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RESEARCH ARTICLE

Development of rNcSRS2 IgG ELISA kit for Serological Investigation of Bovine Neosporosis at Dairy Farms of Punjab, Pakistan

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ABSTRACT

Neospora (N.) caninum is an obligate intracellular protozoan parasite of economic importance for the dairy industry due to its abortifacient nature. This study aimed to develop an *N. caninum* IgG ELISA Kit (N) based on the recombinant antigen (rNcSRS2) to diagnose bovine neosporosis. We cloned C terminal region comprising of 1054 base pairs of NcSRS2 in expression vector pET-28α and expressed it in the prokaryotic expression system (BL21, DE3). The expressed protein was purified using Nickel-affinity-columns and was characterized by immunoblotting. Purified 0.5µg/mL of rNcSRS2 was used to coat flat-bottomed microtiter polystyrene plate to develop the ELISA kit (kit N) for diagnosis of bovine neosporosis. To this end, 400 cattle sera were screened through our newly developed kit N. Out of 400 samples, 72 (34 positive and 38 negative) were selected to be tested with commercially available *N. caninum* Antibody Test Kit, cELISA (E) in comparison with kit N. Out of the 34 samples tested positive with kit N, 33 were also found positive in gold standard (kit E), while all the 38 samples tested negative in kit N were also found negative with gold kit E. Thus, the sensitivity of the kit N was found as 100% and the specificity as 97.4% (95% C.I: 97.277-97.595) and the cut-off value was 0.54. Overall prevalence of neosporosis in three districts of Punjab was 8.5% (34/400; 95% CI: 5.77-11.23). The district wise prevalence of neosporosis in cattle herds in Lahore, Sheikhupura and Narowal was 10.5% (22/210; 95% CI: 6.33-14.63), 4.5% (05/112; 95% CI: 0.64-8.29) and 9.0% (7/78; 95% CI: 2.63-15.32), respectively. The promising sensitivity and specificity of this format of ELISA allows for designing larger scale and more reliable sero-epidemiological surveys of bovine neosporosis across diverse cattle herds in different regions of Pakistan.

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INTRODUCTION

Neospora (N.) caninum is a heterologous coccidian parasite in phylum Apicomplexa and family Sarcocystidae (Tsakmakidis *et al.,* 2024). Due to its structural and antigenic similarity, the parasite mimics *Toxoplasma (T.) gondii,* which is why it has long been misdiagnosed as *T. gondii* until 1988 (Sarfraz-ur-Rahman, 2023). The parasite can cause infection in different types of animals, including sheep, goats, buffalo, horses, dogs, and cattle. The dog and various other canids are the final hosts, and cattle is the main intermediate host. The dogs infected with neosporosis depict neurological signs that gradually lead to paralysis (Alf *et al.,* 2024). In an experimental study, the parasite has also been shown to infect non-human primates, the two *Rhesus macaques*, and a recent study has found evidence in human samples thus potentially an emerging zoonotic pathogen (Barr *et al.,* 1994; Duarte *et al.,* 2020).

Abortions induced by the *N. caninum* may occur in an endemic or epidemic pattern with epidemic occurrences up to 15% in a herd spanning a month or 12.5% in 2 months, due to oocyst contamination through food or water (McAllister *et al.,* 2000). Whereas, in endemic situations, the abortions occur for a much longer duration spanning

months or years, mainly due to trans placental transmission and point exposure of oocyst (Dubey *et al*., 2007; de Souza *et al*., 2022). Economic losses to US industry attributed to Neosporosis have been roughly calculated to be 1.29$ billion (Reichel *et al.,* 2013; Carrillo Parraguez *et al*., 2025), with 15 to 24$ million to the beef industry alone in the Texas state only (Kasari *et al.,* 1999). Losses per abortion have been estimated to be 1865$ elsewhere (Moore *et al.,* 2013).

Tissue cyst visualization is essential for definitive diagnosis of neosporosis through morphometric analysis using microscopy and histopathology, with the limitation that the sample can only be collected during post-mortem. A similar limitation is for obtaining DNA for molecular confirmation through PCR (Liu *et al.,* 2024). The serological diagnostic procedures like ELISA and IFAT remain the only alternate to ante-mortem diagnosis that can be exploited not only for diagnostic purposes meant for therapy, but also for surveillance studies in a disease eradication program (Kamali *et al.,* 2014).

