk factors and measured the associated clinicopathological alterations in Chilika buffaloes, maintained with typical management and feeding practices.

MATERIALS AND METHODS

Study sites: The present study was carried out in 29 herds of Chilika buffaloes belonging to small and marginal farmers in 16 villages of three Chilika Lake adjoining districts of Puri, Khordha and Ganjam, in Odisha, India. Chilika Lake is Asia's largest inland brackish water lagoon and is enlisted as a tentative UNESCO World Heritage site for its rich aquatic flora and fauna. It is situated along the eastern coast line of Indian peninsula in-between Latitude 190 28'N to 190 54' N and Longitude E 850 06' to E 85015', spreading over 1,100 sq. km of wet land area, and has a catchment area of more than 3,500 sq. km.

Owners consent and experimental design: This clinical study was carried out by registered veterinary physicians, observing all CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines, for the welfare and good health of the animals. The study did not involve any invasive or painful procedures. The owner's consent was taken to conduct the buffalo/cow side test on their milch Chilika buffaloes. A total number of 526 lactating Chilika buffaloes were screened by Ross test in milk and Rothera's test in urine during the period from December, 2019 to June, 2020. Briefly, early morning milk samples, collected for buffalo-side test in sterile dry containers was used for Ross test for detection of acetone. Five ml of milk (5ml) was saturated with granules of ammonium sulphate, and few drops of freshly prepared sodium nitroprusside solution (%) were added and mixed by tilting the test tube several times. Then, a flake of sodium hydroxide was added to it. Purple colour (like potassium permanganate) indicated presence of ketone body (Alder *et al.* 1957).

The early morning urine sample, collected in the sterile containers was subjected to Rothera's test for detection of acetone. Five ml of urine sample collected from each Chilika buffalo in a clean and dry glass test tube saturated with granules of ammonium sulphate. Few drops of freshly prepared sodium nitroprusside solution were added and mixed by tilting the test tube several times. Then, concentrated ammonium hydroxide solution (1 ml) was carefully layered over it. Purple colour (like potassium permanganate) ring at the layered interface indicated presence of ketone body (Alder *et al.* 1957).

The screened 526 Chilika buffaloes, were further grouped as per parity or lactation (1st/ 2nd / 3rd /4th / 5th /6th and above), stage of lactation (early, mid, late mid or late lactation) based on the period after parturition (0 to 60 days, 61-120 days, 121 to 180 days or above 180 days, respectively) and amount of milk yield/ day (up to 1L, 1-2L, 2-3L or above 3L) to calculate the prevalence of sub clinical ketosis with respect to parity, stages of lactation and milk yield. The milk yield was recorded through a pre-designed questionnaire.

Collection and processing of blood sample: As a buffalo side test, glucometer (On Call Plus blood glucose meter kit, Right Med Bio System, Chennai) was used for quantitative estimation of blood glucose concentration (mg/ dl). A drop of blood was placed on disposable test strip, which is read

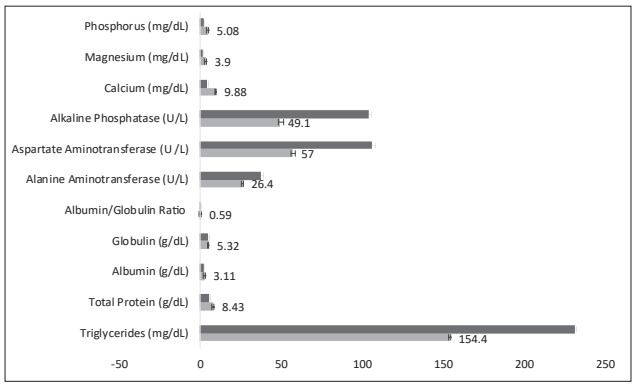

Fig 1. Changes in serum biochemical parameters in lactating Chilika buffaloes with subclinical ketosis

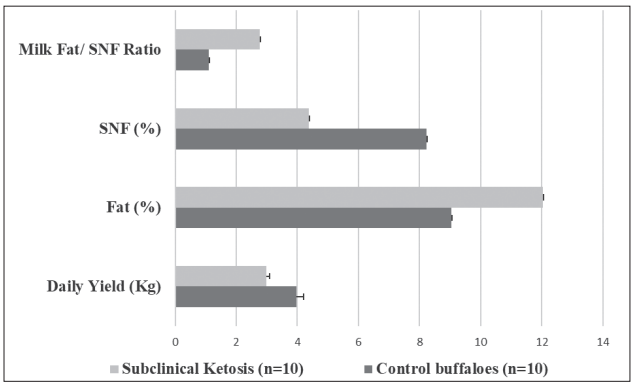

Fig 2. Changes in milk parameters in lactating buffaloeswith subclinical ketosis

by the glucometer within seconds.

