Saliva as an alternative non-invasive biofluid for disease diagnosis in cattle: An evaluation of its reliability

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Abstract

Saliva is a non-invasive fluid that could be used for diagnoses in veterinary practice. However, we still do not know enough about how reliable it is in reflecting serum biochemical parameters and how stable it is when stored. This study observed at the relationship between serum and salivary biochemical parameters and their stability during storage in cattle. Saliva and blood samples were collected at the same time from twenty crossbred Jersey cattle and were processed and stored them at -20 °C in aliquot till analysis. Biochemical parameters, including glucose, total cholesterol, total protein, albumin, creatinine, urea, AST, ALT, total and direct bilirubin, calcium, phosphorus, and uric acid levels were analysed using an automated analyser. Later, the correlation between serum and saliva biochemical parameter was studied using Pearson's coefficient and assessed storage stability with one-way ANOVA at 0, 7, 120, and 180 days. We found strong positive correlations for AST (r = 0.97), ALT (r = 0.92), and creatinine (r = 0.84). Cholesterol, phosphorus, albumin, and total bilirubin showed moderate correlations, while parameters such as glucose, calcium, urea, and uric acid had poor or inconsistent correlations. Even though AST, ALT, and creatinine were found in lower amounts in saliva, they still reflected serum trends. We could estimate them using serum-based methods effectively. Most biochemical parameters in saliva remained stable like serum during storage. However, enzymatic activities (ALT & AST) decreased after 120 days. In conclusion, salivary AST, ALT, and creatinine show strong potential as non-invasive biomarkers in cattle. Other parameters need further testing across different physiological and pathological conditions before saliva can be considered a reliable diagnostic alternative to serum.

Key words: Saliva, Serum, Biochemical Parameters, Correlation, Storage stability

Introduction

Development of non-invasive techniques for health monitoring and disease diagnosis in dairy industry is important for decreasing the losses due to diseases (Darvesh et al., 2023). Saliva is an alternate non-invasive body fluid that has been a choice next to serum and urine for diagnosing many clinical conditions and physiological conditions. For certain metabolites (like PAGs) saliva is reported as even a better fluid than serum and urine in recognising physiological conditions like pregnancy in animals (Mojsym et al., 2022). In addition, few studies have opined that saliva will serve as a better alternate to serum or blood and is advantageous over them as it does not clot and has longer durability due to its major constituent being water (Chojnowska et al., 2018). It has been a diagnostic sample from many years in humans, but its role as a sample for disease diagnosis in veterinary practise is being explored recently and methodologies associated for its use as a sample must be standardised. Saliva is a body fluid whose composition is the result of the secretions of four different glands and it is substantially variable quantitatively from the serum than qualitatively and considered as ultrafiltrate of serum (Singh et al., 2021). However, some molecules are unique to saliva and secreted locally. Some of the metabolites of saliva are similar in concentration to serum and some though vary in their concentration shows strong correlation and few metabolites were either present in very small amounts or absent (Ferrari et al., 2023; Vallejo-Mateo et al., 2024). Despite these variations, Saliva was a choice as a diagnostic sample for disease diagnosis and components of it served as biomarkers for various pathological and physiological conditions. For instance, studies in cattle have reported total esterase activity in saliva showing strong correlation with severity of lameness (Contreras-Aguilar et al., 2020), elevated levels of cortisol, alpha amylase, and decreased cholinesterase in saliva being associated with mastitis (Contreras-Aguilar et al., 2019) and transformed redox status in pregnant cattle and bulls (Bielecka et al., 2021; Uzlu et al., 2016). In addition to cattle, studies in other species, has reported elevated salivary creatinine and urea levels analogous with serum in chronic kidney diseases in dogs (Tvarijonaviciute et al., 2018), increased salivary gGT, CK, Urea TB, TP in acute abdominal disease (Contreras-Aguilar et al., 2019) in horses and altered salivary profile with the physiological status like pregnancy, farrowing and lactation in sows (Contreras-Aguilar et al., 2021). Therefore, there is a need of evaluating the reliability of considering it as an alternative biological sample and the stability of biochemical parameters upon storage in saliva must be explored. Furthermore, the levels of certain biochemical parameters in saliva were low and the methods adapted for the levels of serum may not be suitable for saliva (Pfaffe et al., 2011). So, present study was designed to study the correlation of common biochemical parameters between serum and saliva and further their stability in saliva upon storage adopting the same methods that were used for serum.

