Indian Journal of Experimental Biology
Vol. 61, September 2023, pp. 697-704
DOI: 10.56042/ijeb.v61i09.3872

Development of recombinant sialidase (NanH) protein-based Indirect-ELISA for epidemiological survey of anti-*Pasteurella multocida* antibodies in bovines

Awadhesh Prajapati, Nihar Nalini Mohanty, Revanaiah Yogisharadhya, Mohammed Mudassar Chanda, Arul Dhayalan & Sathish Bhadravati Shivachandra*

ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Ramagondanahalli, Yelahanka, Bengaluru-560 064, Karnataka, India

Received 29 July 2022; *revised 11 July 2023*

Haemorrhagic septicaemia (HS) is a highly contagious and fatal disease of cattle and buffaloes and causes major economic losses to farmers. Though indirect hemagglutination (IHA) test has been used to detect a specific antibody against *P. multocida*, it has low specificity for sero-diagnosis of HS. Therefore, development of a rapid, highly sensitive and specific serological test is a prerequisite for detection of antibodies against HS. In this context, we explored an in-house ELISA method using recombinant antigens for detection of antibodies against *P. multocida* in bovines. *nanH* gene from *P. multocida* B:2 strain P52 was cloned and the recombinant mature protein with a C- and N-terminal truncation was produced as a fusion protein (~63 kDa) in *Escherichia coli*. The immunogenic potential of purified rNanH-Tr was assessed by the Western blot method using specific anti-rNanH-Tr antibody responses in sera collected from immunized rabbits. An indirect-ELISA based on rNanH-Tr was developed and optimized. Furthermore, the rNanH-Tr ELISA was applied to screen bovine serum samples (n=250). The receiver operating characteristic curve analysis for the detection of anti *P. multocida* specific antibodies indicated a diagnostic sensitivity of 86.2 (CI 73.26-96.80%) and specificity of 80.0 (63.06- 91.56%). No cross reactivity was noted with antibodies against other bovine diseases (e.g., foot-and-mouth disease and brucellosis). Screening of random bovine serum samples showed a 22% sero-positivity for anti *P. multocida* specific antibodies.

Keywords: Brucellosis, Foot-and-mouth disease, Haemorrhagic septicaemia, Indirect hemagglutination, Pasteurellosis

Haemorrhagic septicaemia (HS) is a highly contagious and fatal disease of cattle and buffaloes. It is caused by *Pasteurella multocida*, a Gram-negative non-motile, coccobacillus[1,2]. *P. multocida* strains are classified into five serogroups (A, B, D, E and F) based on capsular antigens and 16 serotypes (1-16) based on lipopolysaccharide antigens[3,4]. In Asia, *P. multocida* B:2 is the predominant serotype causing HS outbreaks in cattle and buffaloes leading to major economic losses to the farmers[5,6]. Sporadic outbreaks of HS are often observed after introduction of index cases, which are often healthy carriers within the same herd[7]. *P. multocida* is a mucosal pathogen that colonizes the upper respiratory system and subsequently invades the circulatory system[8]. Many virulence factors have been identified in *P. multocida*[9,10] and sialidases are among them that are involved in host–microbe interactions, infection, and the modulation of host innate immunity[11]. Sialidases/neuraminidase producing genes are ubiquitous in *P. multocida* strains and are produced *in vivo* during an active infection[12]. Two different kinds of sialidase gene *nanB* and *nanH* were identified in *P. multocida* and they have different substrate specificity[13,14]. Neuraminidases (NanH) protein is responsible for bacterial colonization of the epithelium. They are potential targets for both vaccines and small molecule inhibitor drugs[15]. Except for substrate-binding residues, the NanH enzyme exhibits little homology with other sialidases suggesting that it may be useful for the serological diagnosis of pasteurellosis[16,17].

Specific PCR assays or bacterial isolation methods are commonly considered as “gold standard” for detection of infection[18,19]. Conventional methods are with several limitations and often fail due to improper transport of clinical samples[20]. The enzyme-linked immunosorbent assay (ELISA) is a rapid, highly sensitive and specific serological test that has been used in previous studies to detect antibodies against HS[21-24]. Although different antigens of *P. multocida* have been prepared for ELISA, the whole cell crude antigen has limitations such as changes in antigen

*Correspondence:
Ph.: +91 80 23093100 Ext. 133; Fax: +91 80 23093222)
E-Mail: sbshivachandra@gmail.com

moiety during antigen preparation, batch-to-batch variation[24]. Antigens having secretory and immunogenic potential that are expressed only post-infection would be ideal diagnostic candidates and may offer enhanced sensitivity and specificity[25]. Therefore, in the present study, we have made an attempt to develop an in-house ELISA method using recombinant NanH antigens for detection of antibodies against *P. multocida* in bovine.

Materials and Methods

Bacterial strains, vector and primers

Pasteurella multocida serogroup B:2 strain P52 (an Indian HS vaccine strain) maintained at 'Bacterial Epidemiology Laboratory-3', ICAR-NIVEDI, Bengaluru, Karnataka, India, was used for clone construction and challenge studies. For construction and expression of clones, pET32a vector (Novagen, USA), *Escherichia coli* TOP10 and BL21-CodonPlus (DE3)-RIPL cells were used. Primers were synthesized and procured commercially (Eurofins Genomics India).