Neosporosis is prevalent all over the world in variety of hosts (Cao *et al.,* 2022; Nayeri *et al.*, 2022; Costa *et al*., 2023; Hamzavi *et al*., 2023; Dogan *et al*., 2025). It has been reported to be 13.69% in China (Ying *et al.,* 2022), 24.6% in Egypt (Metwally *et al*., 2023) and 24.8% in India (Hebbar *et al*., 2022) 13.14 % in sheep in Mexico (García-Valle *et al.,* 2024) whereas a very high prevalence (39 to 47%) in bovines (Shabbir *et al.,* 2011; Nazir *et al.,* 2013) and 12.3% in horses has been reported in Pakistan (Talib *et al.,* 2025). A recent milk-based serology on small herd found up to 60% of buffaloes seropositive for Neosporosis (Nasir *et al.,* 2018). On the other hand, the seroprevalence of *N. caninum* in sheep and goat was found to be 3.1 and 20.2%, respectively, in Türkiye (Ceylan *et al.,* 2024). A recent study in Egypt found *Neospora* prevalence in cows with aborting history higher than non-aborting cows (Selim *et al.,* 2023). In USA, 92 abortions in a herd of 1700 animals were attributed to *Neospora* indicating it as a major cause of abortions (Melendez *et al.,* 2021).

Given the impact of this disease, diagnosis, prevention and control are essential to contain these losses (Souza *et al*., 2022). Different serological tests are performed to diagnose bovine neosporosis i.e. IFAT (Hebbar *et al.,* 2022) and ELISA (Zimpel *et al*., 2015) based on antigens. The structural and antigenic similarities between *Neospora* and *Toxoplasma* limit the use of total antigen prepared from tachyzoite lysate, thus leading to test results with positive outcomes owing to potential cross-reactivities. For reliable serodiagnosis, several recombinant markers have been identified: P40, N57, N54, NcGRA2, NcGRA6-d, and NcGRA1 to obtain more specific and sensitive results (Huang *et al.,* 2007).

Owing to high prevalence of *Neospora* in Pakistan, the current study was designed to develop and evaluate an indigenous ELISA-based immunodiagnostic kit for *N. caninum* infection screening in bovine populations of Pakistan. The study was also meant to provide an economic diagnostic alternative that should be equally specific and sensitive in comparison to existing costly immunodiagnostic tools practiced or available in Pakistan. The main benefits of our locally developed *Neospora* ELISA kit include easy access and an economic alternative to the costly imported kit.

MATERIALS AND METHODS

Designing and Construction of Recombinant pET-28α for rNcSRS2: Based on antigenic similarity among *Neospora caninum* isolates reported by Sánchez et al. (2009), a consensus sequence was generated from 14 NcSRS2 sequences (AY940480–AY940488, AF160217–AF160220, and JQ410454) aligned using Geneious R8.1.6. Primers were designed to amplify a 1,054-bp C-terminal fragment of NcSRS2, which was cloned into the pET-28a vector as described by Yang et al. (2023). Plasmid orientation was confirmed by restriction digestion. Expression in *E. coli* BL21 (DE3), protein purification by Ni-NTA chromatography (Rahman et al., 2021), quantification using a BCA assay, and verification by Western blotting (Rahman et al., 2023) were performed following established protocols. Details of previously reported different antigens used in development of ELISA for diagnosis of *N. caninum* is given in table 1.

Collection of cattle sera: Cattle blood (*n*=400) were collected from the jugular veins of cattle (Kapale *et al.,* 2008) from commercial dairy farms located in Lahore (31.32°N and 74.22°E), Sheikhupura (31.42°N and 73.58°E) and Narowal (32.2730°N and 75.0611°E) districts of Punjab, Pakistan. Out of 400 sera samples, 210, 112 and 78 samples were from Lahore, Sheikhupura and Narowal districts, respectively. Convenient sampling was done via voluntarily participating farms making this an exploratory survey or point prevalence study. All sampling was done after taking verbal consent from farm owner/manager. All the experimentation was carried out as

Table 1: Previously reported different antigens used in development of ELISA for diagnosis of *Neospora caninum*

Antigen	Antigen size	Animal sera	OD threshold	Sensitivity (%)	Specificity (%)	References
rNcSRS2	40 kDa	Cattle sera	0.54	100	97.4	Current study
NcSRS2 Truncated distal C terminal two thirds	30 kDa	Beef cattle	0.095	95	96	Borsuk *et al.,* 2011
NcSRS2	42 kDa	Bovine sera	0.189	-	-	Nishikawa *et al*., 2001
NcGRA6-d	33 kDa	Cattle sera	_	83.3	78.6	Jenkins *et al*., 2005
NcGRA1	45 kDa	Dog sera	0.008	44.4	-	Abdelbaky and Nishikawa, 2020
NcSRS2 Truncated Middle portion of gene	61 kDa	Dog sera	0.8-2.9	-	-	Gaturaga *et al.,* 2005
NcSRS2 C-terminal 730 BP	-	Bovine sera	-	98.7	88.7	Sinnot *et al.,* 2014
Nc ISCOM antigen	30-43 kDa	Dog sera	0.18	95.6	97.7	Björkman *et al.* 1994
P40	65 kDa	Cattle sera		98.2	98.6	He *et al.,* 2013
N57	20 kDa	Cattle sera	0.03	83	87	Louie *et al*., 1997
N54	29 kDa	Cattle sera	0.04	95	96	
NcGRA2	48 kDa	Dog sera	–	–	–	Strohbusch *et al.* 2009
NcSAG1 truncated	55 kDa	Cattle sera	0.65	88.4	80.7	Udonsom *et al.,* 2024
NcGRA7	66 kDa	Cattle	0.355	94.6	90.3	Hamidinejat *et al*., 2015
		Buffalo	0.384	98.5	86.5	