Materials & Method

A total of twenty cross bred jersey cattle from the local farm maintained under similar managemental conditions were considered for the present study. Salivary and blood samples were collected simultaneously during morning hours before the animals were allowed to grazing. Saliva samples were collected using sterile tubes from the buccal cavity, collected sample is centrifuged at 10000g for 30 min at 4^0 C and stored at -20^0 C till further analysis (Balwant Rai et al., 2010). At the same time, blood was collected from the jugular vein, serum is separated carefully and stored with saliva samples. Samples were made into four aliquots before storing and biochemical parameters were estimated on 0th, 7th, 120th and 180th day of storage.

Biochemical parameters like glucose, total cholesterol, Total protein, albumin, creatinine, urea AST, ALT, total bilirubin, direct bilirubin, calcium, phosphorous and uric acid were estimated in both saliva and serum from respective animals following standard protocols using Autoanalyzer A15. The correlation between serum and saliva biochemical parameters was analysed based on Pearson correlation coefficient obtained using "COR" function and a correlogram was created using packages "CORRPLOT" in R version 4.4.1 software on 0th day of collection. For the strongly correlated parameters the trendline of correlation was plotted using GraphPad Prism version 8.4.3. The storage stability of the biochemical parameters in serum and saliva over 0th day. 7th day, 120th day and 180th day was analysed by one way ANOVA with Tukey's post hoc test for comparing means in graph pad prism 8.4.3

Results & Discussion

Sample collection for disease diagnosis by restraining animals through painful method is a challenge in veterinary practise. An alternate non painful non-invasive sample collection will be convenient and is safe for both animals and sample collectors. Saliva, a biological fluid that can be collected through non-invasive methods would be a better alternate to the fluid that were collected through invasive methods. However, before considering saliva as an alternate to serum, correlation, stability, and methods for the estimation of biochemical parameters in saliva need to be assessed. Present study has assessed them and findings (Table 1, Fig 1 & 2) classified biochemical parameters between saliva and serum as strongly correlated (0.8 to 1), moderately correlated (0.4 to 0.8) and poorly correlated (0.4 and below).

Table 1: Comparative analysis of Biochemical parameters in serum and saliva in cross bred jersey cattle

