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1 PROTOCOLS AND METHODS

Development of B cell epitopes-based enzyme linked immune sorbent assay for detection of bovine anti-Mullerian hormone

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Abstract

The present study aimed to generate antibodies against predicted B cell epitopic peptides encoding bAMH for developing different ELISA models. Sandwich ELISA was determined to be an excellent technique for assessing bAMH in bovine plasma based on sensitivity tests. The assay's specificity, sensitivity, inter- and intra-assay CV, recovery %, Lower limit of quantification (LLOQ), and Upper limit of quantification (ULOQ) were determined. The test was selective since it did not bind to AMH-related growth and differentiation factors (LH and FSH) or non-related components (BSA, progesterone). The intra-assay CV was 5.67%, 3.12%, 4.94%, 3.61% and 4.27% for 72.44, 183.11, 368.24, 522.24 and 732.25 pg/ml AMH levels, respectively. At the same time, the inter-assay CV was 8.77%, 7.87%, 4.53%, 5.76% and 6.70% for 79.30, 161.27, 356.30, 569.33 and 798.19 pg/ml AMH levels, respectively. The average (Mean ± SEM) recovery percentages were 88–100%. LLOQ was 5 pg/ml and ULOQ at 50 µg/ml (CV < 20%). In conclusion, we developed a new highly sensitive ELISA against bAMH using epitope specific antibodies.

Keywords Anti-Mullerian hormone · ELISA · B cell epitope · Antibody · Bovine · bAMH

Introduction

Fertility is an important economic element in the livestock sector and a significant measure for determining the animal's productivity. As a result, it is critical to understand the elements that influence fertility and the signs of an animal's reproductive lifespan. It has been observed that an animal's productive life is mostly determined by the quality and amount of its ovarian follicle reserve (Haldar et al. 2019). In domestic animals, Anti-Mullerian Hormone (AMH) is a direct predictor of ovarian reserve and a prospective biomarker of fertility. It is a dimeric glycoprotein that belongs to the growth and differentiation factor family transforming growth factor (TGF) (Cate et al. 1986). AMH secretion is not dependent on other hormones, particularly gonadotropins, and is expressed at a constant level regardless of the cycle, making AMH an appealing direct marker of ovarian reserve (Hehenkamp et al. 2006; La Marca and Volpe 2006).

So, assessment of AMH levels in animals can benefit the selection of animals. Several kits are available in the market for the detection of bovine AMH. However, all of those are costly and can be used for few numbers of samples. Since 1990, several studies have been conducted to develop assays

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against AMH (Hudson et al. 1990; Long et al. 2000; Al-Qahtani et al. 2005; Groome et al. 2011; Ataman-Onal et al. 2019). Interestingly, all these inventions mainly focused on developing assays for the human to use in clinical diagnosis or treatment. Secondly, the antibodies they used were monoclonal in nature produced through hybridoma technology. As a result, assessing AMH levels in animals may benefit animal selection. There are several kits on the marketplace for detecting bovine AMH (Hudson et al. 1990; Long et al. 2000; Al-Qahtani et al. 2005; Groome et al. 2011; Ataman-Onal et al. 2019), however, all of these are expensive and can only be used for a limited number of samples. Surprisingly, all of these kits aimed to establish assays for human use in clinical diagnosis or therapy. Secondly, the antibodies they used were monoclonal in nature and were made using hybridoma technology.

The present study was designed to develop B cell epitopes-based Enzyme Linked Immune Sorbent Assay (ELISA) to detect bovine Anti-Mullerian hormone (bAMH). Producing antibodies against desired biomolecules through this approach can revolutionise the field of immunoassay by decreasing the time and cost as well as making it easier. This triggered us to conduct research on developing a new, economical assay for the estimation of bovine AMH using epitope specific antibody.

