

Detection of ovine leptospirosis in various agro-climatic zones of Odisha in the aftermath of cyclone Hudhud using a multi-faceted approach

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ABSTRACT

Leptospirosis is an important emerging zoonotic disease responsible for hampering the productivity of ovine husbandry worldwide. A total of 1,877 ovine serum samples were collected from five agro-climatic zones of Odisha following the aftermath of cyclone Hudhud. A multifaceted approach for detection of anti-*Leptospira* antibodies using Microscopic Agglutination Test (MAT), in-house developed rapid spot tests such as recombinant Loa22 antigen-based Latex Agglutination Test (rLoa22 LAT) and rLoa22-based IgG Dot ELISA Dipstick Test (rLoa22 IgG DEDT) were developed. Further, nucleic acid detection of leptospirosis employing Polymerase Chain Reaction (PCR) utilizing LigBF/LigBR and G1/G2 were performed. Additionally, renal histopathology of kidneys from MAT positive sheep was also used for investigating leptospirosis in sheep. MAT results for ovine sera revealed a seropositivity of 16.62% (312/1877) with maximum agglutinins detected against serovar Pomona. Among agro-climatic zones, South Eastern Ghat recorded the highest seroprevalence while Western Central Table Land recorded the lowest seroprevalence for ovine leptospirosis. A total of 296 and 345 sera tested positive by rLoa22 LAT and rLoa22 IgG DEDT respectively. PCR performed on kidneys of MAT positive sheep revealed amplicons for LigBF/LigBR and G1/G2 primers in 104 and 93 sheep respectively. The histopathological evidence such as tubular atrophy, severe tubular degeneration and necrosis with interstitial and periglomerular fibrosis suggestive of chronic leptospirosis was present in 78 sheep. This multi-pronged approach of employing a battery of diagnostic tests would allow the execution of mediation strategies to curb ovine leptospirosis following natural calamities such as cyclones.

Keywords: Dot ELISA dipstick test, Latex agglutination test, Leptospirosis, Microscopic agglutination test, Polymerase chain reaction, Sheep

India has 75 million sheep, second in world (Mazinani and Rude 2020). However, infectious diseases such as leptospirosis have reduced the fecundity and hence profitability of small ruminant husbandry in India (Sabarinath *et al.* 2018). Lack of awareness among small ruminant farmers about leptospirosis vaccine, poor sanitary conditions, lack of management of synanthropic rodents, and lack of field-level diagnostic

tests are among primary reasons. Thus, ‘point-of-care diagnostics’ including LAT and IgG-based DEDT are required to diagnose diseases at field level without time-consuming laboratory tests (Behera *et al.* 2014).

Spirochaetes from pathogenic species *L. interrogans*, that includes 250 pathogenic serovars from 24 serogroups, are source of leptospirosis, a recently discovered prototypical disease of small ruminants (Xu *et al.* 2017). Due to its numerous clinical symptoms, including infertility, abortion, neonatal deaths, stillbirth, embryonic resorption, milk drop syndrome, prolonged lambing intervals, weak lamb birth, increased number of services per conception, along with decreased productivity, leptospirosis is a burden to small ruminant husbandry (Martins *et al.* 2012a, Martins *et al.* 2012b, Martins and Lilenbaum 2014).

Leptospirosis in small ruminants has been associated to strains belonging to the serogroup Sejroe, particularly serovar Hardjo (Lilenbaum *et al.* 2007a, 2007b, Seixas *et al.* 2007). The age of small ruminants and type of farming seem to be potential risk factors since adult animals reared under intensive farming system are most commonly infected (Martins *et al.* 2014). Since animals in tropical climates that

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come into contact with rodents have been demonstrated to triple the probability of developing seroreactive animals against leptospirosis, environmental factors and rodent presence on farm premises also significantly influence incidence of leptospirosis in small ruminants (Lilenbaum *et al.* 2008, Higino *et al.* 2013). Sheep and goats have been demonstrated to excrete *L. interrogans* in their urine, that may transmit the infection to humans and other animals (Hajikolaie *et al.* 2022).

A standard test for serological identification of leptospirosis in small ruminants is known as MAT (Martins *et al.* 2012a). But MAT's inherent flaws have led researchers to switch to other field-oriented assays (Budihal and Perwez 2014). "Latex Agglutination Test (LAT)" is a simple, cost-effective spot test for screening sera samples in endemic areas without advanced technology (Senthilkumar *et al.* 2008). Dot-ELISA Dipstick Test (DEDT) is another prominent pen-side spot assay. It is simple to operate and affordable, and it is often employed for screening several sera samples or a single sample (Tansuphasiri *et al.* 2005, Shekatkar *et al.* 2010a, 2010b).

With a detection limit of 6 leptospire, *Lig* gene-based diagnostic PCR technique has been utilized to identify pathogenic *L. interrogans* in biological specimens (Palaniappan *et al.* 2005). Since positive PCR test results in biological specimens without leptospire isolation by culture methods have been reported, several disease investigators have employed PCR as a diagnostic tool for improving reliability of disease inspection (Fearnley *et al.* 2008, Tulsiani *et al.* 2011). Hence, a diagnostic PCR using *LigB* gene has been employed for the detection of leptospire in kidney specimens of sheep whose results have been further analyzed with a traditional PCR assay using G1/G2 primers.