| S No	Parameter ±	Serum Concentration	Saliva Concentration	P	Pearsons correlation r value	P value	Ratio (SE/SA)
1	Glucose (mg/dl)	61.5 ±6.31	7.166 ±0.30*	0.00005	0.3691	0.471	8.5:1
2	Total Cholesterol (mg/dl)	183.5 ±23.19	21.16 ±0.47*	0.00045	0.6639	0.150	8.7:1
3	Albumin (g/dl)	3.65 ±0.07	0.283 ±0.04*	1.44E-10	-0.5030	0.309	12.9:1
4	Creatinine (mg/dl)	1.39 ±0.13	1.09 ±0.17	0.19671	0.8373	0.037	1.27:1
5	Urea (mg/dl)	37.7 ±1.26	38.65 ±4.06	0.8307	-0.3923	0.441	0.97:1
6	AST (IU/L)	74.11 ±6.58	23.11 ±2.52*	0.0003	0.9720	0.001	3.2:1
7	ALT (IU/L)	35.08 ±4.08	14.7 ±1.94*	0.0027	0.9161	0.01	2.38:1
8	Total Bilirubin (mg/dl)	0.56 ±0.04	1.2 ±0.10*	0.0008	-0.446	0.375	0.47:1
9	Direct Bilirubin (mg/dl)	0.16 ±0.009	0.15 ±0.017	0.5324	-0.3392	0.510	1.08:1
10	Calcium (mg/dl)	8.27 ±0.23	5.17 ±2.40	0.2551	-0.1114	0.833	1.59:1
11	Phosphorous (mg/dl)	6.98 ±0.605	22.10 ±8.54	0.076	0.6789	0.207	0.31:1
12	Uric Acid (mg/dl)	6.573 ±0.5851	3.612 ±0.05689	0.4525	-0.3839	0.115	1.8:1
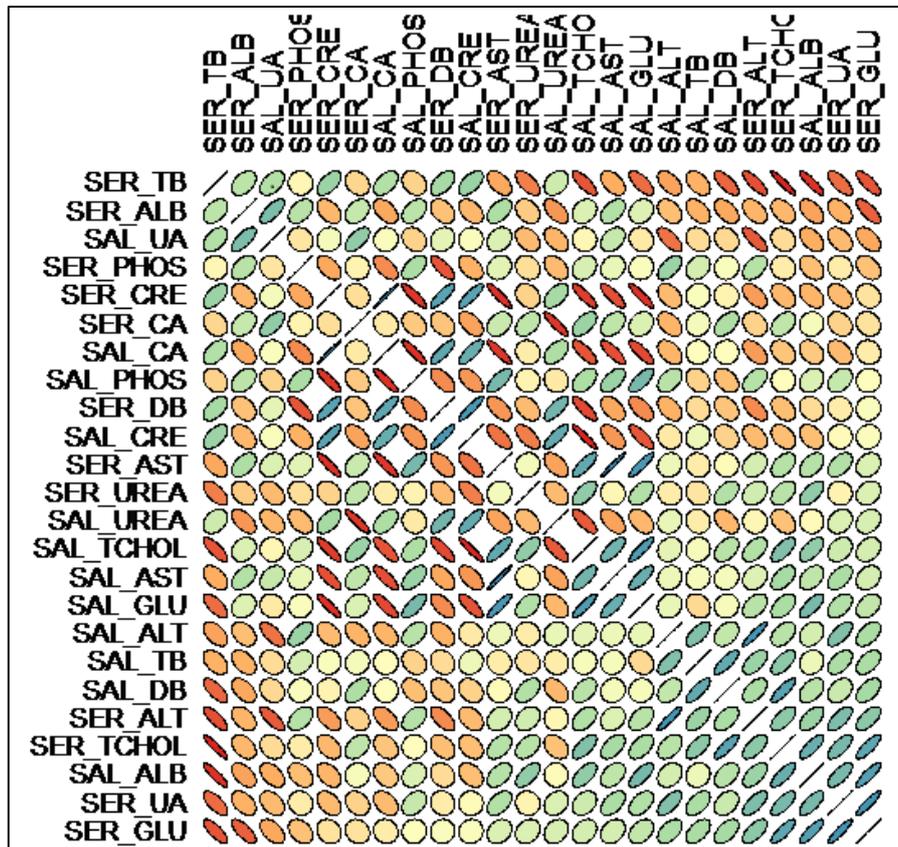
Fig 1: Correlogram of biochemical parameters between serum and saliva
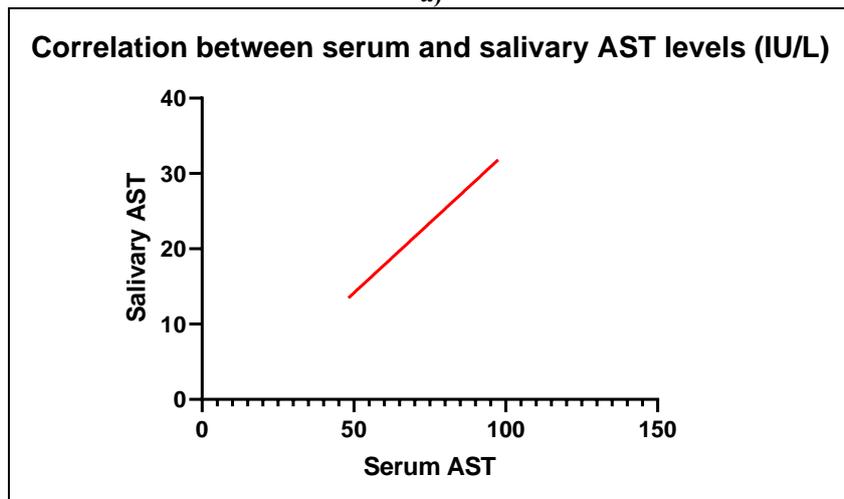
Strongly correlated Biochemical parameters