per the recommendations of the Ethical Review Committee of UVAS, Lahore, for animal experimentations (DR/1137, Dated: 24.10.2017 and DR/1137, Dated: 14.09.2021). Briefly, the animals were humanely restrained by trained personnel in squeeze chute (<5 minutes) to avoid stress and the injury to animal. The jugular vein was located, disinfected with 70% alcohol, and sterile disposable syringe was used to collect 3-4 mL of blood into the vacutainer with clot activator. The samples were refrigerated overnight and on the next day, clear sera were separated and stored at -20ºC until further use.

Development of *N. caninum* IgG ELISA kit (kit N): For optimization of the new indirect ELISA kit N, various concentrations of rNcSRS2 (1, 0.25 and 0.50µg/mL), serum dilutions (1:200, 1:100, and 1:50), and incubation periods (5, 10, 15 and 30 minutes) were used until the optimum results were obtained (Dong *et al*., 2012; Udonsom *et al*., 2023). Thus, 0.5µg/mL coating concentration of rNcSRS2 protein, 1:50 level of dilution of cattle sera, and 15 minutes interval between substrate addition and OD reading was found as optimum for the developed ELISA kit N. Eventually, 2 hours incubation time was determined as optimum for antibodies and sera at 37ºC.

Screening of cattle sera with *N. caninum* IgG ELISA kit (N): The flat-bottomed microtiter polystyrene plate (96-well, JET BioFil, Hong Kong, China) was coated with rNcSRS2 (0.5µg/mL in 50 mM Na_2CO_3) and incubated for 12 hours at 4ºC. After five washings with 300µL/well washing buffer, plate was blocked with 4% bovine serum albumin (BSA) in 0.01 MPBS with 200µL/mL for 2h at 37ºC followed by washing (5x). Duplicate wells for positive (found positive in commercial kit) and negative sera (found negative in commercial kit) and blanks were added and incubated for 2h at 37ºC, followed by washing (5x). Alkaline Phosphatase (AP) conjugated Anti-bovine-IgG (Sigma-Aldrich, St. Lois, MO, USA Cat#A0705) with 1:10000 dilution was added 100µL/well and incubated for two hours at 37ºC followed by washings (5x). p-nitrophenyl phosphate (pNPP) prepared as 1mg/mL in Diethanolamine buffer (Thermo Scientific ™ Pierce™, Rockford, IL, USA, Cat # 34064) was added and incubated for 15 minutes at 37ºC. Reaction was stopped with 100µL/well of stop solution (3 M NaOH) and OD was measured at 405 nm (BioTek, Winooski, VT, USA ELX-800).

Defining the criteria positive and negative sera and validation of kit N: The study panel was first screened using kit N to establish a practical cut-off for classifying samples. This cut-off was derived empirically from the baseline OD values: We averaged the lowest readings, which represented near-negative signals, and then multiplied this mean by a factor of 2.5 (antibody-titre heuristic), resulting into the provisional cut-off OD value. Samples with readings at or above this value were considered positive, while those below were classified as negative for kit N. To ensure reliability, all samples were tested in duplicate, and the assay's reproducibility was confirmed by repeating the procedure on independent plates.

Validation of kit N: A total of 72 sera sample (34 positive and 38 negatives by kit N) were used to validate the sensitivity and specificity of the kit N against a commercial *N. caninum* antibody test kit (hereafter referred as kit E) used as the study reference test. Kit E is competitive ELISA kit (VMRD, Inc, 4641 Pullman-Albion Road, Pullman, WA 99163 USA Cat # 280-2).

Statistical Analysis: For statistical analysis, MedCalc statistical program (MedCalc Version 20.015 Mariakerke, Belgium) was used. Sensitivity and specificity of the kit N in comparison with the kit E was determined by plotting ROC curve. Statistical significance of κ was determined at $P<0.05$. (Rahman *et al.,* 2021). The Likelihood Ratio (LR) and diagnostic ratio (DOR) were determined as described previously (Glas *et al.,* 2003, Trevethan, 2017).