With 480 km of coastline along Bay of Bengal, cyclone-prone Indian state of Odisha has seen multiple destructive cyclones that have been associated with post-cyclone

spikes in leptospirosis incidence (WHO 2000, Sehgal *et al.* 2002, Jena *et al.* 2004). Cyclone Hudhud that occurred during October 2014, devastated property and lives in 15 districts of Odisha.

The present study for seroprevalence of ovine leptospirosis in five agro-climatic zones of Odisha was conducted immediately following the aftermath of cyclone Hudhud using a battery of diagnostic tests to confirm the intricate association between cyclones and leptospirosis.

MATERIALS AND METHODS

Ovine serum sample collection: During exsanguination of sheep at the time of slaughter, 1877 ovine sera, from five agro-climatic zones of eastern Indian state of Odisha, were collected. The study sample was calculated as per sample size estimation formula for finite population (Grande 2016). Fig.1 illustrates district-wise locations of slaughterhouses from where sera samples have been collected in each of Odisha's agroclimatic zones. Ovine sera samples were collected from four cyclones Hudhud affected districts of Odisha such as Malkangiri, Puri, Gajapati, and Kalahandi as well as from cyclone Hudhud unaffected district of Balangir from November-December, 2014 to study the intricate relationship between cyclone and Leptospirosis. Serum tubes (Becton Dickinson, 106 Franklin Lakes, NJ, USA) have been employed to extract 5 ml of blood, that had been centrifuged at 2,000 rpm for 20min then stored at -80°C.

Microscopic agglutination test (MAT): As previously stated, MAT has been employed for detecting presence of anti-*Leptospira* antibodies in sheep (USDA 1987). By adding an amount of leptospiral antigen equivalent to 1:50 diluted serum volume, final serum dilution has been achieved 1:100 in MAT. Sixteen live leptospiral serovars (leptospiral cultures roughly 4-8 days' old containing about 200 million leptospire/mL) such as *L. borgpetersenii* serovar Hardjo

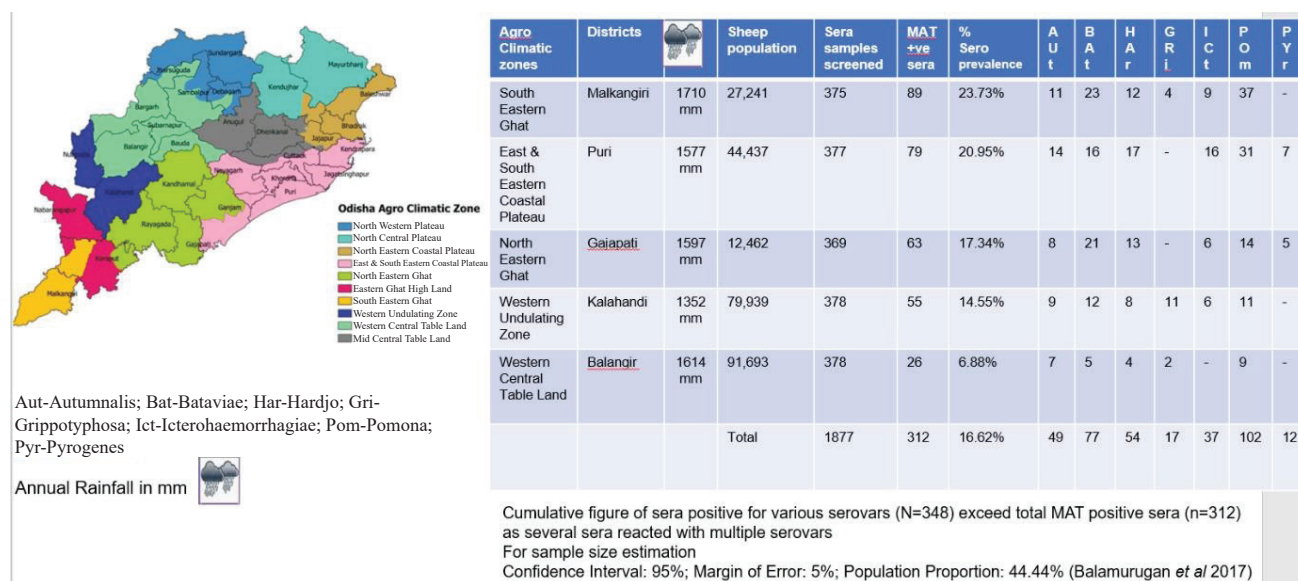


Fig.1. Seroprevalence of ovine leptospirosis in various agro-climatic zones of Odisha, India