Pearson's correlation analysis has revealed that there is a strong positive correlation between serum and salivary levels of AST (0.9720), ALT (0.9161) and creatinine (0.8373). However, except creatinine which is almost in similar concentrations (1.27:1) other two parameters AST and ALT were 2.38 to 3.2 times and significantly different ($P<0.05$) from that of their serum levels. This strong correlation suggests that AST, ALT, and Creatinine appear in saliva mostly from serum and concentration gradient driven mechanisms could be basis for their transport from serum to saliva, the effect of local metabolism or salivary glands secretion over the concentration of these molecules in saliva might be limited, particularly in healthy animals. Likewise, studies in other species

like buffaloes (Hefnawy et al., 2013) and dogs (Tvarijonaviciute et al., 2018) has reported a strong correlation of creatinine levels between saliva and serum in various pathophysiological conditions. In contrast, in certain other studies in cows suffering with metritis a poor correlation of creatinine levels between serum and saliva is reported (Valleo-Mateo et al., 2024). However, the same study (Valleo-Mateo et al., 2024) in cows has reported AST and ALT levels in saliva were sensitive and correlated with the levels in serum in accordance to present study findings. Although the correlations were in concurrence with most of the studies, but the ratios of their levels between saliva and serum varied with species. Therefore, salivary levels of AST, ALT and Creatinine may serve as an alternate non-invasive method to understand their variations in serum, by taking into consideration of species and pathophysiological variations. Further, the methods adopted for their estimation in serum were suitable and could detect their salivary levels.
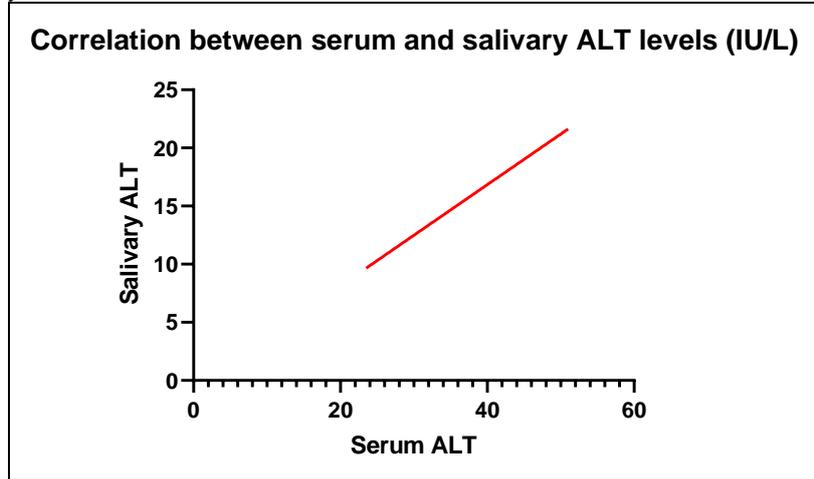
a)

b)
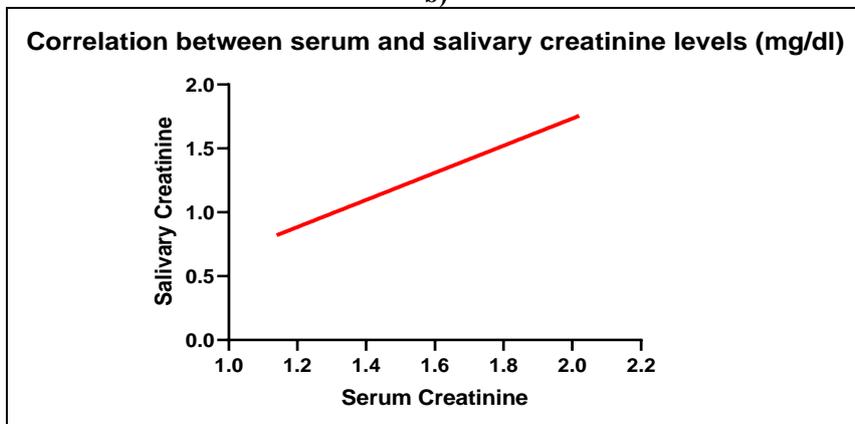
c)

Fig 2: Correlation trendlines of strongly correlated parameters between serum and saliva (a, b and c)