Construction of pNanH-Tr clone

Initially, the *nanH* gene from *P. multocida* B:2 (strain P52) was analyzed by predicting the mature NanH protein characteristics using various online/offline bioinformatics tools such as PROTEAN program (DNASTAR), PSIPRED as well as Protoparam tools from the ExPASy website. Furthermore, SVMTriP (Support vector machine tripeptide similarity and propensity scores) online tool (Sysbio.unl.edu/SVMTriP) was used for prediction of the most probable epitopic region[26]. For construction of pNanH-Tr clone, a primer set [targeting the *nanH* gene sequence from *P. multocida* B:2 strain P52 encoding mature NanH ($_{59}$P to M$_{463}$) without the signal peptide sequence] was designed using the reference sequence available at GenBank. The sequences of the oligonucleotides are as mentioned below:

NANHF 5′CGCGGATCCATGCCGAATGGCGA3′
NANHR 5′GTGCTCGAGTTACATTTTGGCGCTTA3′

Chromosomal DNA, purified from *P. multocida* B:2 (strain P52) using standard procedures, was used as a template (50 ng) for amplifying *nanH*-Tr gene fragments. The PCR mixture consisted of 25 pmol of each primer along with other reagents as described earlier[27]. PCR included 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The PCR product (50 µg) was digested with *Bam*HI and *Xho*I enzymes before ligation into pET32a vector. The prokaryotic cells such as *E. coli* TOP10 and BL21-CodonPlus (DE3)-RIPL were successfully transformed with pET32a (*nanH*-Tr) and selected using ampicillin (50 mg/mL) and chloramphenicol (35 mg/mL). For overexpression and purification of rNanH-Tr fusion protein, *E. coli* BL21-CodonPlus (DE3)-RIPL cells containing the recombinant plasmid (pNanH-Tr) were cultured at 37°C in 1L Luria Bertani (LB) broth with appropriate antibiotics and chemically induced with 1.0 mM IPTG. After induction (3 h), the cell pellet was harvested and solubilized using a denaturing buffer. The recombinant protein was purified under denaturing conditions by affinity chromatography using Ni-NTA superflow cartridges (Qiagen) as described previously[28]. The concentrated rNanH-Tr protein was quantified by using Qubit 3.0 (Thermo Scientific, USA) and stored at −80°C until further use.

Raising of hyperimmune sera

Two New Zealand white rabbits were immunized with two antigens (rNanH-Tr protein and sonicated formalin killed *P. multocida* B:2 strain P52) following standard protocols[29]. Briefly, 250 µg of each antigen was mixed with equal volume of Freund's Complete Adjuvant (FCA) (Sigma) and was injected subcutaneously at multiple sites of rabbits. Subsequently, booster doses of antigens along with Freund's incomplete adjuvant (FIA) were given at 2nd and 3rd week. The animals were bled on 0, 14, 21, 28 and 42 days post immunization and sera were collected and stored at −20°C until use.

The animal experiments were performed according to the norms following the approval by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India, Ministry of Environment and Forests, Animal Welfare Division, and Institute Animal Ethics Committee (IAEC) (Approval # NIVEDI/IAEC/2019/01) as well as Institute Biosafety Committee (IBSC), ICAR-NIVEDI, Bengaluru, Karnataka, India. Adequate veterinary care and husbandry practices were followed during the course of the experiment.

Western blot analysis of rNanH-Tr protein

For confirmation and immune-reactivity, purified rNanH-Tr protein was electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose membrane

using Bio-Rad, Mini-PROTEAN® Tetra system. Following blocking of each membrane at 37°C for 1 h with phosphate-buffered saline 0.05% Tween 20 (PBST) containing 5% skimmed milk powder, the membrane strip with rNanH-Tr protein was incubated with rabbit hyperimmune sera (1:100) raised against rNanH-Tr antigen. Upon washing with PBST, each membrane was incubated for 1h at 37°C with anti-rabbit, IgG-HRP conjugate (Sigma, USA) at 1: 5000 dilutions in blocking buffer and was finally developed with 3,3′-diaminobenzidine tetrahydro-chloride (DAB) substrate.

Indirect haemagglutination assay (IHA)