strain *Hardjoprajitno*, *L. borgpetersenii* serovar *Javanica* strain *Veldrat* *Batavia* 46, *L. borgpetersenii* serovar *Tarassovi* strain *Perepelitsin*, *Leptospira interrogans* serovar *Australis* strain *Ballico*, *L. interrogans* serovar *Autumnalis* strain *Akiyami* A, *L. interrogans* serovar *Ballum* strain *S102*, *L. interrogans* serovar *Bataviae* strain *van Tienen*, *L. interrogans* serovar *Canicola* strain *Hond Utrecht IV*, *L. interrogans* serovar *Djasiman* strain *Djasiman*, *L. interrogans* serovar *Hebdomadis* strain *Hebdomadis*, *L. interrogans* serovar *Icterohaemorrhagiae* strain *RGA*, *L. interrogans* serovar *Pomona* strain *Pomona*, *L. interrogans* serovar *Pyrogenes* strain *Salinem*, *L. kirschneri* serovar *Cynopteri* strain *3522C*, *L. kirschneri* serovar *Grippotyphosa* strain *Moskva* V and *L. noguchii* serovar *Louisiana* strain *LSU 1945* have been utilized to conduct MAT. Antigen-antibody combination has been examined under dark field microscope following three hours of incubation of microtiter plates in a BOD incubator. A seropositivity/exposure for leptospirosis indicated by MAT is explained as any sera showing >50% agglutination in the field of dark field microscope in contrast to control at serum dilution of 1:100 for a minimum of one leptospiral serovar. Second incubation with two-fold serial dilutions of positive ovine sera for leptospirosis is required to determine endpoint titre.

Histopathology: A sheep with MAT+ve leptospirosis kidney tissue has been fixed in 10% Neutral Buffered Formalin. Standard histopathological methods have been implemented following buffered formalin treatment (Luna 1968). An automatic tissue processor “(Leica Biosystems, Wetzlar, Hesse, Germany)” has been employed for preparing renal tissues following overnight submersion in tap water. Renal tissue paraffin blocks have been designed after the tissues had been dried in progressively higher grades of ethanol, cleaned with xylene, then embedded in molten paraffin wax (Merck, Darmstadt, Hesse, Germany). A microtome (Thermo Shandon, Woonsocket, RI, USA) has been employed for cutting and segmenting renal tissue blocks at 5 µm thickness. Once on glass slides, they received staining with hematoxylin and eosin.

Polymerase chain reaction of sheep renal tissue: DNA was drawn out from kidney samples of sheep (25 mg each), which tested positive for leptospirosis by MAT. Two sets of primers were used to carry out PCR viz. G1/G2 (5'-CTGAATCGCTGTATAAAAGT-3' and 5'-GGAAAACAAATGGTCGGAAG-3') and LigBF/LigBR (F3/B3) (5'-AACCGGTCTGGTAGGATT-3' and 5'-GAATCGGGGACGATGGAT-3'). Reaction conditions for G1/G2 and LigBF/LigBR (F3/B3) have been followed as already described (Gravekamp *et al.* 1993, Ali *et al.* 2017). Saprophytic leptospires (*L. biflexa* serovar *Patoc*) served as negative control in 2 PCR reactions, while *L. interrogans* serovar *Icterohaemorrhagiae* served as positive control. After completing agarose gel electrophoresis, PCR product could be detected utilizing UV trans-illuminator (Bio-Rad, Hercules, CA, USA).

Induction of expression and purification of

recombinant Loa22 antigen: BL21 strain of *Escherichia coli* possessing recombinant pET28b-Loa22 plasmid was utilized for rLoa22 protein synthesis. The recombinant pET28b-Loa22 plasmid was custom synthesized (Biotech Desk Pvt Ltd, Hyderabad, India) by inserting codon-optimized synthetic Loa22 gene at NdeI and XhoI restriction sites of pET28b plasmid vector. To cultivate the *E. coli* BL21 strain, Luria Bertani broth (Difco, Sparks, MD, USA) had been utilized. *E. coli* BL21 cells had been stimulated with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) when spectrometric reading reached 0.5-0.7 at OD 600nm, then they had been left to grow overnight at 37°C. Proteins have been investigated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when cells have been harvested. Recombinant protein has been purified through Ni-NTA agarose affinity chromatography (Qiagen, Hilden, Germany) following manufacturer's instructions. To analyze isolated recombinant protein, SDS-PAGE had been employed.

Western blot analysis: Following conventional procedure, Western blot analysis had been performed for assessing immunogenicity of purified recombinant Loa22 antigen utilizing high-titred ovine sera (Towbin *et al.* 1979). The purified recombinant Loa22 protein had been separated on 15% SDS-PAGE then transferred onto “nitrocellulose membrane (NCM) (Thermo Fisher, Waltham, MA, USA)” utilized for transferring buffer with current at 0.8 mA/cm² in a blotting device (BioRad, Hercules, CA, USA) to perform western blot. NCM has been stained with Ponceau S stain to determine insertion of the recombinant Loa22 protein. Membrane initially subjected to a positive ovine serum control (1:400 MAT titre against *L. grippotyphosa*) then to known negative sera (1:50 MAT dilution). Loa22 protein on membranes had been immuno-stained with species-specific secondary antibodies coupled to horse radish peroxidase at 1:2500 dilution upon overnight blocking using 3% skimmed milk. Finally, reaction had been developed with 3,3',5,5'-TMB (Sigma-Aldrich). For determining if field sera with high MAT titre against other leptospiral serovars react with rLoa22 antigen produced by expressing the Loa22 gene of *L. grippotyphosa*, employed western blot analysis on ovine field sera reactive to serovars that include *L. icterohaemorrhagiae*, *L. Pomona*, and *L. autumnalis*.