RESULTS

Cloning and transformation: The ligation of NcSRS2 fragment and plasmid (pET-28α- NcSRS2) was confirmed by sequencing. The BL21 was transformed with pET-28α-NcSRS2 and transformed colonies were confirmed on kanamycin added Luria-Bertani agar (Fig. 1).

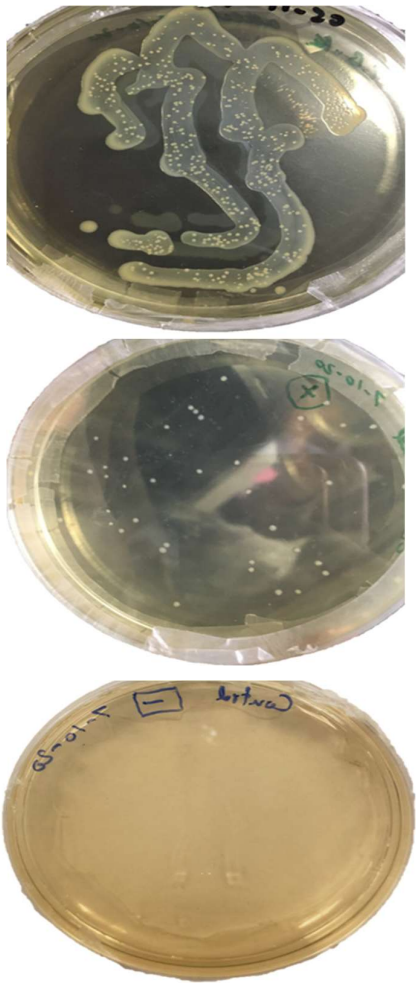

Fig 1: (A): Transformation of BL21 (DE3): BL21 grown on LB Agar after 24 hours of incubation at 37°C temperature, **(B):** BL21 colonies transformed with pET-28α-NcSRS2 (White spots) grown post incubation on LB-K agar. Only transfected clones are visible because of their kanamycin resistance while non transfected clones were killed with kanamycin **C:** Control negative, BL21 culture without plasmid did not show any growth on LB-K agar.

Restriction analysis of pET-28α-NcSRS2: The sequence of pET-28α-NcSRS2 was analyzed for other restriction sites using Snap-Gene software (Version 5.3.2 from GSL Biotech, Chicago, IL, USA; available at snapgene.com) and it was found that restriction enzyme *SmaI* has two restriction sites, one present in pET-28α and other was in NcSRS2. The correct orientation of NcSRS2 in pET-28α was confirmed by treating the plasmid with *SmaI* enzyme and it gave two bands of 4876 bp and 1493 bp on 0.9% Agarose gel confirming the correct orientation of NcSRS2 gene in the pET-28α plasmid (Fig 2). The Snap-Gene software simulation showed that if the orientation would have been incorrect, then one band would have been at 4299 bp and other at 2070 bp.

SDS-PAGE and Western Blot analysis: Recombinant NcSRS2 examined with SDS-PAGE on a 12 percent polyacrylamide gel stained with Coomassie blue stain after expression and purification showed a 40 kDa band. The appearance of band on expected size and absence of any extra band in Coomassie blue stained gel confirms the purification. The expressed and purified rNcSRS2 was also reacted with anti-His antibody on Western blotting which showed reactive band of 40 kDa size (Fig. 3).

Validation of kit N against a gold standard competitive ELISA kit E: Out of 400 cattle sera which were examined with *N. caninum* IgG ELISA Kit (N) 34 (8.5%) were positive by N while 366 (91.5%) samples were negative by N. 72 sera (34 positive and 38 negative) were screened with the gold standard kit E to compare the results of kit N. An excellent degree of agreement was found between the kit N and the gold standard (kit E). Out of the 34 samples tested positive in kit N, 33 were also tested positive in gold standard (kit E), while all the 38 samples tested negative in kit N were also tested negative in gold standard (kit E). Thus, the sensitivity and specificity of Kit N was found 100 and 97.4%, respectively and the cut-off value as 0.54. However, the comparison was done with only 72 Gold-standard-validated sera which could be considered a limitation of current study and further validation with a larger number of sera is suggested for future (Fig 4). The kappa value was calculated to be 1.00. The LR +ve, LR -ve, DOR, PPV and NPV values for the kit N in comparison with the kit E were also calculated and found as PPV (97.05), NPV (100), LR+ (26.27), LR- (0.0153) and DOR (1717.0) (Table 2 & 3).