IHA was performed as reported earlier[30]. 10 % (v/v) suspension of sheep RBC (sRBC) were prepared in chilled NSS and stored at 4°C. Equal volume of chilled 1% (v/v) glutaraldehyde solution in NSS and 10% washed RBC suspension were mixed and kept at 4°C for 30 min. After three washes in NSS resuspended in the same buffer containing 0.1% sodium azide to yield a 10% suspension of sRBC and then stored at 4°C. A 10% suspension of gluteraldehyde fixed sRBC (G sRBC) was mixed with an equal volume of PBS containing 0.005% tannic acid and incubated at 30 min with occasional shaking. The tanned G sRBC were washed thrice with PBS and resuspended to yield a 10% suspension. The antigen is prepared as after seeding the BHI agar plates with overnight P52 culture and incubated overnight at 37°C. Lawn culture was collected in 3 mL/plate NSS, pooled and heated at 56°C for 30 min in a water bath with frequent shaking. Heat treated suspension was then centrifuged at 8000 rpm for 15 min at 4°C. The clear supernatants were separated and stored at –20°°C. One volume of packed TGs RBC and 15 volumes of antigen were mixed and incubated for 1-2 h at 37°C. The sensitized cells prepared as described above were washed thrice with NSS by centrifuge at 2000 rpm for 5 min. Packed cells were resuspended in chilled NSS to obtain a 1% suspension. All the test serum samples (3 volumes) were absorbed with packed sRBC (1 volume) for 2 h at 37°C with periodic shaking before the test, to remove the heterophilic antibodies and RBC was removed by centrifugation.The supernatant sera was collected and used for the test after centrifugation at 2000 rpm for 15 min at 4°C. Normal saline (150 µL) and inactivated absorbed serum (50 µL) were added to the first well (1 in 5 dilution) and 100 µL of NSS was added to all the other wells of 96-well U bottom microtiter plates. Two-fold serial dilution of serum was made (final volume 100 µL). After that, 100 µL of the 0.5 % sensitized RBC suspension was added to all the wells. The plates were gently shaked and left room at temperature (25°C) for 2 h. The coarse agglutination of RBC (matt) indicated a positive result and formation of small button of deposited cell was considered as a negative result. Appropriate antigen, RBC, and serum controls were also incorporated in the test.

Positive and negative reference serum

Positive reference sera

Buffalo calf (Bidar District, Karnataka) suspected of HS was found positive in *P. multocida* and HS (serogroup B: 2) specific PCR in samples (nasal swab, blood)[31]. Blood was collected after 14 days post infection and serum was separated after recovery of the animal. IHA test was performed and 1: 128 dilutions were found in the serum. A serum was considered as the reference HS sera and was further used as positive control.

Negative reference sera

Field sera samples collected from 6-month old calf reared at isolated farm having no history of vaccination. Animals were found negative by deep nasal screening by PM-PCR and having IHA titre <1:4.

Positive sera panel

Random bovine serum without vaccination received from National Livestock Serum Repository of ICAR-NIVEDI, Bengaluru, Karnataka. All serum samples screened by IHA test and serum having IHA titre >1:160 were considered positive[20].

Negative sera panel

Field sera samples collected from 6-8 month old calf and found negative by nasal screening PM-PCR and having IHA titre <1:4 were considered as HS negative.

Standardization of optimum concentration of antigens and antibody dilution

Checkerboard ELISA was performed as described earlier with slight modifications[32]. Coating of ELISA plates was done with the recombinant antigen in carbonate buffer in two-fold serial dilution starting from 500 ng/well and the last row was considered as negative control (coating buffer without antigen) and incubated for 18 h at 4°C. Wells were washed three times with wash buffer and then each well was filled

with blocking buffer 200 µL and were incubated 37°C for 1 h. Two-fold serially diluted serum (positive and negative reference sera) added in each half plate (6 columns) starting from 1:20 dilution in the blocking buffer. Plate was washed using a wash buffer after 1 h incubation at 37°C and 100 µL of antibovine HRP conjugate was added to each well (1:5000). The plate was washed after incubation at 37°C for 1 h and reaction was developed by adding 100 µL of OPD substrate and the plate kept for 15 min in darkness at room temperature (25°C). Finally, 50 µL of a stop solution (H_2SO_4) was added and the plate was read at a wavelength of 492 nm by an ELISA plate reader (Multiskan, Thermoscientific, USA). Optimum concentrations of antigen, antibody and conjugate were determined based on the ratio of positive and negative optical density (OD).

Determination of cut-off value and receiver operating characteristic analysis

Indirect ELISA was performed using rNanH-Tr coating antigen with a panel of 70 samples (35 positive and 35 negative) with positive and negative reference sera. OD was recorded at a wavelength of 492 nm by an ELISA plate reader. To enable comparison of data between different ELISA plates, OD values were routinely normalized using a positive control serum and expressed as percent positivity (mean corrected test [(OD value/Mean positive control OD value) × 100].The ROC analysis for recombinant protein for screening of negative and positive sera sample for HS was performed by the empirical method using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). The ROC curve analysis was expressed as the area under curve (AUC) for the recombinant proteins for bovine sera. Area under curve (AUC) represents summary statistics of ROC analysis and quantifies the diagnostic accuracy of the test along with the test likelihood ratio. The specificity of the indirect ELISA was evaluated by immunoblotting using negative control (bovine sera from FMD and brucellosis disease).

Results

Preparation of recombinant antigens

After *in silico* analysis of the overall protein sequence, and considering probable epitopes at the amino-terminal, the truncated construct of NanH protein was designed using the SvmTrip prediction program. Regions with high and intermediate scores were predicted and the region from 59-463aa between the N- and C- termini was used for construction of the recombinant protein. The N and C-termini region with low scores was not included (Table 1). For recombinant NanH-Tr protein, the expression construct was designed such that the NanH-Tr region was fused to hexa-histidine tag on its N-terminus (Fig. 1). Upon PCR amplification, a ~1100 bp product was obtained (Fig. 2).This was cloned into pET32a vector and recombinant clone (pNanH-Tr) was

Table 1 — Prediction of epitopes in NanH protein of *Pasteurella multocida* using the SVMTriP Web server