Materials and methods

Designing and synthesis of peptide

The amino acid sequence of Mullerian inhibiting substance (MIS)/Anti-Mullerian Hormone, *Bos taurus* (GenBank: AAA98765.1) was retrieved from NCBI. The protein sequence in FASTA format was scanned to predict linear B cell epitope using antigen sequence properties by Immune Epitope Database Analysis Resource (IEDB) (Vita et al. 2010). For the identification of the B cell epitope region in protein, we used Bepipred Linear Epitope Prediction 2.0 (Jespersen et al. 2017). The MIS protein was analysed with the help of various indices viz. Jameson–Wolf antigenic index, surface probability, and hydrophilicity index, Karplus and Schulz flexibility index, Emini surface probability index (Emini et al. 1985; Hopp and Woods 1981; Karplus and Schulz 1985). The antigenicity propensity score for the protein was determined by the online tool SCRATCH Protein Predictor (http://scratch.proteomics.ics.uci.edu/). The bioinformatics tools used the MIS protein sequence for the generation of hydrophilicity/hydrophobicity and antigenicity/antigenicity propensity scores. The MIS protein sequence was primarily analysed on a hydrophilicity scale by Hopp and Woods, Kyte, and Doolittle; on a hydrophobicity scale by Manavalan and Sweet/Eisenberg and antigenicity scale by Welling, Jameson-Wolf, Parker and Hopp and Woods antigenicity plot. The antigenicity propensity score was accessed by the SCRATCH Protein Predictor (http://scratch.proteomics.ics.uci.edu/) weblink. The predicted antigen propensity score was determined using ANTIGENpro under SCRATCH and other properties of the protein like relative solvent accessibility and predicted secondary structure was also determined suing ACCpro and SSpro8 respectively. Additionally, SOPMA secondary structure prediction tool was used for predictive secondary structure analysis of the peptides (Geourjon and Deleage 1995) and the Protean Software was used for MIS protein sequence analysis using the comprehensive secondary structure, amphiphilicity, hydropathy, antigenicity, surface probability, and flexibility indices-based analysis. The selected peptide sequences (20 mg each with > 98% purity) were purchased from Genscript®, supplied by Biotech Desk®, India.

Interaction of the designed peptides with the bovine AMH receptor

Sequence of AMH protein (P03972.1) was downloaded from NCBI and 3D structure was built using ab initio modelling using Phyre2 suite. The model was then submitted to PDB-sum sever to check its stereochemical quality. After AMH receptor protein was docked with peptides separately, to see the interactions using pep-dock server.

Preparation of conjugated peptides for immunisation

To stimulate antibody responses for smaller peptides, the peptides need to be covalently conjugated to a larger immunogenic carrier protein prior to immunisation. Peptides were conjugated with Keyhole limpet hemocyanin (KLH) as per the methodology described by Lateef et al. (2007). Conjugated peptides were mixed with equal volume of Freund's Complete /Incomplete adjuvant (FCA/FIA) for preparation of emulsion.

Immunisation

250 µg of emulsified and conjugated peptides were administered subcutaneously in rabbits ($n = 3$). The first injection was given with FCA emulsion and the subsequent booster doses emulsion was prepared with FIA. Immunisations were done on day 0, 14, 28, 42, 70 (Lee et al. 2016). Blood samples were collected from ear vein of all the animals using Nipro® 1 ml Syringe with Needle (26 G) before immunisation and 10 days after each booster dose and the final booster dose in serum collecting tubes (VACUETTE® TUBE, 9 ml CAT Serum Clot Activator 16 × 100 red cap-black ring, non-ridged, Catalog no- 455,092). It was allowed to clot and

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Journal : Large 13205 | Article No : 3622 | Pages : 16 | MS Code : 3622 | Dispatch : 13-6-2023

138 the serum was separated through centrifugation (3000 rpm, 139 10 min). The serum samples were divided into aliquots and 140 stored in (– 20 °C) for future use.

141 Determination of antibody titer

142 Antibody titer was determined by Indirect ELISA as 143 described by Lee et al. (2016). The wells of a mictrotiter 144 plate were coated with 300 µl of 0.2–2.5 µM synthetic pep-145 tides, leaving wells at the end as blanks. Incubated overnight 146 at 4 °C. The unbound synthetic peptide was discarded and he 147 wells were washed three times with PBST. The unoccupied 148 sites were blocked with 300 µl/well of blocking solution and 149 incubated for 1 h at room temperature. The wells were again 150 washed three times with PBST. Serial dilutions of antiserum 151 were prepared with PBST ranging from 1:300 to 1:300,000. 152 300 µl/well of the antiserum serial dilutions were added to 153 the wells and incubated for 2 h at 37 °C. The wells were 154 washed three times with PBST. The anti-rabbit secondary 155 antibody was diluted 1:7000 with PBST. 300 µl/well of the 156 secondary antibody was added to the wells and incubated at 157 37 °C for 2 h. The wells were washed three times with PBST. 158 50 µl enzyme substrate (OPD) was added in each well. Incu-159 bated for 10–30 min at 37 °C. Yellow colour developed. The 160 colour development was terminated by addition of 100 µl of 161 stopping solution. Colour changed into brownish. Absorb-162 ance was measured at 490 nm with a microtiter plate reader.