Moderately correlated Biochemical parameters

Nonetheless, other parameters like total cholesterol and phosphorous in serum and saliva were moderately positively correlated (0.663 and 0.6789), albumin and total bilirubin is moderately negatively correlated (-0.5030 and -0.446) despite this correlation their levels in serum were many times higher (12.9 times for albumin , 8.5:1 time for cholesterol) or many times lower (0.31:1 for phosphorous, 0.47 :1 for total bilirubin) when compared to their levels in saliva. This suggests that these metabolites are either actively secreted or excreted from saliva beyond concentration gradients or governed and having a unique metabolism for these molecules in salivary glands that judges their levels in saliva apart from serum's contribution. A similar correlation (r=0.63) of serum and salivary cholesterol levels were reported earlier (Kale et al., 2017) and further a similarly low levels of salivary cholesterol when compared to serum was observed in previous studies (Fricaudet et al., 2023). Though a moderate correlation of salivary and serum phosphorous level has been observed in present study, previous reports states that saliva is a major phosphorous reservoir and helps in maintaining ruminal pH (as salivary phosphorous serves a key buffer in regulating ruminal pH) (Castillo-Lopez et al., 2021). Therefore, salivary phosphorous levels are independent and their levels in saliva vary and it is the effect of the above and other factors like amount of dry matter intake (Qureshi et al., 2019). In the similar lines a wide variation in the salivary phosphorous levels has been observed among the individual animals in the present study. Some of the animals' salivary phosphorous levels has fallen out of range (considered as missing values in data analysis) and method adopted for serum phosphorous levels estimation was unable to detect such high salivary phosphorous levels. Therefore, samples must be diluted based on the phosphorus concentration before adopting molybdenum blue method for salivary samples (considering the linearity limit as 15 mg/dl).

No works has reported on the correlation of serum and salivary albumin, but the levels of albumin in saliva was lower when compared to serum and the average levels in saliva was 0.3 mg/dl in present study. Few previous studies have reported similar salivary albumin levels (Abd Ellah et al., 2015). Likewise, very few studies have reported salivary total bilirubin and correlation with serum, in contrast to present study findings of moderate negative correlation, in equines a moderate positive correlation was observed for salivary and serum total bilirubin (Contreras Aguilar et al., 2019)

Poorly correlated Biochemical parameters

Urea (r = -0.3923) and direct bilirubin (r = -0.3392) in saliva and serum were negatively correlated and the correlation is poor and non-significant, though their average concentrations in serum and saliva were almost similar (0.97:1 and 1.08:1). In contrast to our findings a strong correlation between serum and salivary urea levels were reported in dogs suffering with kidney disease (Tvarijonaviciute et al., 2018). In addition, parameters like glucose, calcium, and uric acid levels were poorly correlated between serum and saliva and their levels are many times higher in serum when compared to saliva (8.5, 12.9, 1.59 and 1.8 times). However, in certain other studies moderate to strong correlations were reported for these parameters (Hefnawy et al., 2013, Soukup et al., 2012)

In spite the salivary average levels of calcium and phosphorous has been observed to be on par with serum levels, the individual animal data indicates that the levels of both calcium and phosphorous were either very high or very low when compared to their levels in serum as reported in few other studies (Abd Ellah et al.,, 2015) and surprisingly it is found that there is strong negative correlation between salivary calcium and phosphorous levels (r=-0.8907). This further endorses that saliva acts a reservoir for these electrolytes and helps in buffering and maintaining their concentrations in different body compartments as per the requirements.

In present study an attempt has been made to estimate the total protein by Biuret method. However, the method failed to detect and estimate the lower levels of protein present in saliva. Previous studies in cows by using commercial kits have reported that the salivary protein concentration in the range of 0.1 to 0.3 g/dl (Contreras Aguilar et al., 2019). However, Biuret method adopted in present study (automatic analyser), a commonly used method for serum total protein estimation was found to be not effective for the lower salivary protein concentrations.

Storage stability of biochemical parameters serum *vs* saliva

The storage stability of most of the biochemical parameters in serum and saliva is comparable. Significantly altered levels of these parameters were observed on 180th day of storage when compared to 0th day in both serum and saliva (Table 2 &3). However, certain enzymatic parameters like AST and ALT have significantly reduced by 120th day of storage and few parameters like creatinine and urea levels have increased non significantly upon storage in both serum and saliva by 180th day. Whereas, calcium and phosphorous levels increased significantly in serum and non-significantly in saliva. Despite assumptions of poor stability of metabolites in saliva due to oral microbiota metabolism and other factors upon storage (Ferrari. et al., 2023), stability of most of the biochemical parameters in saliva was comparable to that of serum