Recombinant: Loa22-based Latex agglutination test (rLoa22-based LAT): With a few minor adjustments, rLoa22 antigen coating of latex beads has been conducted as previously described (Dey *et al.* 2007). 1.0 µm diameter polystyrene latex beads (Sigma-Aldrich, St. Louis, MO, USA) had been washed thrice with glycine buffered saline (Glycine 0.1M, NaCl 10.17M; pH 8.2). An equivalent volume of rLoa22 protein (1 mg/mL), that is equally diluted in glycine buffered saline, has been added to polystyrene beads that had been cleaned with glycine buffered saline. To ensure even mixing, protein-latex bead mixture had been kept in a shaking apparatus at room

temperature overnight. After being blocked with 0.04% BSA (Difco, USA), protein-coated polystyrene beads had been incubated overnight. After centrifuging, latex beads had been reconfigured as a 2% solution in glycine-buffered saline with 0.02% sodium azide added as preservative. Prior being utilized, rLoa22-coated polystyrene particles had been stored at 4°C. To perform LAT, equal amounts of serum (20 µL) and protein-coated latex beads (20 µL) had been combined on glass slides. Test's results were observed within two minutes. When agglutination is observed, sera is deemed positive for leptospirosis. Sera samples which agglutinated within 30 sec, 30 sec to 1 min, and within 2 min were given scores of 3, 2 and 1+ve respectively as mentioned earlier (Smits *et al.* 2000). Samples had been deemed negative in absence of agglutination.

Recombinant Loa22-based IgG dot ELISA dipstick test: With a few minor adjustments, IgG-based DEDT has been conducted applying previously disclosed methodology (Tansuphasiri *et al.* 2005). NCM (Thermo Fisher, Waltham, MA, USA) attached to tips of plastic combs had been dotted with 2 µL of rLoa22 antigen in carbonate-bicarbonate buffer at different concentrations (250 ng-1 µg) then allowed to air dry to standardize IgG DEDT. Through blocking buffer solution consisting of 3% bovine serum albumin in PBS-T (0.05% Tween-20 in PBS), NCM's vacant sites have been blocked overnight at 4°C. Dotted NCM had been cleaned utilizing wash buffer that included 0.05% Tween-20 in PBS. Wells of a microtiter plate have been filled with 200 µL of varying dilutions of ovine sera (1:50-1:400) that had been diluted in blocking buffer. Following being submerged in micro-titer plate's wells, combs speckled with Loa22 antigen had been incubated for 1 h at 37°C. Wash buffer was used at least thrice for at least 5 min each for washing the Loa22 coated combs. The cleaned combs have been incubated for an hour at 37°C after being submerged in varied dilutions (2500-10,000) of rabbit anti-ovine IgG HRP conjugate (Sigma-Aldrich). 200 µL of substrate buffer containing 3,3',5,5'-TMB (Sigma-Aldrich) has been added to combs after they had been cleaned three times with wash buffer. They were then left in the dark for fifteen minutes. For stopping reaction, NCM strips had been rinsed with PBS. Appearance of purple dots on NCM indicates positive reaction. Negative test result could be determined by absence of a purple color dot on NCM. All ovine sera samples had been tested utilizing known positive and negative controls.

Statistical analysis: For serodiagnosis of leptospirosis in sheep, relative specificity, sensitivity, and accuracy (in percentage) of IgG-based DEDT and rLoa22-based LAT in comparison with MAT have been evaluated in detail below.

$$\text{Specificity} = d/(b+d) \times 100$$

where d is number of serum samples negative by MAT and rLoa22 based LAT/IgG DEDT, 'b' number of serum samples negative by MAT however positive by rLoa22 based LAT/IgG DEDT"

$$\text{Sensitivity} = a/(a+c) \times 100,$$

here a is number of serum samples positive by both MAT and rLoa22 based LAT/IgG DEDT, c is number of serum samples positive by MAT but negative by rLoa22 based LAT/IgG DEDT

$$\text{Accuracy} = a+d/(a+b+c+d) \times 100$$

following standard method positive and negative results from tests have been calculated intuitively for determining predictive values (in percent) (Jacobson 1998).

$$\text{Positive Predictive Value (PPV)} = a/(a+b) \times 100$$

$$\text{Negative Predictive Value (NPV)} = d/(c+d) \times 100$$

Kappa statistics has been employed for comparing rLoa22-based LAT/ IgG DEDT with MAT for small ruminant anti-*Leptospira* antibody detection (Deneke *et al.* 2014).