Rank	Location	Epitope	Score
1	72-91	SRIPAMTITDDNKMVVMFDL	1
2	134-153	AMDPTLLFNSFDGSLYVMHG	0.707
3	544-563	FLALNTPLDFSKYIKQGEKL	0.693
4	627-646	VRAQYTKGDNVLAPFLRYRT	0.595
5	682-701	GAYTSLSSRTLLDEDVAVNK	0.485
6	290-309	GREDNGKKTRWAYYTEDLGQ	0.477
7	4-23	PVFLLSLLALSTSMAVHGNS	0.457
8	580-599	VEYDSVIKNSQHRPTIALGL	0.449
9	112-131	GGHSWKRITAWNFNDSKISL	0.416
10	457-476	ETLSAKMRRANDNAVAESNV	0.337

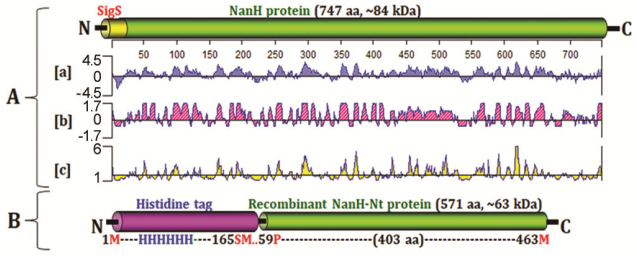

Fig. 1— Schematic of NanH protein construct. Panel A: Schematic of full length NanH protein with functional motifs. Panel B: Schematic of partial length rNanH with N- and C-terminus truncation having N- terminal histidine tag

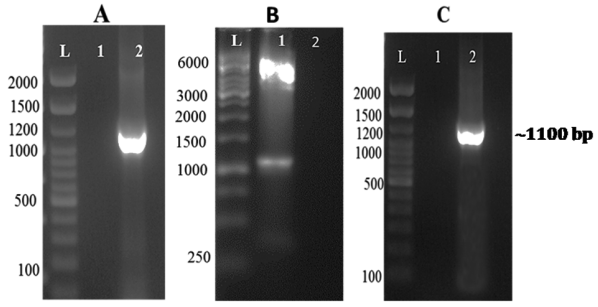

Fig 2 — PCR amplification and clone confirmation of *nanH-Tr* gene of *P. multocida*. (A) PCR amplification of *nanH-Tr* gene of *P. multocida*. (Lane L: 100 bp DNA ladder, Lane 1: Non template control, Lane 2: *P. multocida* serotype B:2); (B) Confirmation of NanH-Tr clone by restriction enzyme digestion (Lane L: 1 kb DNA ladder, Lane 1: RE (*Bam*HI and *Xho*I) digest of recombinant plasmid vector); and (C) Confirmation of recombinant NanH-Tr clones by colony PCR. (Lane L: 100 bp DNA ladder, Lane 1: Non template control, Lane 2: Positive recombinant *E. coli* clone)

confirmed by colony PCR. After transformation and chemical induction, the rNanH-Tr was over expressed (molecular weight ~63 kDa). Of this, ~45 kDa was NanH-Tr and ~18 kDa was the coding region of pET32a vector including the N-terminus hexa-histidine tag. Solubility analysis after cell lysis indicated the partitioning of over-expressed rNanH-Tr into the insoluble fraction of the lysate. Furthermore, rNanH-Tr was purified under urea denaturing conditions. After dialysis, the final concentration of the protein was 250 ng/mL. This protein appeared as a single band at ~63 kDa during 12% SDS-PAGE (Fig. 3). The rNanH-Tr protein was detected by rabbit hyperimmune sera (Fig. 3).

Formation of positive and negative serum panel

Serum samples found negative in PCR screening of nasal swab and serum samples having titre<1:4 were considered 'negative' for HS. Serum samples having IHA titre>1:160 titre were considered 'positive' for HS (Fig 4). A panel of 35 each positive and negative sera sample was formed for determination of the cut-off value.

Diagnostic sensitivity and specificity of rNanH-Tr protein based indirect ELISA

Based on the checker-board titrations, 250 ng of antigen and 1:40 dilutions of test sera per well in rNanH-Tr ELISA showed maximum or optimum P/N ratio while using positive and negative reference sera (Table 2 and Fig 5). These antigen and sera dilutions were used for screening of sera samples in indirect ELISA. Percent positivity cut-off value for rNanH-Tr ELISA was 30.50, and it resulted in diagnostic sensitivity of 86.2 (CI 73.26-96.80%) and specificity of 80.00 (CI 63.06-91.56%) (Table 3). Cross-reactivity of rNanH-Tr was not detected in Western blot analysis when sera against other diseases such as brucellosis and foot-and-mouth disease (FMD) were tested, indicating that the rNanH-Tr antigen possessed good analytical specificity. The ROC curve analysis was expressed in terms of area under the curve (AUC) for the rNanH-Tr ELISA test for known naïve and positive test sera (Fig. 6). AUC represents summary statistics of ROC analysis and it quantifies the diagnostic accuracy (discriminatory power) of a test along with test likelihood ratios (+/−), which are summarized in Table 3.