163 Antibody purification and its characterisation

164 Ig G antibodies were procured from the sera samples 165 using Protein A Antibody Purification kit (Catalog Num-166 ber PURE1A). Following purification, the concentration 167 of antibody was determined with Nanodrop® Spectro-168 photometer (ND 1000). Nitrocellulose paper was cut into 169 6.0 cm × 1.5 cm dimensions. Then circles are drawn by 170 pencil and 3.0 µl of antigens (AMH peptide- higher and 171 lower concentration, KLH and BSA) was applied and left 172 to dry. After that, the membrane was dipped in 5% BSA for 173 blocking and kept at shaker for 1 h at room temperature. 174 Then it was washed with TBST on the shaker for 10 min 175 three times. After that, it was dipped in primary antibody 176 (20 µl diluted in 14 ml of 3% BSA in TBST) and incubated 177 for 30 min to one hour at room temperature. Then, it was 178 washed with TBST for minutes three times. Next, the mem-179 brane was dipped and incubated for 1 h at room temperature 180 in an HRP conjugated secondary antibody (7 µl diluted in 181 10 ml TBST). After that, it was again washed three times 182 with TBST. Then it was taken into a dark room for the devel-183 opment of X-ray film. X ray films were cut in the size of the 184 membrane. Enhanced Chemiluminescence (ECL) substrate 185 was prepared. The membrane was dried in air and 1 ml of 186 ECL substrate was added. When fluorescence was observed, 187 the X ray film was applied on it for 20–50 s. Then the film 188 was dipped in developer 2–3 times, washed in water for 2–3 189 times and dipped in the fixer solution 2–3 times. Finally, the 190 film was dried and observed for the black spots indicating 191 the binding of developed antibody with antigens.

Conjugation of purified antibody 192

193 Purified IgG antibodies were conjugated with horse reddish 194 peroxidase (HRP) enzyme using ab102890 – HRP Conjuga-195 tion kit. In brief, 1 µl of modifier reagent was added to each 196 10 µl of antibody to be labelled and mixed gently. The cap 197 from vial of HRP conjugation mix was removed and the 198 antibody sample was pipetted directly onto the lyophilised 199 material. Then, it was resuspended gently by withdrawing 200 and re-dispensing the liquid once or twice using a pipette. 201 After that, the cap on the vial was replaced and left standing 202 for 3 h in the dark at room temperature (20–25 °C). Longer 203 incubation times, such as overnight, have no negative effect 204 on the conjugation. After incubating for 3 h, 1 µl of quencher 205 reagent was added for every 10 µl of antibody used and 206 mixed gently.

Development of direct, competitive and sandwich 207 ELISA 208

Direct ELISA 209

210 The wells of a mictrotiter plate were coated with 300 µl of 211 bAMH of different concentrations and unknown serum sam-212 ples, leaving wells at the end as blanks. Incubated overnight 213 at 4 °C. The unbound bAMH and unknown samples were 214 discarded. The wells were washed three times with PBST. 215 The unoccupied sites were blocked with 300 µl/well of 216 blocking solution and incubated for 1 h at room temperature. 217 The wells were washed three times with PBST. 300 µl/well 218 of the HRP conjugated primary antibody was added to the 219 wells and incubated for 2 h at 37 °C. The wells were washed 220 three times with PBST. The OPD tablet was dissolved in 221 100 ml citrate–phosphate buffer, and 40 µl fresh hydrogen 222 peroxide was added. 50 µl enzyme substrate was added in 223 each well and incubated for 10–30 min at 37 °C. The colour 224 development was terminated by addition of 100 µl of stop-225 ping solution. Absorbance was measured at 490 nm with a 226 microtiter plate reader.