RESULTS AND DISCUSSION

MAT results demonstrated seroprevalence of 16.62% (312/1877) for ovine leptospirosis with maximum agglutinins detected against Pomona followed by Bataviae, Hardjoprajitno, Autumnalis, Icterohaemorrhagiae, Grippityphosa, and Pyrogenes (n=102,77,54,49,37,17,12 respectively) (Fig. 1). All MAT-tested ovine sera lacked antibodies to serovars "Australis, Ballum, Canicola, Cynopteri, Djasiman, Hebdomadis, Louisiana, and Tarassovi". Leptospiral serovar MAT titres varied from 1:100-1:1600. The MAT results indicate that Malkangiri (south eastern ghats) district recorded the highest seroprevalence (23.73%) for ovine leptospirosis (Fig. 1). The landlocked Balangir (Western Central Tableland) district recorded the lowest seroprevalence for ovine leptospirosis (Fig. 1).

The present study conducted immediately following the aftermath of cyclone Hudhud confirms the complex connection between cyclones and leptospirosis. South Eastern Ghat was the agro-climatic zone which recorded the highest seroprevalence of 23.73% for ovine leptospirosis. The reason could be attributed to the fact that Malkangiri district located in South Eastern Ghat was among the 15 districts affected by cyclone Hudhud. Further, south eastern ghat receives the highest annual rainfall of 1710 mm annually among all the ten agro-climatic zones of Odisha (DAHVS, Odisha 2025). This high precipitation results in flooding which leads to sewage-contaminated water laden with pathogenic leptospires entering into public water supply system which would be consumed by farm animals. Moreover, the unfettered transboundary movement of sheep from adjoining Indian state of Andhra Pradesh, which was the worst-hit Indian state during cyclone Hudhud, could be an exacerbating factor responsible for highest seroprevalence of ovine leptospirosis in this agro-climatic zone.

East and south eastern coastal plateau and north eastern ghat had the second and third highest ovine leptospirosis seroprevalences of 20.95% and 17.34%. Cyclone Hudhud impacted 15 Odisha districts, including Puri and Gajapati in the East and South Eastern Coastal Plain and North Eastern Ghat.

The two landlocked agro-climatic zones such as Western Central Tableland and Western undulating zone recorded lowest and second lowest seroprevalence for ovine leptospirosis respectively. Balangir, being a non-coastal inland district in Odisha, and located in Western Central Table land, was unaffected by cyclone Hudhud and recorded the least seroprevalence of 6.88%. Another reason for the lowest seroprevalence can be attributed to the fact that even though Western Central Table land receives precipitation of 1614 mm annually, this agro-climatic zone has no water-holding capacity and is mostly a dry area out of all the 10 agro-climatic zones of Odisha. Even though Kalahandi located in Western undulating zone was one of the districts affected by cyclone Hudhud, this agro-climatic zone did not show a sharp spike in the incidence of leptospirosis partly since this region receives a precipitation of 1352 mm annually that is the lowest among all 10 agro-climatic zones of Odisha (DAHVS, Odisha 2025).

Gross examination of several slaughtered sheep's kidneys revealed pale white necrotic spots on their subcapsular surface. Gross kidney results in this investigation resemble those in a previous investigation (Azizi *et al.* 2012). The kidneys of sheep that tested positive by MAT were subjected to histopathological examination. Major histopathological results of renal tissue observed in 78 sheep include tubular atrophy, severe tubular degeneration, and necrosis with interstitial as well as periglomerular fibrosis which was suggestive of chronic leptospirosis. (Fig. 2A and 2B). Author of mouse model of chronic *L. interrogans* infection (Fantom d'Andon *et al.* 2014) proposed that leptospiral colonization of kidneys

caused renal fibrosis, that had pathologically accumulated on ECM components that compromised renal functions.

Lymphocyte-dominated multifocal lymphoplasmacytic inflammatory infiltration had been observed (Fig. 2A and 2B). Two prior investigations demonstrated multifocal lymphoplasmacytic inflammatory infiltration with a bias toward lymphocytes (Almeida *et al.* 2019, Mathesh *et al.* 2021).

PCR amplicons of 219 bp and 285 bp were observed in 104 and 93 sheep when PCR amplification of kidney samples from MAT⁺ve sheep was performed using LigBF/LigBR (F3/B3) and G1/G2 primers respectively (Fig. 2C). This proves that PCR employing LigBF/LigBR (F3/B3) were more sensitive than PCR done using G1/G2 primers. The reason for the lower sensitivity of G1/G2 primers compared with LigBF/LigBR (F3/B3) primers is since these primers fail to detect *L. kirschneri* species, which can be detected using B641 and B651 primers (Gravekamp *et al.* 1993) and also by LigBF/LigBR (F3/B3) primers. We have found that the sera of 11 kidney samples which G1/G2 primers failed to detect were seropositive only for serovar Grippotyphosa which belongs to *L. kirschneri* species.