Discussion

Haemorrhagic septicaemia is an acute, fatal disease of cattle and buffaloes with high morbidity and mortality rates. Bacterial isolation and confirmation is considered the "gold standard" for diagnosis of HS. Culture techniques for the isolation and identification of *P. multocida* are time-consuming and often fail because of the labile nature of the organism. Several diagnostic tests have been used to assess immune-responses against HS in either naturally infected or vaccinated animals. These include the passive mouse protection test (PMPT), the IHA test, and ELISA[20]. ELISA based on crude antigen (LPS or OMP) are available as a screening test for detecting humoral

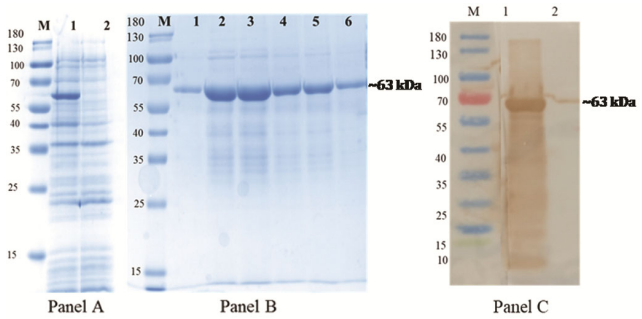

Fig. 3 — Expression and purification of recombinant rNanH-Tr protein and confirmation of expressed His-tagged protein rNanH-Tr by Western blot using the rabbit hyper immune sera from recombinant *E. coli* clone. (Panel A) Lane M: Protein Marker, Lane 1: induced (2.30 h) *E. coli* cell lysate, Lane 2: non-induced *E. coli* cell lysate. (Panel B) Elution of rNanH-Tr proteins from the Ni-NTA column, Lane M: Protein Marker, Lane 1- 6: Different elutes showing varying concentrations of rNanH-Tr proteins; and (Panel C) Lane M: Prestained protein ladder; Lane 1: Induced *E. coli* cell lysate; Lane 2: Purified rNanH-Tr protein

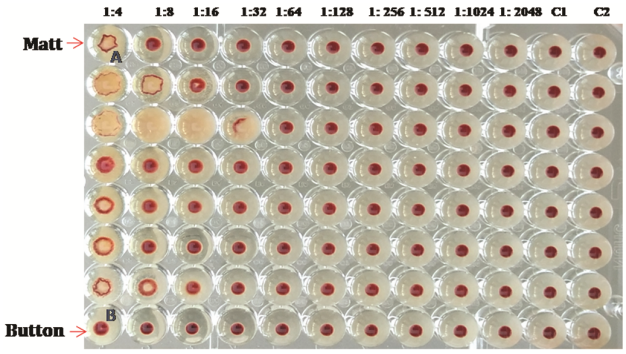

Fig. 4 — Indirect Haemagglutination Test using two-fold dilution of serum. (A) Mat formation – positive reaction; and (B) Button formation - negative reaction. [C1, Antigen control; C2, Serum control]

Table 2 — The ratio of OD, serum dilution and conjugated protein values using positive and negative bovine sera samples in checkerboard of rNanH-Tr antigen based indirect-ELISA.

Proteins conc.	P/N ratio	Serum dilution	Conjugate dilution
250 ng	8.5	1:40	1:5000

Table 3 — Receiver operating characteristic (ROC) curve and determination of the cut-off and sensitivity and specificity of indirect ELISA using rNanH-Tr protein

Cut off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio	Area
>30.50	86.2	73.26-96.80%	80.00	63.06-91.56%	4.43	0.9249 (0.8570-0.9928) (*P*= <0.0001)

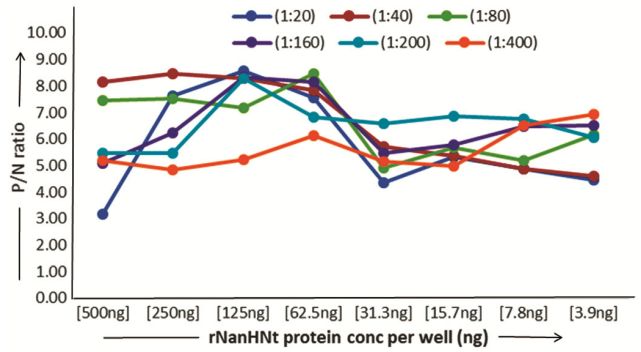

Fig. 5 — Chequer board titration to optimize the rNanH-Tr protein concentration and serum dilution in indirect ELISA. [Positive/negative ration of control positive and negative bovine sera dilutions against the rNanH-Tr protein dilutions]

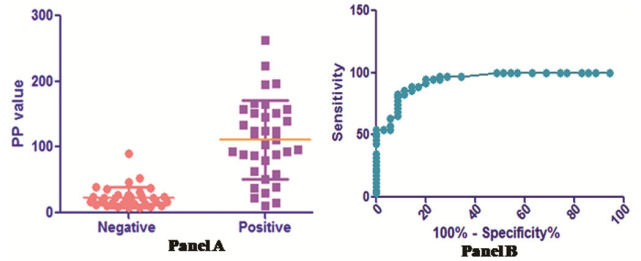

Fig. 6 — Panel A: Determination of the cut-off for anti-*P. multocida* antibodies in *P. multocida* negative and positive animals by indirect rNanH-Tr ELISA. Cut-off values were determined based on the ROC curve (PP > 30.50); and Panel B: ROC curves based on the test results obtained using ELISA. Sensitivity and specificity of the test were determined based on the cut-off values of these panels of sera. The area enclosed under the curve with diagonal base present AUC.