Competitive ELISA 227

228 The wells of a mictrotiter plate were coated with 5000 ng/µl 229 bAMH, leaving wells at the end as blanks and incubated over-230 night at 4 °C. The wells were washed three times with PBST. 231 Then, the unoccupied sites were blocked with 300 µl/well of 232 blocking solution and incubated for 1 h at room temperature.

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Journal : Large 13205 | Article No : 3622 | Pages : 16 | MS Code : 3622 | Dispatch : 13-6-2023

233 After that, the wells were washed three times with PBST and
234 dried using blotting paper. Serial dilutions of bAMH were
235 prepared with PBST to be used as standard. 100 µl/well of the
236 unconjugated antibody were added to the wells. Then 100 µl
237 of standards were added in standard wells and 100 µl of sam-
238 ples in sample wells. It was incubated for 2 h at 37 °C. After
239 incubation, the wells were washed three times with PBST and
240 dried. The HRPO conjugated anti-rabbit secondary antibody
241 was diluted 1:10,000 with PBST and 300 µl/well of it was
242 added to the wells and incubated at 37 °C for 2 h. Absorb-
243 ance was measured at 490 nm with a microtiter plate reader
244 as described earlier.

245 ### Sandwich ELISA

246 The wells of a mictrotiter plate was coated with 300 µl of
247 unconjugated antibody, leaving wells at the end as blanks. It
248 was incubated overnight at 4 °C. The unbound antibody was
249 discarded. The wells were washed three times with PBST. The
250 unoccupied sites were blocked with 300 µl/well of blocking
251 solution and incubated for 1 h at room temperature. The wells
252 were washed three times with PBST. Serial dilutions of bAMH
253 with known concentrations were prepared with PBST. 100 µl
254 of bAMH of different concentrations and 100 µl unknown
255 serum samples, leaving wells at the end as blanks were added
256 to the wells and incubated for 2 h at 37 °C. The wells were
257 washed three times with PBST. 300 µl/well of the HRP conju-
258 gated primary antibody was added to the wells and incubated
259 at 37 °C for 2 h.

260 ## Validation of assay

261 Among all the three ELISA types, sandwich ELISA was found
262 to be best and was validated through the following tests.

263 ### Specificity test

264 Specificity was examined by measuring samples with known
265 supra-physiological amounts of the growth and differentiation
266 components most closely connected to AMH as well as unre-
267 lated members. We introduced BSA and progesterone as unre-
268 lated members and LH and FSH as related members at sup-
269 raphysiological quantities (at least 1–2 times higher than in a
270 healthy body). As unknowns, all the chemicals were measured
271 against the standard curve. According to the equation below,
272 the percentage of cross-reactivity was estimated (Kumar et al.
273 2010; Long et al. 2000).

274

$$\%\text{Cross-reactivity} = (\text{Observed concentration} \div \text{Estimated concentration}) \times 100$$

275 ### Sensitivity test

276 Lowest concentration of AMH determined by the newly
277 developed assay was calculated. The results were also
278 compared with data estimated through a commercially
279 available AMH kit (Bioassay technology laboratory, Cata-
280 log no- E0241Bo).

281 ### Inter- and intra-assay coefficient of variation

282 Plasma was isolated from blood samples taken from cows.
283 Using a devised assay, the levels of AMH were estimated,
284 and the inter- and intra-assay coefficient of variation was
285 calculated. In order to achieve this, we collected five
286 samples with bAMH concentrations ranging from 70 to
287 800 pg/ml to assess the levels in several wells on the same
288 plate and on other plates. The CV was determined and
289 compared to ELISA kits that had already been created.

290 ### Recovery test

291 Six samples of bovine plasma with varying levels of
292 endogenous AMH were spiked with known concentra-
293 tions of AMH to determine recovery. AMH levels in the
294 sample were measured both before (endogenous) and after
295 (observed) the injection of exogenous AMH. We combined
296 100 l of known-concentration plasma samples (98, 188,
297 228, 356, 546) with known-concentration bAMH (5 l and
298 10 l of 1000, 5000, and 50,000 pg/ml). The selectivity
299 was established by comparing the expected and observed
300 values. Expected concentration was calculated per the cal-
301 culation by Kumar et al. (2010).