The recombinant Loa22 protein showed an expression level of approximately 20 mg/L purified protein (Fig. 3A). Expected molecular weight of rLoa22 had been 22kDa, however, SDS-PAGE demonstrated 29 kDa. SDS-PAGE revealed rLoa22 protein following 2h IPTG induction. At 8 h after IPTG induction, rLoa22 protein expression peaked. In western blot analysis, rLoa22 protein had been highly reactive against anti-*Leptospira* antibodies in ovine

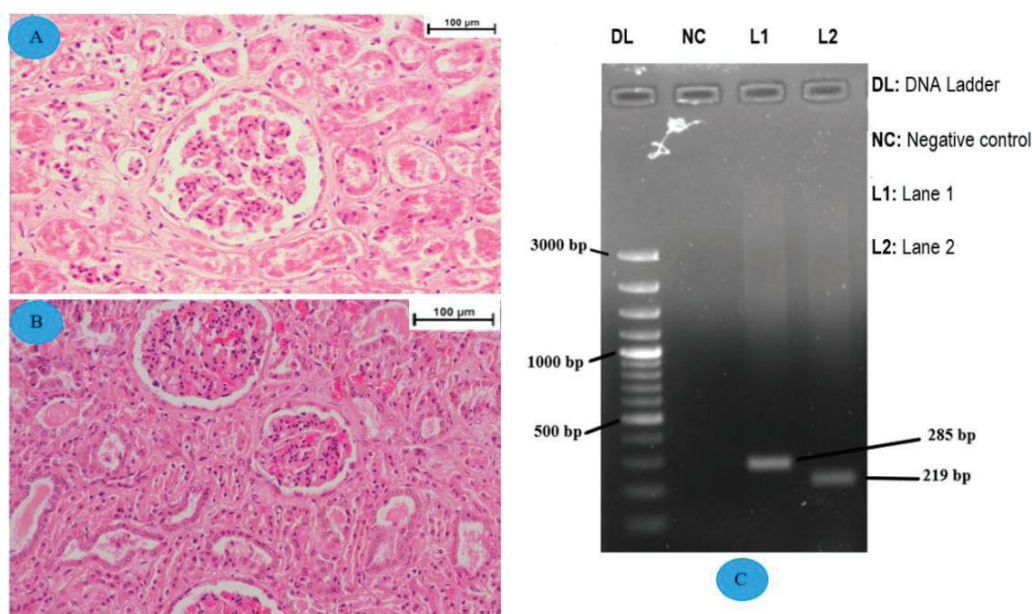


Fig. 2. (A) Histopathology of kidney in chronic case showing severe tubular degeneration and necrosis with interstitial as well as periglomerular fibrosis HE x200. (B) Histopathology of sheep kidney in chronic case showing severe tubular degeneration HE x200. (C) PCR amplification of leptospira DNA in sheep kidney tissue using leptospira specific primers DL: 100 bp plus DNA Ladder, NC: Negative control (*L. biflexa* serovar Patoc), L1: PCR amplicon (285 bp) using G1/G2 primers, L2: PCR amplicon (219 bp) using LigBF/LigBR (F3/B3) primers

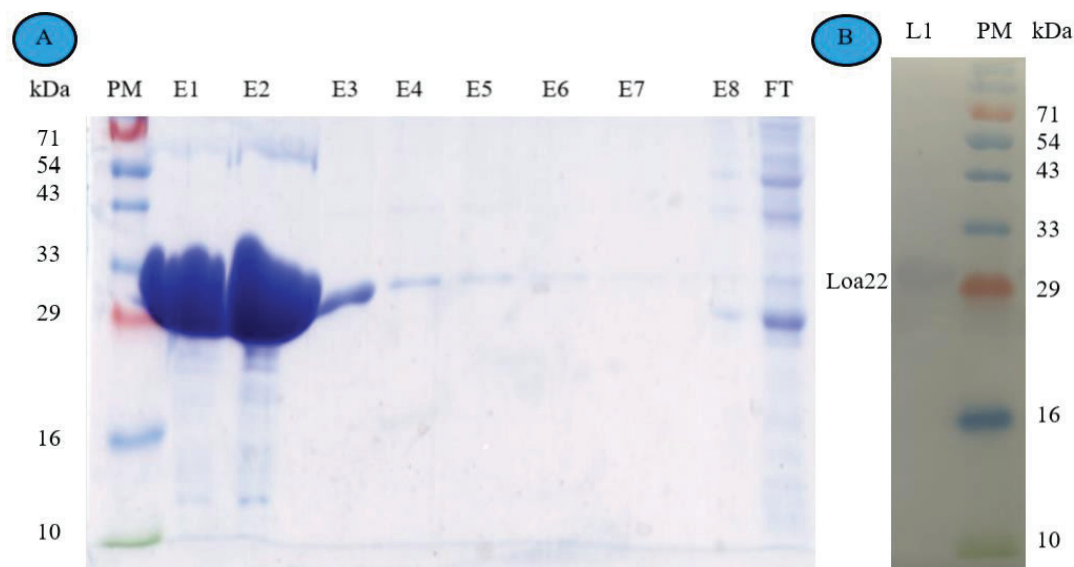


Fig. 3. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of elution fractions of purified recombinant Loa22 protein obtained after Ni-NTA affinity chromatography. (B) Western blotting of recombinant Loa22 protein against ovine field serum sample

sera, resulting in brownish NCM bands (Fig. 3B). We additionally discovered that rLoa22 protein responded in western blot to sera positive for *L. grippityphosa* in MAT (Positive control) and various leptospiral serovars “(*L. icterohaemorrhagiae*, *L. Pomona* and *L. autumnalis*)”, demonstrating its presence in all pathogenic serovars.