immune response to vaccination. Previous studies have successfully demonstrated that the test can be used to quantify the response to vaccination[21,24]. However, the technique is limited due to the fact that the antigen used was a crude, boiled extract composed of lipopolysaccharides. Furthermore, there was also cross-reactivity among HS-causing *P. multocida* serotypes. A better target for the detection of anti *P. multocida* antibodies would be a homogeneous stable antigen that could be easily generated and purified. Hence recombinant antigen-based ELISA could be a better alternative for diagnosis/sero-surveillance of HS antibodies[33]. In this study, a membrane-associated protein of *P. multocida* was cloned and expressed using a prokaryotic expression system. The expressed protein was found in the insoluble fraction of *E. coli*. Therefore, the proteins were solubilized under denaturing conditions before further purification. The yield of the refolded protein is decreased by the presence of contaminants like polypeptide, phospholipids, and inclusion bodies[34]. Presence of proteins in inclusion bodies leads to low yield and increase the cost of production of recombinant proteins. However, the expression of protein as inclusion bodies has some advantages such as very high level of protein expression and resistance to protease attack. We have used detergent washing in order to remove cell debris from inclusion bodies and to increase the yield. Aggregation of proteins due to the use of sub-optimal refolding procedures leads to low protein recovery. The best method for maximizing protein recovery from inclusion bodies is mild solubilization of the aggregates using denaturants, followed by pulsatile refolding of the solubilized protein[35]. We have also used gradation of the buffer (6, 4 and 2 molar of urea) for renaturing the protein before final elution. The purified proteins were found to be stable during storage at −80°C or 4°C, further indicating its suitability for use in diagnostics.

The present immunoblotting results for rNanH-Tr revealed a major band at approximately 63 kDa. Antibody kinetics showed that rNanH-Tr protein is immunogenic and can be used in ELISA. A 1:5000 dilution of bovine HRP conjugate and an optimized concentration of different antigens were chosen for the testing since a subsequent dilution indicated a satisfactory difference between the positive and negative reference sera. The IHA test has previously been recommended to detect immunity in animals and for the purpose of diagnosis IHA titers from 1:160 to 1:1280 or higher among in-contact animals surviving in affected herds are reported to be indicative of recent exposure to the pathogen[20]. A strong antibody response can be detected using the IHA test in naturally infected animals, and antibodies will be detectable within 10–14 days post-exposure, rising to a peak in 3-4 wk. Determination of the cut-off value for ELISA OD/percentage positive is critical to differentiate the positive and negative sera samples. Cut-off point is being determined for the standardization of an ELISA because a continuous

variable such as OD must be transformed into a qualitative response (positive or negative). Previous studies have used many criteria to determine the cut-off value for ELISA, such as a mean of sample-to-positive ratio value plus two standard deviations of the control sera. In this study, cut-off values were chosen as negative serum plus 3 standard deviations due to the ease of calculation and effectiveness in practice. Moreover, this method was also used to calculate the cut-off value of antibodies against *P. multocida* in ducks in a previous study[36]. The cut-off value was determined using a receiver operating characteristic (ROC) curve analysis, and it yielded optimum high Se and Sp values for the ELISA. In the present study using ELISA for sero-surveillance of HS, the sensitivity and specificity values of rNanH-Tr were 86 and 80%, respectively. The results indicate that rNanH-Tr ELISA has moderate sensitivity and specificity for the sero-surveillance of HS. The sensitivity of the serological test is dependent on the *P. multocida* antigen used[23]. The sensitivity and specificity of the test can vary depending upon the criterion (OD) defined, and it also depends on the prevalence of the disease. Here, we optimized the test for moderate sensitivity and specificity. Previously, ELISA based on crude antigens for the detection of antibodies against *P. multocida* has shown the highest sensitivity in apparently healthy, diseased, and emergency animals[22]. A recombinant antigen-based ELISA using the NanH sialidase of *P. multocida* was developed using a similar strategy, and was found to detect antibodies against *P. multocida* rabbits experimentally infected with *P. multocida*[17]. Standardized rNanH-Tr based ELISA was used for screening of 250 random bovine sera samples, and antibodies against *P. multocida* were detected in 22% of them. In contrast, a sero-prevalence study of HS in North-East India using a commercial ELISA kit recorded 35.7% herd prevalence[37].

Conclusion

In the present study, we cloned the recombinant rNanH-Tr protein and expressed in a prokaryotic expression system. The recombinant protein was expressed as inclusion bodies and solubilized using urea before purification under denaturing conditions. An indirect-ELISA using purified rNanH-Tr was developed and was found to have moderate sensitivity and specificity. Screening of random bovine sera samples revealed 22% sero-positivity of antibodies against *P. multocida*.

Acknowledgements

Authors are thankful to the Indian Council of Agricultural Research (ICAR), New Delhi, and ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Benglauru, Karnataka, India, for providing financial assistance to carry out the research on *Pasteurella multocida.* This study was a part of doctoral research carried out by AP under the institute funded project (Code: IXX12176) on '*Epidemiology of Haemorrahgic septicaemia (HS) in India'* granted to SBS.