Table 3. LR+, LR-, DOR values for the newly developed *Neospora* IgG ELISA Kit (N) against the commercially available *N. caninum* Antibody Test Kit, cELISA (E) used as gold standard

Neospora IgG ELISA Kit (N)	Gold standard commercially available *Neospora caninum* Antibody Test Kit, cELISA (E)		LR	DOR
	Positive	Negative		
Positive	33.5(a)	1.5(b)	26.27	1717.0
Negative	0.5*(c)	38.5(d)	0.0153	
	34	40		

a: = True Positive
b: = False Positive
c: = False Negative
d: = True Negative
LR+ of *Neospora* IgG ELISA Kit (N)= [a/{a+c}] / [b/{b+d}]= [33.5/{33.5+0.5}] / [1.5/{1.5+38.5}]= [33.5/{34}] / [1.5/{40}]= 0.9853/0.0375 = 26.27
LR- of *Neospora* IgG ELISA Kit (N)= [c/{a+c}] / [d/{b+d}] = [0.5/{33.5+0.5}] / [38.5/{1.5+38.5}]= [0.5/{34}] / [38.5/{40}]= 0.0147/0.9625 = 0.0153
Diagnostic Odds Ratio (DOR) = LR+ / LR- = 26.27/ 0.0153= 1716.99 =1717.0
*The contingency table with zero values in it requires addition of 0.5 in each cell of contingency table for calculating LR, DOR, Therefore, 0.5 was added to every positive and negative value to calculate LR, DOR.

Table 2: Determination of Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Kit N in diagnosing bovine Neosporosis in comparison with Kit E

	Kit E[$] +ve	Kit E -ve	Predictive Values
Kit N +ve	33 (a)	1 (b)	97.05 (PPV)
Kit N -ve	0 (c)	38 (d)	100 (NPV)

$: Kit E considered as Gold Standard in this comparison
a: = True Positive
b: = False Positive
c: = False Negative
d: = True Negative
Sensitivity of Kit N= [a/{a+c}] x 100= [33/ {33+0}] x 100= [33/ {33}] x 100= 100%
Specificity of Kit N= [d/{b+d}] x 100= [38/ {01+38}] x 100= [38/{39}] x 100=97.4%
PPV of Kit N= [a/{a+b}] x 100= [33/{33+1}] x 100= [33/34}] x 100= 97.05
NPV of Kit N= [d/{c+d}] x 100= [38/{38+0}] x 100= [38/{38}] x 100= 100%

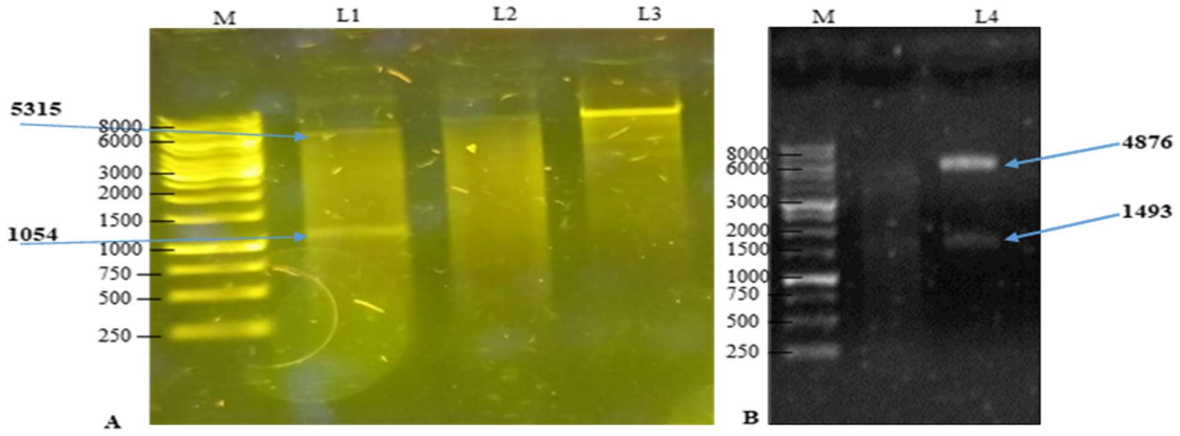

Fig 2: Restriction analysis of pET28a-NcSRS2: **(A)**: The Molecular weight marker 1 kb DNA ladder is shown in Lane M (ThermoFisher, USA, Cat# SM0311), Lane 1 shows two fragments of 1054 Base pair (bp) and 5315 bp after performing double digestion with *XhoI* and NheI. It confirms the cloning of our gene in pET28a which appeared on expected base pair size (1054 bp). Lane 2 shows single digestion with *XhoI* and Lane 3 shows single digestion with *NheI.* In figure, both single digestions have linearized the plasmid which appeared on expected size 6 kbp **(B):** Confirmation of cloning of NcSRS2 gene in correct orientation in pET28a. Lane M shows 1kb DNA Ladder (and lane L4 shows Plasmid digested with *SmaI* restriction enzyme showed two bands of 4876 bp and 1493 bp confirming the correct orientation. If the orientation had been incorrect, then the bands would have appeared at 4299 bp and 2070 bp