About 8 ml blood samples from each of the ten randomly selected subclinically ketotic and ten apparently healthy lactating Chilika buffaloes were collected by jugular vein puncture with the help of sterilized 1.5-inch, 18 mm gauge needle after application of rectified spirit on the collection site. Approximately, 4 ml of blood was collected in clot activator vial (Accuvet plus) for separation of serum for biochemical analysis. The biochemical profile like serum metabolites (plasma glucose, triglycerides, cholesterol), serum proteins (total protein, albumin, globulin), serum minerals (calcium, magnesium, phosphorus) and serum enzymes for liver function test namely, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were estimated in serum samples. The plasma glucose was estimated by glucose oxidase-peroxidase (GOD/ POD) method, using reagent kit, supplied by Crest Biosystem, Goa and the results are expressed in mg/dL (Trinder, 1969). The serum triglyceride was estimated by Glycerol Phosphate Oxidase-Peroxidase 4-Aminoantipyrine (GPO/PAP) method, using Crest Biosystem reagent kit (Tietz, 1994). The serum total protein and albumin were estimated by Biuret and Bromocresol Green (BCG) dye method, respectively (Trinder, 1969; Lubran,1978). The activity of serum alanine aminotransferase (ALT) was estimated by IFCC method, using the Crest Biosystem reagent kit (Schiele *et al.* 1992). The activity of serum aspartate amino transferase (AST) was estimated by IFCC method using Crest Biosystem reagent kit (Bruns *et al.* 1981). The activity of serum alkaline phosphatase (ALP) was estimated by pNPP kinetic method using Crest Biosystem reagent kit (Tietz, 1994). The serum calcium, magnesium and inorganic phosphorus were estimated by using Crest Biosystem reagent kits (Tietz, 1994).

Analysis of milk and urine samples: The fresh milk after milking was kept in milk can/ vessel for 2 hours, and then filtered to remove the foreign particles. The milk was stirred well for 5 minutes so that the lower layers are reached and mixed properly before taking sample for analysis. Milk was slowly heated to 35-40 °C, simultaneously shaken slowly and then poured several times for proper mixing of cream and cooled to dislodge fats that got stuck to the walls of the container upon storage for a long time. Samples for testing were used only once, and were not returned to the main vessel, rather discarded (FSSAI, 2016).

The Gerber method was used as a routine screening test for milk fat (IS: 1223, 2001). The milk sample was mixed with sulphuric acid in a special Gerber tube/ butyrometer in order to dissolve protein and release fat, and iso-amyl alcohol (IS: 360, 1964) was added for separation of fat (IS: 1224 Part 1, 1977). The lactometer was used to measure milk specific gravity. Richmond's formula was used to calculate the total solid not fat (SNF) and total solid of milk based on fat % and specific gravity. Pre-warming of milk was done to bring fat to a liquid state and to ensure variation-free specific gravity reading (IS: 9385, 1980). The estimation of fat and SNF % in milk was done using Indiz milk stirrer and milk analyser. The urine samples from these buffaloes were tested using multi-diagnostic urinalysis strip.

Statistical analysis: The data were analysed to express values in mean ± standard error. One way and repeated measure ANOVA and post-hoc analysis was conducted by Duncan multiple range test using SPSS -22 computer package.

RESULTS AND DISCUSSION

The present study is the first report on the prevalence of subclinical ketosis and its associated risk factors in Chilika buffaloes. Subclinical ketosis, otherwise termed as the 'silent killer', is one of the most important production diseases in dairy buffaloes that adversely affects the quantity and quality of milk yield, mostly in an unnoticed manner, that does not show overt clinical signs, noticeable by the owners. The Chilika buffaloes get into the lake to graze on the natural grown flora and fauna, returns in the evening for milking and are not provided with concentrate, vitamins and minerals and supplements, thus predisposing dairy animals to imbalances in carbohydrate and fat metabolism during transition period and milk production. The subclinical ketosis remains in a hidden form and brings huge economic losses through changing quality and quantity of milk for a pretty long time, impacting the rural household economy of the poor dairy farmers.