302

$$\text{Expected concentration} = \left[\begin{array}{l}(\text{Endogenous concentration} \times \text{Volume of sample added}) \\ +(\text{Spike concentration} \times \text{Volume of spike added})\end{array}\right] \div (\text{Volume of sample added} + \text{Volume of spike added}).$$

303

304

$$\text{The percent recovery} = (\text{Observed concentration} \div \text{Expected concentration}) \times 100$$

305

Journal : Large 13205 | Article No : 3622 | Pages : 16 | MS Code : 3622 | Dispatch : 13-6-2023

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306 ### Lower limit of quantification (LLOQ) and upper limit
307 ### of quantification (ULOQ)

308 To determine the genuinely acceptable limit of the stand-
309 ard curve, LLOQ and ULOQ were determined. The analyte
310 response must be five times larger than the blank response
311 to detect LLOQ, and the response must also be reproduc-
312 ible and identifiable with 20% precision or 80–120% accu-
313 racy (O'Kennedy and Murphy 2017).

314 ## Results and discussion

315 ### Determination of antigenic region, epitope
316 ### prediction and optimisation of epitope sequence

317 In the present study, we use the Immune Epitope Database
318 (IEDB) database to predict the linear B cell epitopes in
319 the AMH protein. The reason for taking predicted B cell
320 epitopes for designing ELISA kit is that if the capture anti-
321 bodies can only recognise the linear AMH epitopes, then a
322 portion of the circulating antigen may be ignored as antibod-
323 ies normally recognise conformational epitopes. We used the
324 BepiPred linear B cell epitope prediction tool to identify lin-
325 ear B cell epitope. The BepiPred-2.0 server predicts the anti-
326 genic sequence using the Random Forest algorithm trained
327 on epitopic and non-epitopic amino acids determined using
328 the crystal structure of proteins (Jespersen et al. 2017). For
329 the selection of the antigenic peptide region, we used the 0.5
330 thresholds with expected 58.56% sensitivity and 57.158%
331 specificity of the prediction method. The BepiPred 2.0 anal-
332 ysis predicted 8 different antigenic sequences of variable
333 length ranging from 9 amino acid to 45 amino acid long
334 peptides. Out of these 9 peptides we selected three regions
335 encoding the amino acid ranges in between 20 and 43
336 (region 1), 266–308 (region 2) and 445–474 (region 3) hav-
337 ing high threshold regions. The increase in threshold value
338 improves the specificity of the method and the peptide from
339 region three shows a threshold value as high as 0.7, indicat-
340 ing 99.9% specificity in the prediction method. Amino acid
341 numbers of IEDB-Bepipred-2 predicted sequence and opti-
342 mised sequence of the three peptides derived from bAMH
343 are presented in Table 1.

Table 1 IEDB-Bepipred-2 predicted sequence and optimised sequence of the three peptides derived from bAMH

Peptide	Optimised sequence used in study	IEDB-Bepipred-2 predicted sequence
AMH-1	20 amino acids	24 amino acids
AMH-2	27 amino acids	43 amino acids
AMH-3	16 amino acids	30 amino acids

344 The AMH protein was also analysed using the SCRATCH
345 protein predictor and protean software. This two software were
346 used to study the protein and generate information about the
347 secondary structure, hydropathy, antigenicity, amphiphilicity,
348 surface probability, and flexibility. This analysis helped us in
349 optimising the antigenic peptide sequence and get better pre-
350 dictive regions in the selected three peptide sequences The
351 ANTIGEN pro in SCRATCH gives a sequence-based, align-
352 ment-free, and pathogen-independent predictor antigen predic-
353 tion of protein. We used a high antigenic propensity scoring
354 region with a Net Vote score + 10 and above. This helped us in
355 optimising the peptide sequence obtained from the Bepipred
356 2.0. Further, the Protean analysis was done to study the fea-
357 sibility of optimised peptide sequence using various indices
358 scores such as secondary structure, hydropathy, antigenicity,
359 amphiphilicity, surface probability, and flexibility. The Protein
360 analysis indicated that the optimised peptide sequences were
361 in the range of higher antigenic index (JamesonWolf). The
362 optimised AMH peptide sequences were subjected to multiple
363 sequence alignment and the specificity of the peptide to MIS
364 was ascertained. The results obtained through Protean analysis
365 is depicted in Fig. 1 and the Immune Epitope Database Analy-
366 sis Resource Based B cell epitope prediction analysis showing
367 antigenicity score of various regions of bAMH protein has
368 been shown in Fig. 2. The Ramachandran plot shows that 92%
369 of residues lie in the favourable region (Fig. 3).