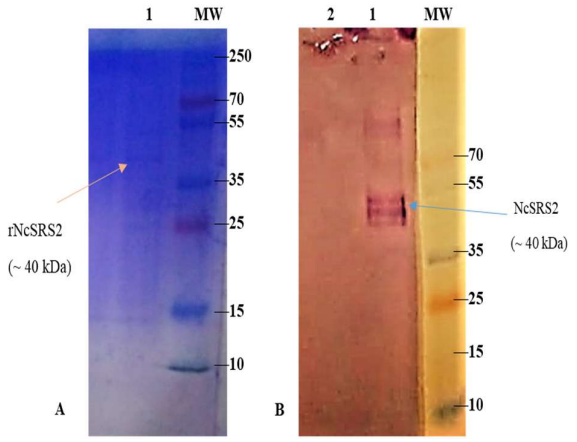

Fig 3: The SDS-PAGE analysis of expression of rNcSRS2 protein using 12% acrylamide gel. **(A):** The Lane MW shows protein marker with size range of 10-250 kDa (Thermo Scientific, Cat# 26619), Lane 1 shows purified rNcSRS2 around expected size of 40 kDa on a Coomassie blue stained 12 percent polyacrylamide gel. **(B)**: WB analysis of the rNcSRS2 using an Anti-6x His Tag Antibodies (ThermoFisher, USA, Cat # A190-114P). The Lane MW shows protein marker (Thermo Scientific, Cat# 26619). Lane 1 shows rNcSRS2 protein purified though Nickle affinity chromatography detected by anti-His antibody. The band in WB showed upward shift as compared to Coomassie blue stained gel which might be due to anti body recognition of isoforms or minor post translational modification. Lane 2 shows control negative.

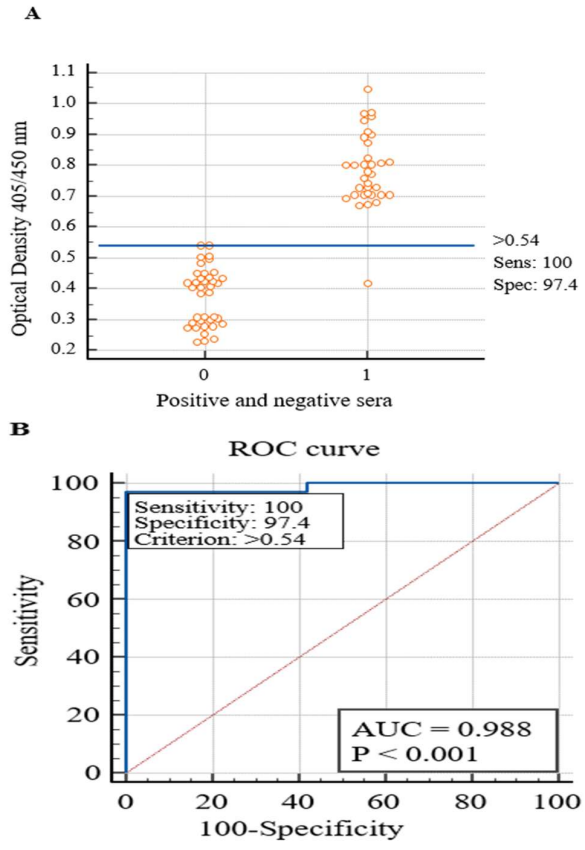

Fig 4: Sensitivity (Sens), Specificity (Spec) and cut-off value fitted to N with display of *Neospora*-positive and negative sera as per kit N. **(A):** Sensitivity and Specificity were 100% and 97.4 % (95% C.I.: 97.277- 97.595) respectively. The cut-off value was 0.54 **(B):** Receiver Operating Characteristic (ROC) curve for kit N applied to positive versus negative cattle sera identified by kit E to determine the cut-off value. The area under curve (AUC) of 0.988 at 95% confidence interval of 0.9-1.0 indicated that test could correctly discriminate positive and negative sera. (B).

Prevalence of Neosporosis determined by *N. caninum* IgG ELISA Kit (N) post-validation: The total number of cattle sera screened with *N. caninum* IgG ELISA Kit (N) was 400. Out of which 8.5% (34/400; 95% CI 5.77-11.23), showed positive test outcome while 91.5% (366/400; 95% CI 88.32-94.04), with a negative test outcome. The prevalence of Neosporosis in cattle herds in Lahore, Sheikhupura and Narowal was 10.5% (22/210; 95% CI 6.33-14.63), 4.5% (05/112; 95% CI 0.64-8.29), and 9.0% (7/78; 95% CI 2.63-15.32), respectively.