LAT agglutinated positive ovine sera (Fig. 4B, 4C, 4D) and distinguished negative sera with uniform suspension (Fig. 4A). MAT titre and LAT scores have been similar. Ovine sera (n=52) with MAT titre $\geq 1:400$ had LAT score of 3+ve and spontaneously exhibited high-intensity agglutination (within <30 sec), resulting in immediate visible flakes upon addition (Fig. 4D). Sera samples (n=69) with MAT titre of 1:200 and LAT score of 2+ve demonstrated fair intensity of agglutination, forming ring at periphery and an empty centre during glass slide revolving and spanning 30 sec to 1 min. Sera samples (n=158) with a MAT titre of 1:100 and LAT score of 1+ve

showed feeble level of agglutination which took 2min to occur (the outer ring at the periphery not elevated) (Fig. 4B). 33 MAT+ve sera (1:100 titre) failed to agglutinate in LAT (Fig. 4A), whereas 17MAT-ve sera scored 1+. LAT and MAT detected 296 (15.77%) and 312 (16.62%) of 1877 sera samples. Specificity, sensitivity, accuracy, and Kappa value of rLoa22-based LAT against MAT are displayed in Table 1.

For ovine leptospirosis detection, rLoa22-based LAT satisfied MAT specifications regarding specificity, sensitivity, and accuracy. This investigation revealed LAT negative in 33 sera with MAT titres of 1:100. This indicates that LAT is slightly less sensitive (89.42%) in comparison to MAT. However, LAT gave high specificity of 98.91% in comparison to MAT mainly due to employment of recombinant protein which lack non-specific moieties found in whole-cell preparations (Hoelzle *et al.* 2007). Moreover, LAT produces limited amount of biohazardous

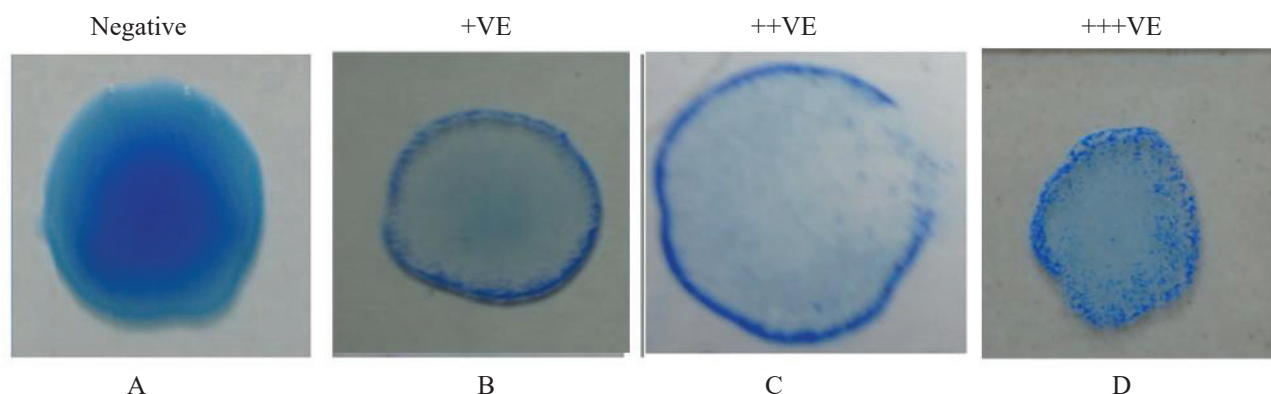


Fig. 4. Latex agglutination test results of ovine sera samples using recombinant Loa22 antigen. (A): Negative ovine sera showing homogenous suspension; (B, C and D): Ovine sera showing a LAT score of 1+ve, 2+ve and of 3+ve respectively.