Conflict of interest

Authors declare no competing interests.

References

1 Almoheer R, Abd Wahid ME, Zakaria HA, Jonet MAB, Al-Shaibani MM, Al-Gheethi A & Addis SNK, Spatial, temporal, and demographic patterns in the prevalence of hemorrhagic septicaemia in 41 countries in 2005–2019: a systematic analysis with special focus on the potential development of a new-generation vaccine. *Vaccines*, 10 (2022) 315.

2 Verma H, Rawat M, Verma R, Gandham R, Tiwari AK, Khan RIN, Praharaj MR & Smith E, First report of whole genome sequence of septicemic *Pasteurella multocida* serovar B: 2 'Soron' strain isolated from swine. *Braz J Microbiol,* (2023).

3 Harper M & Boyce JD, The myriad properties of *Pasteurella multocida* lipopolysaccharide. *Toxins*, 9 (2017) 254.

4 Calderón BJM, Fernández A, Arnal JL, SanzTejero C, Fernández-Garayzábal JF, Vela AI & Cid D, Molecular epidemiology of *Pasteurella multocida* associated with bovine respiratory disease outbreaks. *Animals*, 13 (2022) 75.

5 Govindaraj G, Krishnamoorthy P, Nethrayini KR, Shalini R & Rahman H, Epidemiological features and financial loss due to clinically diagnosed haemorrhagic septicaemia in bovines in Karnataka, India. *Prev Vet Med*, 144 (2017) 123.

6 Verma H, Rawat M, Samal A, Upmanyu V, Durairajan & Verma R, Standardization and development of *Pasteurella multocida* inactivated adjuvanted vaccine against septic pasteurellosis in pigs. *Indian J Exp Biol*, 57 (2019) 315.

7 D'Amico F, Casalino G, Bozzo G, Camarda A, Lombard, R, Dimuccio MM & Circella E, Spreading of *Pasteurella multocida* infection in a pet rabbit breeding and possible implications on healed bunnies. *Vet Sci*, 9 (2022) 301.

8 Yang W, Li M, Zhang C, Zhang X, Guo M & Wu Y, Pathogenicity, colonization, and innate immune response to *Pasteurella multocida* in rabbits. *BMC Vet Res*, 18 (2022) 416.

9 Kubatzky KF, *Pasteurella multocida* toxin–lessons learned from a mitogenic toxin. *Front Immunol*, 13 (2022) 1058905.

10 Truswell A, Laird TJ, Jones S, O'Dea M, Blinco J, Abraham R, Morison D, Jordan D, Hampson DJ, Pang S & Abraham S, Antimicrobial resistance of and genomic insights into *Pasteurella multocida* strains isolated from Australian Pigs. *Microbiol Spectr*, 11 (2023) e03784.

11 Sudhakara P, Sellamuthu I & Aruni AW, Bacterial sialoglycosidases in virulence and pathogenesis. *Pathogens*, 8 (2019) 39.

12 Peng Z, Wang X, Zhou R, Chen H, Wilson BA & Wu B, *Pasteurella multocida*: genotypes and genomics. *Microbiol Mol Biol Rev*, 83 (2019) e00014.

13 Nugroho CMH, Kurnia RS, Tarigan S, Silaen OSM, Triwidyaningtyas S, Wibawan IWT, Natalia L, Takdir AK & Soebandrio A, Screening and purification of NanB sialidase from *Pasteurella multocida* with activity in hydrolyzing sialic acid Neu5Acα (2–6) Gal and Neu5Acα (2–3) Gal. *Sci Rep*, 12 (2022) 9425.

14 Lin H, Liu Z, Zhou Y, Lu W & Xu Q, Characterization of resistance and virulence of *Pasteurella multocida* isolated from pet cats in South China. *Antibiotics*, 11 (2022) 1387.

15 Su A, Tong J, Fu Y, Müller S, Weldearegay YB, Becher P, Valentin-Weigand P, Meens J & Herrler G, Infection of bovine well-differentiated airway epithelial cells by *Pasteurellamultocida*: actions and counteractions in the bacteria–host interactions. *Vet Res*, 51 (2020) 140.

16 Mizan S, Henk A, Stallings A, Maier M & Lee MD, Cloning and characterization of sialidases with 2-6′ and 2-3′ sialyl lactose specificity from *Pasteurella multocida*. *J Bacteriol*, 182 (2000) 6874.

17 Sanchez S, Mizan S, Quist C, Schroder P, Juneau M, Dawe D, Ritchie B & Lee MD, Serological response to *Pasteurella multocida* NanH sialidase in persistently colonized rabbits. *Clin Diagn Lab Immunol*, 11 (2004) 825.

18 Abera D & Mossie T, A review on pneumonic pasteurellosis in small ruminants. *J Appl Anim Res*, 51 (2023) 1.

19 Hashem YM, Mousa WS, Abdeen EE, Abdelkhalek HM, Nooruzzaman M, El-Askary A, Ismail KA, Megahed AM, Abdeen A, Soliman EA & Wareth G, Prevalence and molecular characterization of Mycoplasma species, *Pasteurella multocida*, and *Staphylococcus aureus* isolated from calves with respiratory manifestations. *Animals*, 12 (2022) 312.