DISCUSSION

In the current study, *N. caninum* IgG ELISA Kit (N) was developed using recombinant protein rNcSRS2 of *N. caninum.* We choose this antigen because it is highly immunogenic, abundantly expressed on tachyzoites, and consistently recognized by antibodies in naturally infected cattle (Wang *et al.,* 2022). It has not only showed higher sensitivity and specificity (>90%) in bovines (Sinnott et *al.,* 2020; Yang et *al.,* 2022) but also in dog and sheep (Pinheiro *et al*., 2015). Therefore, this protein could not only be used for cattle but also carries potential to be used for dogs, sheep and equines. The antigen was produced from 1054bp long sequence of *NcSRS2*. This 40kDa recombinant protein NcSRS2 produced in BL21, transformed with pET28α-rNcSRS2, was characterized through SDS-PAGE and Western Blotting. The ELISA plate was coated with rNcSRS2 at a concentration of 0.5µg/mL. The 400 cattle sera were screened through the kit N for anti-*N. caninum* IgG antibodies detection.

Serological assays based on the recombinant proteins of *N. caninum* can be employed for accurate ante-mortem diagnosis and surveillance studies meant for disease control programs as well as for investigating the risk factors and disease transmission. Commonly used serological tests for *N. caninum*-specific antibodies in bovine are Indirect Fluorescent Antibody Test (IFAT), ELISA and Immunoblotting. IFAT is considered the most accurate test for serodiagnosis of *N. caninum* (Bahavarnia *et al.,* 2020) but cannot be used for a large-scale screening program because it is labor intensive and time consuming (Abdelbaky *et al.,* 2020). ELISA, on the other hand, is considered more appropriate diagnostic tool for mass scale screening of bovine commercial herds for this disease (Tagwireyi *et al*., 2024). Serological assays based on whole *N. caninum* antigen or *Neospora*-Lysate antigen show lower specificity due to cross-reactivities owing to the conserved antigen between closely related species like *Toxoplasma* (Nishikawa *et al.,* 2002). Therefore, recombinant antigens are considered better choice for ELISA.

Various antigens of *N. caninum*, i.e., NcGRA1, NcGRA2, NcGRA6, NcGRA7, NcSAG1, N54, N57, P57 and NcSRS2 have been evaluated for serodiagnosis of bovine neosporosis. Earlier work comparing rN54 and rN57, showed that rN54 has higher sensitivity and specificity (95 and 96%) than rN57 (83 and 87%) for bovine Neosporosis (Louie *et al.,* 1997). Double purified granular rNcGRA6 showed lower sensitivity and specificity (83 and 78%) than surface derived rNc40 (98.2 and 98.6%) (Jenkins *et al.,* 2005; He *et al.,* 2013). Another granular protein rNcGRA7 demonstrated better sensitivity

and specificity (94.6 and 90.3%) in cattle (Hamidinejat *et al*., 2015) but there is potential for improvement with surface antigens. Surface antigen can also lose their key epitope potentially due to their expression with Glutathione S-Transferase (GST) fusion protein, as indicated by a recent study of rNcSAG1 demonstrating 88.4% sensitivity and 80.7% specificity (Udonsom *et al.,* 2024).

Several studies have also assessed the rNcSRS2 proteins of varying lengths and expression systems. A truncation of both N and C terminal of rNcSRS2 (730 bp coding sequence demonstrated 95% sensitivity and 96% specificity (Borsuk *et al*., 2011). A truncation of only N-terminal part of rNcSRS2 (also 730bp CDS) increased sensitivity to 98.7% but decreased specificity to 88.7% (Sinnott *et al.,* 2015). Variation of fragment length of CDS of NcSRS2 affects the antigenic repertoire and thus epitope availability for antibodies from natural infection. The CDS construct from (Borsuk *et al.,* 2011) potentially had some crucial epitopes which were absent in CDS construct by (Sinnott *et al.,* 2015) which resulted in relatively lower specificity. A recent study also predicted the distribution of B-cell and T-cell epitopes on n terminal part of NcSRS2 (Asghari *et al.,* 2022) prompting its testing as a vaccine candidate.

The current study used a larger CDS (1054 bp) of distal two third part of the NcSRS2 to incorporate the all the important amino acids missed in construct by Sinnott *et al.* (2015) to diagnose Bovine Neosporosis. The ELISA developed in the present study showed relatively higher combination of sensitivity (100%) and specificity (97.4%) compared to above-mentioned studies. However, the limitations of the current study include: use of small sample size of validated sera (72 samples), sera from limited geographical areas (three districts of Punjab) and employing cELISA kit instead of IFAT as gold standard. Despite these limitations, the study provides important evidence for developing an economical diagnostic kit under local conditions.