Table 1. Relative sensitivity, specificity and accuracy values of recombinant Loa22 based latex agglutination test for detection of anti-leptospiral antibodies in ovine sera as compared to MAT

MAT				Low 95% C.I.		High 95% C.I.	
rLoa22 based LAT	Positive	279 (a)	17 (b)	Kappa	0.90	0.88	0.93
	Negative	33 (c)	1548 (d)	Sensitivity	89.42%	84.84%	92.11%
				Specificity	98.91%	98.27%	99.37%
				Accuracy	97.34%	96.39%	97.93%
				† PPV	94.27%	91.08%	96.35%
				‡ NPV	97.91%	97.00%	98.37%

‘a’ represents both MAT⁺ve and rLoa22 Con1-5 based LAT⁺ve, ‘b’ represents MAT⁺ve/ rLoa22 based LAT⁺ve ‘c’ represents MAT⁺ve and rLoa22 based LAT⁻ve ‘d’ represents MAT⁺ve/ rLoa22 based LAT⁻ve, † PPV- Positive Predictive Value, ‡ NPV- Negative Predictive Value”

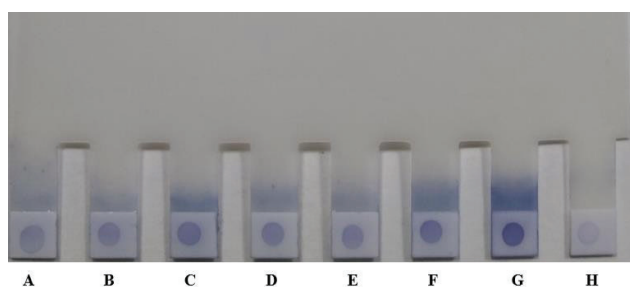


Fig. 5. Recombinant Loa22 antigen-based IgG dot ELISA dipstick test (IgM DEDT) for the detection of anti-leptospiral antibodies in ovine sera. A-F: Purple dots produced by ovine field sera containing antibodies against leptospirosis, (G): Known positive ovine sera, H: Known negative ovine sera

waste and its attractive assets include its simplicity and transportability (Hull-Jackson *et al.* 2006). In a field trail initiated by our research team using rLoa22-based LAT, it was shown that reagents of LAT assay had a life span of at least three months under refrigeration in tropical climate due to presence of 0.02% sodium azide as preservative. To standardize IgG-based DEDT, 1µg of purified rLoa22 antigen has been dotted on NCM at tip of each plastic comb. Ideal field sera and anti-ovine IgG HRP conjugate dilutions were 1:50 and 1:2500. Positive reactors had purple NCM dots, but non-reactors didn't (Fig. 5). MAT and IgG Dot-ELISA Dipstick tests demonstrated seropositivity for 312(16.62%) and 345(18.38%) of 1877 sera samples. Table 2 compares rLoa22-based IgG Dot ELISA Dipstick

test specificity, sensitivity, accuracy, and Kappa value to MAT.

IgG-based DEDT showed a better sensitivity (97.16%) against MAT in comparison to LAT (89.42%). However, the main advantage of LAT over IgG-based DEDT is its rapidity in giving test results (within 2 min) in comparison to IgG DEDT, which require at least 4 hours to complete the test owing to several steps in its protocol (Shekatkar *et al.* 2010). Even with this minor glitch, IgG-based DEDT is still a much sort after spot test which can be performed easily in comparison to MAT (Behera *et al.* 2021), which is a test restricted to specialized laboratories that requires highly skilled technical staff and expensive instruments including dark field microscope (DFM) and biological oxygen demand (BOD) incubator (Behera *et al.* 2022). Hence, IgG-based DEDT is ideally suited for screening of ovine leptospirosis at the peripheral level of animal health care system as a ‘point-of-care test’ since sheep worldwide are reared mostly in rural and remote locations where screening by MAT becomes impractical. In conclusion, the intricate connection between cyclones and incidence of leptospirosis is an undeniable fact based on evidence obtained in the present study regarding seroprevalence of ovine leptospirosis in various agro-climatic zones of Odisha. High sensitivity and specificity of recombinant Loa22 antigen-based LAT and DEDT for anti-*Leptospira* antibody detection in ovine serum samples makes it suitable for resource-poor application in field. Multipronged approach employed in current research

Table 2. Relative sensitivity, specificity and accuracy values of recombinant Loa22 based IgG dot ELISA dipstick test for detection of anti-leptospiral antibodies in ovine sera as compared to MAT

MAT				Low 95% C.I.		High 95% C.I.	
rLoa22 based DEDT	Positive	303 (a)	42 (b)	Kappa	0.91	0.88	0.93
	Negative	09 (c)	1523 (d)	Sensitivity	97.16%	94.59%	98.67%
				Specificity	97.32%	96.39%	98.06%
				Accuracy	97.28%	96.44%	97.97%
				† PPV	87.83%	84.25%	90.68%
				‡ NPV	99.41%	98.89%	99.69%

‘a’“‘represents both MAT⁺ve and rLoa22 based LAT⁺ve, ‘b’ represents MAT⁺ve/ rLoa22 based LAT⁺ve ‘c’ represents MAT⁺ve and rLoa22 based LAT⁻ve ‘d’ represents MAT⁺ve/ rLoa22 based LAT⁻ve, † PPV- Positive Predictive Value, ‡ NPV- Negative Predictive” Value

to diagnose leptospirosis in sheep could assist with implement intervention strategies for small ruminants that involve early case detection and timely antimicrobial therapy during national emergencies, especially cyclones.

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