The farmers still follow age-old practices of their forefathers for their management including housing and feeding. The lactating buffaloes thrive on grazing on submerged flora and fauna. Its diet is devoid of commonly used rural cattle feeds like paddy straw, broken pulses, oil cake, wheat/ rice bran, common salt, and mineral mixtures.

The prevalence of subclinical ketosis largely depends on the amount of milk produced per day and the drainage of energy though milk. The feed intake and body energy reserves constitute the main sources of the energy required for milk production. There is a steady increase in energy demand during peak lactation period on daily basis through increased quantity of milk production. The negative energy balance is attributed to higher energy output required for milk production than the energy received from feed intake.

Table 1. Prevalence of subclinical ketosis in lactating Chilka buffaloes by Rothera's test in urine and Ross test in milk samples

Diagnostic test	No. of lactating Chilka buffalo screened	Positive for subclinical ketosis	Prevalence rate	Colour index*
Rothera's test	526	41	7.79%	±, +, or ++
Ross test in milk	526	nil	nil	-

No colour index - -ve; Very slightly/ faintly purple - ±; Slightly purple - +; Moderately purple - ++; Purple - +++; Deep purple - ++++

Table 2. Prevalence of subclinical ketosis in lactating Chilika buffaloes with respect to risk factors

Risk factors	Group	No. of ketotic buffaloes screened	Frequency of Positive animals	Prevalence percentage
Lactation stage	Early (0-60 days)	157	26	16.56%
	Mid (61-120 days)	185	12	6.49%
	Late Mid (121-180 days)	148	3	2.03%
	Late (Above 180 days)	36	0	0.00%
Parity / Lactation number	1st lactation	56	1	1.79%
	2nd lactation	65	3	4.62%
	3rd lactation	125	14	11.20%
	4th lactation	104	16	15.38%
	5th lactation	67	6	8.96%
	6th lactation and above	109	1	0.92%
Range of milk yield (Kg/day)	Up to 1	132	0	0
	1-2	125	6	4.80%
	2-3	187	21	11.23%
	3 and above	82	14	17.07%
Irrespective of criterion		526	41	7.79%

Parity – calving number, the number of times a buffalo has given birth to a calf, regardless of whether the calf is born alive or stillborn; Lactation stage refers to period after calving

However, this is considered as usual metabolic condition in high yielding buffaloes. Therefore, lactating Chilika buffaloes with higher daily milk yield, any stress like changes in environmental condition could lead to anorexia, making them susceptible to subclinical ketosis.

Prevalence of Sub clinical ketosis in lactating Chilika buffaloes: The screening of 526 lactating Chilka buffaloes by Rother's test on urine samples diagnosed subclinical ketosis in 41 buffaloes, representing 7.79% of screened animals (Table 1). However, Ross test on any of the milk samples from the same 526 lactating Chilika buffaloes did not reveal positive reaction. There were 185 Chilika buffaloes in mid lactation stage, those were between 61 to120 days of lactation, and 12 out of those were positive, indicating a prevalence rate of 6.49% during mid lactation. This was followed by prevalence rate of 2.03% among 148 Chilika buffaloes in the late mid lactation stage, and only 3 animals were positive in this group. All the 36 lactating Chilika buffaloes in the late lactation stage, after 180 days of parturition, were negative for both the Rothera's test on urine and Ross test on milk samples.

The highest occurrence (16.56%) of the subclinical ketosis was recorded during early lactation in 26 Chilika buffaloes followed by mid lactation in 12 (6.49%) Chilika buffaloes, late mid lactation in 3 (2.03%) buffaloes (Table 2). No cases were recorded in late lactation beyound 180 days postpartum. The peak milk production occurs during the first month of lactation, and there is a steady increase in energy demand during this period on daily basis through increased milk production. The energy intake during early lactation, is usually insufficient to meet the energy output through milk, so the animal continues in a negative energy balance resulting in the highest incidence of subclinical ketosis from day 0 to 60 of lactation. Environmental stress and reduced diet can further attribute to vulnerability of these animals to subclinical ketosis.