20 Shivachandra SB, Haemorrhagic septicaemia (Pasteurella multocida serotypes 6:B and 6:E) In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. 12th edn, (OIE Terrestrial Manual 2021, World Organisation for Animal Health–WOAH/OIE), 2021.

21 Singhla T, Tankaew P & Sthitmatee N, Validation of a novel ELISA for the diagnosis of hemorrhagic septicemia in dairy cattle from Thailand using a Bayesian approach. *Vet Sci*, 7 (2020) 163.

22 El-Jakee JK, Ali SS, El-Shafii SA, Hessain AM, Al-Arfaj AA & Mohamed MI, Comparative studies for serodiagnosis of haemorrhagic septicaemia in cattle sera. *Saudi J Biol Sci*, 23 (2016) 48.

23 Tankaew P, Singh-La T, Titaram C, Punyapornwittaya V, Vongchan P, Sawada T & Sthitmatee N, Evaluation of an In-house indirect ELISA for detection of antibody against haemorrhagic septicaemia in Asian elephants. *J Microbiol Methods*, 134 (2017) 30.

24 Mahboob S, Iqbal M & Rahman M, Development of ELISA-based diagnostic methods for the detection of haemorrhagic septicaemia in animals. *J Microbiol Methods*, 204 (2023) 106652.

25 Yang R, Gong M, Jiao S, Han J, Feng C, Pei M, Zhou Z, Du Y & Li J, Protein engineering of *Pasteurella multocida* α2, 3-Sialyltransferase with reduced α2, 3-sialidase activity and application in synthesis of 3′-sialyllactose. *Catalysts*, 12 (2022) 579.

26 Yao B, Zheng D, Liang S & Zhang C, SVMTriP: A method to predict B-Cell linear antigenic epitopes. In: *Immunoinformatics*. *Methods in Molecular Biology*, Vol. 2131 (Tomar N; Humana, New York, NY), 2020, 299.

27 Yogisharadhya R, Mohanty NN, Chacko N, Mondal M, Chanda MM & Shivachandra SB, Sequence and structural analysis of OmpW protein of *Pasteurella multocida* strains reveal evolutionary conservation among members of *Pasteurellaceae*along with its homologues. *Gene Rep*, 14 (2019) 36.

28 Shivachandra SB, Kumar A, Yogisharadhya R & Viswas KN, Immunogenicity of highly conserved recombinant VacJ outer membrane lipoprotein of *Pasteurella multocida*. *Vaccine*, 32 (2014) 290.

29 Memon AM, Chen F, Khan SB, Guo X, Khan R, Khan FA, Zhu Y & He Q, Development and evaluation of polyclonal antibodies based antigen capture ELISA for detection of porcine rotavirus. *Anim Biotechnol*, 5 (2022) 1.

30 Getnet K, Abera B, Getie H, Molla W, Mekonnen SA, Megistu BA, Abat AS, Dejene H, Birhan M & Ibrahim SM, Serotyping and seroprevalence of *Mannheimia haemolytica*, *Pasteurella multocida*, and *Bibersteinia trehalosi* and assessment of determinants of ovine pasteurellosis in West Amhara Sub-region, Ethiopia. *Front Vet Sci*, 9 (2022) 866206.

31 Prajapati A, Chanda MM, Yogisharadhya R, ParveenA, Ummer J, Dhayalan A, Mohanty NN & Shivachandra SB, Comparative genetic diversity analysis based on virulence and repetitive genes profiling of circulating *Pasteurella multocida* isolates from animal hosts. *Infect Genet Evol*, 85 (2020) 104564.

32 Mohanty NN, Hemadri D, Munivenkatarayappa A, Shetty N, Subramanyam V, Biswas SK, Chanda MM & Shivachandra SB, Development of recombinant NS1-NS3 antigen based indirect ELISA for detection of bluetongue antibodies in sheep. *J Immunol Methods*, 490 (2021) 112959.

33 Joycee J, Nayak A, Rai A & Dubey A, Immunoprotection in mice immunized with native OmpH, recombinant OmpH and HS alum precipitated vaccine of *Pasteurella multocida* P52 against *P. multocida* challenge. *Indian J Exp Biol*, 61 (2023) 436.

34 Singhvi P & Panda AK, Solubilization and refolding of inclusion body proteins. *Methods Mol Biol*, 2406 (2022) 371.

35 Oyeleye AO, Mohd Yusoff SF, Abd Rahim IN, Leow AT, Saidi NB & Normi YM, Effective refolding of a cysteine rich glycoside hydrolase family 19 recombinant chitinase from *Streptomyces griseus* by reverse dilution and affinity chromatography. *PLoS ONE*, 15 (2020) e0241074.

36 Poolperm P, Varinrak T, Kataoka Y, Tragoolpua K, Sawada T & Sthitmatee N, Development and standardization of an in-house indirect ELISA for detection of duck antibody to fowl cholera. *J Microbiol Methods*, 142 (2017) 10.

37 Shome R, Deka RP, Sahay S, Grace D & Lindahl JF, Seroprevalence of hemorrhagic septicemia in dairy cows in Assam, India. *Infect Ecol Epidemiol*, 9 (2019).