370 ### AMH–peptides interaction

371 The protein structure of *Bos taurus* AMH receptor protein
372 was modelled using ab initio modelling as its homologous
373 structure is not available in protein data bank. Protein struc-
374 ture is modelled using Phyre2 suite. The 3D structure of
375 protein is validated on PDBSUM for its stereochemical qual-
376 ity through Ramachandran Plot. The peptides were docked
377 with AMH receptor protein using pepdock server. The inter-
378 actions were visualised using Discovery studio visualiser.
379 The amino acids Arg187-Glu1, Asp135, Arg37, Asn140,
380 Thr185, Gln41, Ala183, Gly15, His16, Gly14, Gln41 and
381 Gln186 of AMH are involved in interaction with petide1
382 (Fig. 4). Detailed interactions showed that Arg187 of AMH
383 protein is forming two bonds with Glu1 of ppetide1. Asp135
384 of AMH receptor is also making 2 bonds with Glu1 of pep-
385 tide1 as well as Asn140 and Thr185 is also making one bond
386 each with Glu1 of peptide1. Trp2 of peptide1 is forming
387 bonds with Thr185, Asp135 and Gln186 of AMH receptor.
388 In addition, Agr3 of peptide1 is making four bonds, one with
389 Asp135, two bonds with Gln41 and one bond with Asn140.

390 The amino acids involved in interactions between AMH
391 receptor protein and peptide2 are Arg12, Glu245, Glu182,
392 Gln139, Gln186, Gln239, Ala244, Phe238, Glu182, Pro171,
393 Arg12, Pro174 and Ser172. Amino acids Asp12 of peptide2 is
394 forming bond with Arg12 of AMH receptor. Arg1 of peptide2

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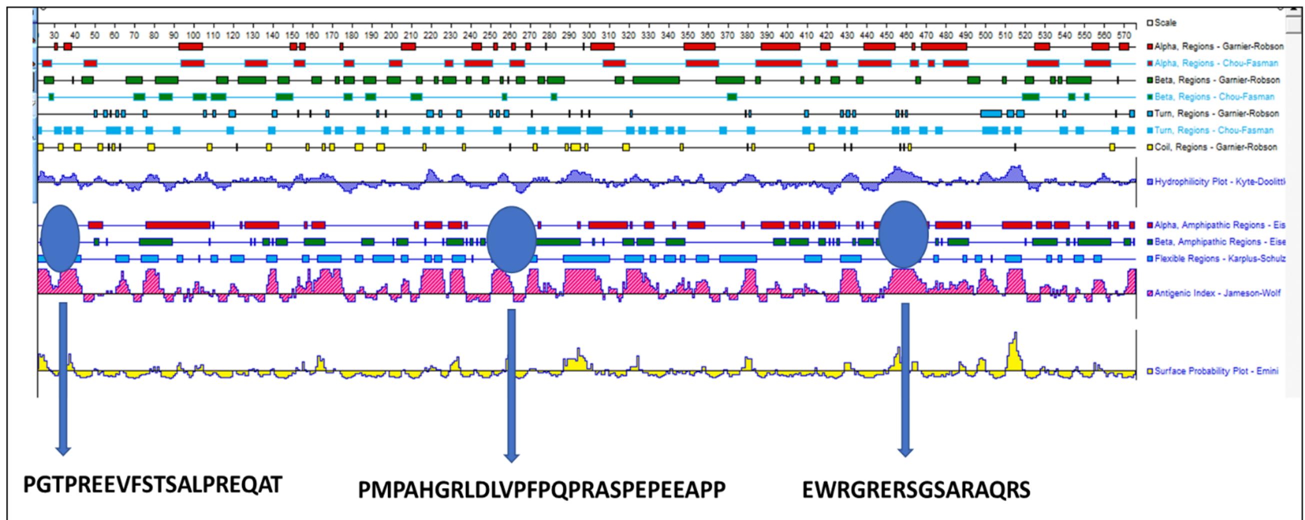