The parity number influenced the occurrence of subclinical ketosis of lactating Chilika buffaloes. Animals in 4th parity were most susceptible (15.38%), followed by 3rd (11.20%), 5th (8.96%) and 2nd (4.62%) parity. Out of 56 Chilika buffaloes in 1st lactation, only one buffalo was found positive that constitutes only 1.79% of buffaloes of this group. This may be attributed to greater body reserves, efficient physiological adaptability to the adverse changes of young lactating Chilika bubaline mothers. The diverse physiological processes gradually weaken with the advancement of age. Therefore, the incidence of ketosis in Chilika buffaloes gradually increased with peak production capacity up to 4th parity; thereafter, the decline commenced.

The clinical signs observed in this study such as drop in daily milk yield, reduced feed and fodder intake, debility, incoordinated gait, sweet smelling breath, and excitatory nervous signs were similar to those reported in dairy cattle, and are attributed to hypoglycemia and hyper acetonemia (Baird and Heitzman, 1969; Schultz, 1974; Singh and Kasaralikar, 1988; Lean *et al.* 1994; Mir and Malik, 2003; Constable *et al.* 2017). The decreased serum total protein and albumin level in Chilika buffaloes in ketosis might be attributed to injury to the liver, as seen in fatty liver syndrome in cattle (Baird and Heitzman, 1969; Grummer, 1995; Marcos *et al.* 1990; Mandali *et al.* 2002; Youssef *et al.* 2010). The utilization of adipose tissue as a source of energy and subsequent production of acetyl CoA leads to a

state of ketosis, as utilization of acetyl CoA in TCA cycle is not optimum resulting in accumulation and formation of free fatty acids (FFA) and ketone bodies (Devi, 1997; Sevinc *et al.* 2003; Roy *et al.* 2007; Lean and DeGaris, 2011; Simonov and Vlizlo, 2014). The mean serum triglyceride concentration in the current study, was significantly higher than the apparently healthy controls (154.4±0.81 mg/dl). The breakdown of body fat releases non-esterified fatty acids which are re-esterified to triglycerides or oxidized to acetyl CoA in the liver (Youssef *et al.* 2010). The hormonal imbalances involving insulin: glucagon ratio, also contribute to increased lipolysis and subsequent increase in plasma NEFA levels (Beitz, 2014).

The mean serum total protein concentration of buffaloes with ketosis was found to be significantly lower than the apparently healthy buffaloes. Significant decrease in serum total protein was noted in clinical ketosis in Chilika buffaloes compared to healthy control animals in the present investigation that supports the earlier findings in bovine ketosis (Roy *et al.* 2007; González *et al.* 2009). The reduction in total protein in this study might be attributed to hepatic dysfunction, reducing protein synthesis (González *et al.* 2009). Besides, the protein catabolism for gluconeogenesis might be another contributing factor for the decline in total protein levels. The energy deficient animals with ketosis, utilize the labile pool of body protein as an alternative source of energy for the synthesis of milk lactose and protein (Radostitis *et al.* 2007; Kumar *et al.* 2015; Bali *et al.* 2016). The present observations are contrary to the earlier findings reported in high yielding ketotic cows (Devi, 1997; Ghanem and El-Deeb, 2010; Elitok *et al.* 2010).

The mean serum albumin and globulin concentration in ketotic buffaloes was significantly lower (67.85% and 90.04% of mean control level) than the apparently healthy controls (3.11±0.02 g/dl and 5.32± 0.05 g/dl, respectively). Significantly decreased blood glucose and increased AST, GGT and urea concentrations have been reported in Anatolian Black cows with primary ketosis (Elitok *et al.* 2010). Increased serum BHBA, decreased glucose and calcium concentrations, with statistically similar concentrations of vitamin C and E have been reported in clinically ketotic cows (Zhang *et al.* 2009). The reduction in serum albumin and globulin is suggestive of hepatic dysfunction/ injury (West, 1990). In energy lacking animals with ketosis, the body protein pool provides energy for the synthesis of milk lactose. The gluconeogenesis from the protein metabolism results in low serum albumin levels (Kumar *et al.* 2015; Bali *et al.* 2016). The high protein intake exacerbates the energy deficit because of energy losses resulting from its metabolism and excretion (Cote *et al.* 1969). In this present experiment, the activity of serum alanine aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) was significantly higher than that of control animals, which may be attributed to impaired liver function in animals with ketosis (Dann *et al.* 2005; Kumar *et al.* 2015; Bali *et al.* 2016). A significant hypocalcaemia was recorded in

Table 3. Frequency distribution of clinical signs in subclinical ketosis in lactating Chilika buffaloes