Likelihood ratios (LRs) present the sensitivity and specify of a test in single measure. The test with the value of LR-ve near to or equal to zero (>0.1) gives strong evidence to rule out a disease while LR+ve value (>10) gives strong evidence to rule in a disease. The value of Diagnostic odds ratio (DOR) with range from 0 to infinity (∞) combines these measures. The higher the value of DOR from 1 means the better the reliability of test (Šimundić, 2009). Current study ELISA kit (N) had the LR +ve, LR -ve, and DOR values as 26.27, 0.0153 and 1717.0, respectively. Therefore, the kit N can be considered as a reliable diagnostic kit for detecting true-positives (to rule-in the disease) and true-negatives (to rule-out the disease) from the sera of cattle for the diagnosis of Bovine Neosporosis, with a higher degree of reliability.

Rahman *et al*. (2021) have shown the Receiver Operator Characteristic (ROC) to find Area Under Curve (AUC) and cut-off value for assessing the efficiency of diagnostic kits (Rahman *et al.,* 2021). The AUC in ROC curve provides basis for objective selection of cut off value balancing the sensitivity and specificity with values closer to 1.0 means the test can excellently discriminate between the diseased and non-diseased subjects. In the current study, the cut-off value was found 0.54 and AUC was 0.988 with highly significant P value as <0.01. The >0.98 value of AUC in the current study indicates the higher diagnostic reliability and accuracy of the developed ELISA kit for use in serodiagnosis of *N. caninum* in cattle populations.

Pakistan has relatively higher prevalence of bovine Neosporosis 54% in buffalo in Lahore (Nasir *et al.,* 2011), 43% in cattle in Punjab (Shabbir *et al*., 2011) and 43% in cattle of Punjab and Sindh combined (Nazir *et al*., 2013). All these studies were done with imported ELISA kits from international manufacturers, which are relatively expensive (>220k PKR) and limit widespread epidemiological survey. The kit N developed locally in this study will make large-scale surveillance studies of Neosporosis, more economical, more affordable, more accessible and more feasible (31K PKR).

The current study tested limited number of sera with 8.5% seropositivity among cattle. The point prevalence of Neosporosis in dairy farms from Lahore, Sheikhupura and Narowal was found to be 10.5, 4.5 and 9.0%, respectively. The major objectives of convenient and volunteer sampling in this study were testing the diagnostic reliability of the developed kit in parallel to the point prevalence- rather than conducting a large scale Sero-epidemiological study on Neosporosis. Nonetheless, the findings of current study could be seen as baseline information and can guide broader surveys that are more comprehensive.

Conclusions: The seropositivity of neosporosis in cattle herds in Lahore, Sheikhupura and Narowal is 10.5, 4.5 and 9.0%, respectively. We believe that this study reports promising results by producing and assessing the potential of this particular length (350 AA) of rNcSRS2 for serodiagnosis of *N. caninum* infection in bovines. The high sensitivity, specificity and DOR values of this format of ELISA allow for Sero-epidemiological survey of bovine neosporosis on larger scale. However, small sample size, collection of sera from limited geographical area and use of cELISA instead of IFAT as gold standard test for comparison, may limit the generalizability of this study. Despite these limitations, the study provides important preliminary evidence for developing an economical diagnostic kit under local conditions. We propose testing of kit in large studies across different cattle herds in different regions and the use of this recombinant protein for development of indirect ELISA for serodiagnosis in canines and other mammalian hosts including human beings as well.

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Authors contribution: Shafqat Shabir has conducted this research with the help of Adeel Mumtaz Abbasi and Sarfraz-ur-Rahman, and collected the data. Shafqat Shabir wrote the early draft of the MS. Naveed Hussain, Muhammad Imran Rashid and Haroon Akbar have involved in conceptualization, grant acquisition, experimental design, data analyses and interpretation, manuscript reviewing and editing. Naveed Hussain, Muhammad Imran Rashid, Muhammad Younus and Haroon Akbar have actively contributed in grant acquisition, data collection, manuscript reviewing and editing. Muhammad Younus helped in sample collections.

Muhammad Suleman, Muhammad Younas and Sakandar Khan have also involved in data collection, manuscript reviewing, editing and formatting. All authors have reviewed the manuscript.

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Data availability: The data supporting the findings of this study are contained within the manuscript. The raw data are available by the corresponding author when requested.

Consent for publication: Verbal consent was obtained from all participants.

Animal ethics: All the experimentation was carried out as per the recommendations of the Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore, for animal experimentations (DR/1137, Dated:24.10.2017 and DR/1137, Dated: 14.09.2021).

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