Table 2: Stability of various biochemical parameters in serum during storage

S. No	Biochemical Parameter	0th Day	7th day	120th day	180th day	P Value
1	Glucose (mg/dl)	61.50 ±6.318	57.67 ±5.914	48.83 ±5.212	19.83* ±2.34	P<0.001
2	Total Cholesterol (mg/dl)	183.5 ±23.20	177.2 ±22.93	187.3 ±20.55	148.8 ±19.08	NS
3	Albumin (g/dl)	3.650 ±0.076	3.683 ±0.10	3.533 ±0.111	2.467 ±0.21*	P<0.001
4	Creatinine (mg/dl)	1.395 ±0.137	1.388 ±0.132	1.483 ±0.118	1.083 ±0.082	NS
5	Urea (mg/dl)	37.70 ±1.263	36.93 ±1.645	36.93 ±1.645	40.67 ±2.290	NS
6	AST (IU/L)	74.12 ±6.587	58.95 ±5.328	13.70 ±1.467*	8.817 ±1.73*	P<0.0001
7	ALT (IU/L)	35.08 ±4.089	31.58 ±3.630	7.733 ±1.675*	7.500 ±1.33*	P<0.001
8	Calcium (mg/dl)	8.277 ±0.232	8.190 ±0.270	8.242 ±0.151	13.81 ±1.50*	P<0.001
9	Phosphorous (mg/dl)	6.980 ±0.605	6.522 ±0.518	8.13 ±0.67	10.10 ±0.72*	P<0.001
10	Uric acid (mg/dl)	6.573 ±0.585	6.493 ±0.568	6.325 ±0.454	5.392 ±0.29	NS

Table 3: Stability of various biochemical parameters in saliva during storage

S. No	Biochemical Parameter	0th Day	7th day	120th day	180th day	P Value
1	Glucose (mg/dl)	7.167 ±0.3073	7.667 ±0.3333	7.667 ±0.3333	5.333 ±0.6667*	P<0.05
2	Total Cholesterol (mg/dl)	21.17 ±0.4773	22.83 ±0.3073	22.83 ±0.3073	35.67 ±0.8819	NS
3	Albumin (g/dl)	0.2833 ±0.04773	0.2667 ±0.08028	0.2667 ±0.08028	0.1667 ±0.03333	NS
4	Creatinine (mg/dl)	1.092 ±0.1708	1.073 ±0.2407	1.073 ±0.2407	1.120 ±0.1340	NS
5	Urea (mg/dl)	38.65 ±4.064	39.67 ±3.783	42.33 ±3.913	42.03 ±5.273	NS
6	AST (IU/L)	23.12 ±2.522	16.05 ±1.494*	5.933 ±0.4876*	2.367 ±0.6515*	P<0.001
7	ALT (IU/L)	14.70 ±1.942	11.57 ±0.6222	3.800 ±0.5177*	2.133 ±0.5155*	P<0.001
8	Calcium (mg/dl)	5.175 ±2.403	6.578 ±2.732	6.578 ±2.732	7.903 ±0.9873	NS
9	Phosphorous (mg/dl)	18.69 ±7.768	19.94 ±8.164	23.44 ±8.919	35.09 ±9.395	NS
10	Uric acid (mg/dl)	3.612 ±0.05689	3.602 ±0.05630	3.767 ±0.04080	3.767 ±0.04080	NS

Conclusion

This study suggests that biochemical parameter like creatinine, AST and ALT levels in saliva were analogous and reflect their levels in serum. The key metabolites like glucose, total cholesterol and albumin levels in saliva are very low when compared to their levels in serum and remains to be constant and a very low variance has been observed across the animals, this may be due to a strict barrier for these compounds between the two fluids and suggests that their levels are tightly regulated in saliva. Based on all the findings it is advisable that except few biochemical parameters like AST, ALT, Creatinine, Urea, and direct bilirubin levels. The other parameters in saliva must be analysed to great extent about the conditions, molecular insights and other factors that control their levels in saliva before considering their levels in saliva as an alternate of serum levels. Further it is not warranted that a similar correlation may exist with different pathological conditions as some studies has reported a good correlation in such case. For example, urea which is observed to be poorly correlated in present study was reported to be strongly correlated in various chronic kidney conditions in other species. Therefore, there is a need of an elaborate study in a large population considering various pathophysiological conditions, prior to establishing and considering saliva as an alternative diagnostic fluid in veterinary practise. Further, though saliva serves as a non-invasive biofluid for disease diagnosis certain hazards of saliva collection like exposure to zoonotic pathogens present in animal saliva, risk of bites or scratches during animal handling, stress or injury to the animal during restraint or collection, sample contamination and environmental biohazard exposure has to be properly addressed during its collection.

Ethical statement

Sample collection was approved by institutional animal ethics committee and methods are as prescribed by the committee

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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