Clinical findings	Number of buffaloes exhibiting signs	% of total buffaloes (n= 41) with subclinical ketosis showing a particular clinical sign
Drop in daily milk yield	41	100
Reduced feed/ fodder intake	24	59
Debility	19	46
In-coordinated gait	4	10
Sweet smelling breath	1	2
Excitatory nervous signs	0	0

Table 4. Urinary changes in subclinical ketosis in Chilka buffaloes

Sl. No.	Parameters	Test reaction in urine from subclinical ketotic buffaloes			
		++	+	-	Total
1	Leucocytes	3	2	36	41
2	Nitrate	0	6	35	41
3	Urobilinogen	38 (3.5)	17 (3)	-	41
4	Protein		3	38	41
5	pH				7.88±0.073
6	Presence of blood			41	41
7	Specific gravity				1.02±0.002
8	Ketone bodies	16	25	0	41
9	Bilirubin			41	41
10	Glucose			41	41

clinical cases of primary ketosis in buffaloes as compared to control animals (Dann *et al.* 2005; Bali *et al.* 2016). The lowered serum calcium in such cases can be attributed to a general state of sub-clinical hypocalcaemia, which is a normal consequence of high metabolic demand of this mineral for higher milk yield. The reduced feed intake also results in secondary hypocalcaemia (Beitz, 2014).

A total of 132 Chilika buffaloes yielding less than 1 kg milk/ day were screened in the study. However, none of those was positive for subclinical ketosis (Table 2). Out of 125 Chilika buffaloes yielding 1-2 kg of milk/day, only 6 animals (4.80%) were positive for subclinical ketosis. Chilika buffaloes with a daily milk yield of 2-3 kg (n=187) had a prevalence of 11.23% (21/187) for subclinical ketosis. The highest prevalence percentage of 17.07% subclinical ketosis (14/ 82) was recorded among the Chilika buffaloes yielding more than 3 kg of milk/ day.

Urinalysis using dip strips: Table 3 shows the changes in the urine in subclinical ketosis in lactating Chilika buffaloes. Forty one out of 526 urine samples from the lactating Chilika buffaloes were positive for ketone bodies, showing ++ reaction in 16 cases, and + reaction in 25 cases. The urine samples from all the positive buffaloes were negative for the presence of glucose, blood and bilirubin. This dipstick for multi-diagnostic urinalysis strip reaction test also revealed positive values for leucocytes, nitrates, urobilinogen and protein. The mean pH of the urine from buffaloes with subclinical ketosis was 7.88±0.073, while mean specific gravity was 1.02±0.002. Multi diagnostic urinalysis strip reaction showed urine samples positive for glucose in all 41 Chilika buffaloes, diagnosed positive for subclinical ketosis.

Early lactation-associated negative energy balance causes mobilization of body fat to fulfil energy requirement (Cote *et al.* 1969). The mean milk fat (%), in the present study, in subclinical cases of ketosis in buffaloes was significantly higher than the apparently healthy controls. The mobilised fatty acids in subclinical ketosis of Chilika buffaloes are straight away integrated into milk fat, thereby increasing milk fat% (Gantner *et al.* 2009; Zhang *et al.* 2012). The mean milk solid not fat percent in buffaloes with subclinical ketosis was significantly lower than the apparently healthy controls. The milk protein % falls to some extent in ketotic animals for the reason of energy supply diminution (Elitok *et al.* 2010). This is in contrast to the milk fat percentage increase as a result of fat mobilization. The increased trend of milk solid not fat percent might be due to the alleviating hypoglycaemic condition and normalization of various enzyme activities. The mean daily milk yield (Kg) in the present study, was significantly lower than the apparently healthy controls. The increase in blood ketone bodies and decrease in availability of lactogenic precursor to the mammary gland in ketosis results lesser milk synthesis (Andersson and Lundstrom, 1985; Lean *et al.* 1992). However, the decrease in milk production is not proportionate to energy status reduction due to excessive hormonal stimuli (Radostitis *et al.* 2007). Urinary changes include strong positive test reaction for ketone bodies in subclinically affected ketotic buffaloes, attributed to acetonemia (Andersson and Lundstrom, 1985).

Subclinical ketosis accompanied with hepatic insufficiency prevailed in 7.79% of lactating Chilika buffaloes, and was more common during early lactation, in 4th parity (15.38 %) and with milk production of ≥ 3lts / day (17.07%).

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