Fig. 1 With the help of PROTEAN, alpha, beta, turn and coil regions were identified by Garnier–Robson and Chou–Fasman methods. Amphipathic regions in alpha and beta, as well as flexible regions, were predicted by Eisenberg and Karplus–Schultz methods. The epitope prediction was done on the basis of Jameson–Wolf antigenic index, Kyte–Doolittle hydrophilicity, and Emini surface probability plots. The identified peptide regions are shown in blue circles with their respective sequences

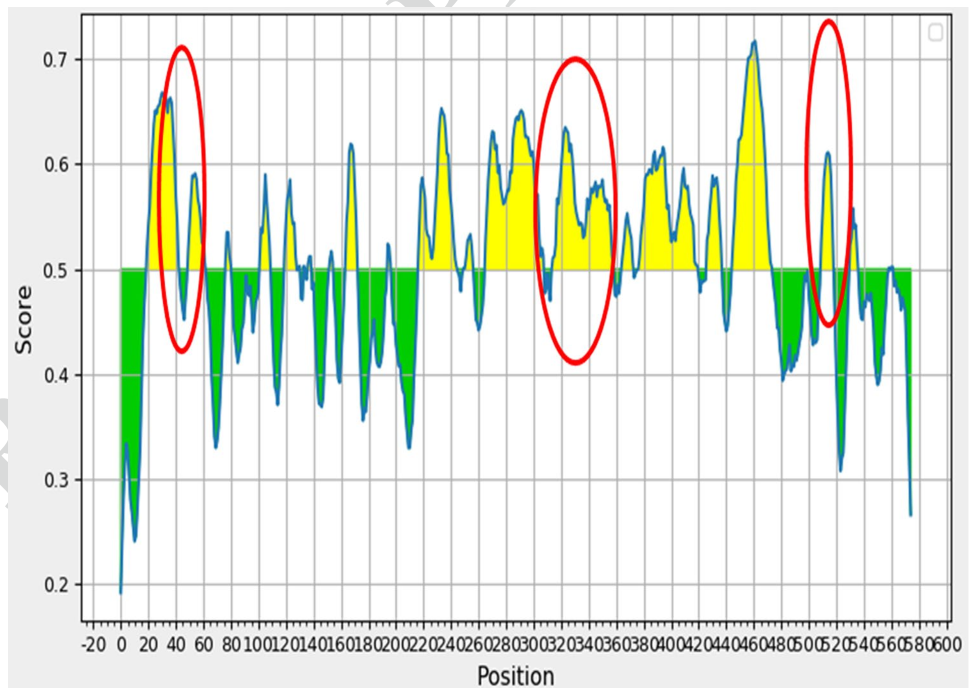

Fig. 2 Immune Epitope Database Analysis Resource Based B cell epitope prediction analysis showing antigenicity score of various regions of bAMH protein. The prediction was done using sequence characteristic using Hidden Markov Model (HMM), A region with high predictive antigen score was selected as antigenic peptides. Red encircled region shows the selected peptide regions in the study

is making 5 bonds, out of that, two bonds with Glu245, two bonds with Ala244 and one with Gln239 of AMH receptor. Leu11 of peptide 2 is interacting with Gln139 of AMH receptor via two bonds. Additionally, Asp12 of peptide2 is making two bonds with Arg12 of AMH receptor. Arg20 of peptide2 is interacting to AMH via two H-bonds with Glu182. Pro15, Ala7, Gln18, Glu27, and Pro30 of peptide 1 is interacting to Gln186, Phe238, Glu182 and Ser172 of AMH receptor via single H-bond, respectively. Whereas, Glu26 of peptide2 is forming two H bonds with Pro171, Pro174 of AMH receptor.

The amino acids involved in interactions between AMH receptor protein and peptide3 are Arg37, Arg187, His91, Gln139, Gln186, Glu13, Ala183, Pro174, Gly176, Pro250 and Glu182. Detailed analysis of interaction between peptide3 and AMH receptor revealed that, Glu6 of peptide3 is interacting to AMH via two H bonds with Arg37. Glu5

Journal : Large 13205 | Article No : 3622 | Pages : 16 | MS Code : 3622 | Dispatch : 13-6-2023

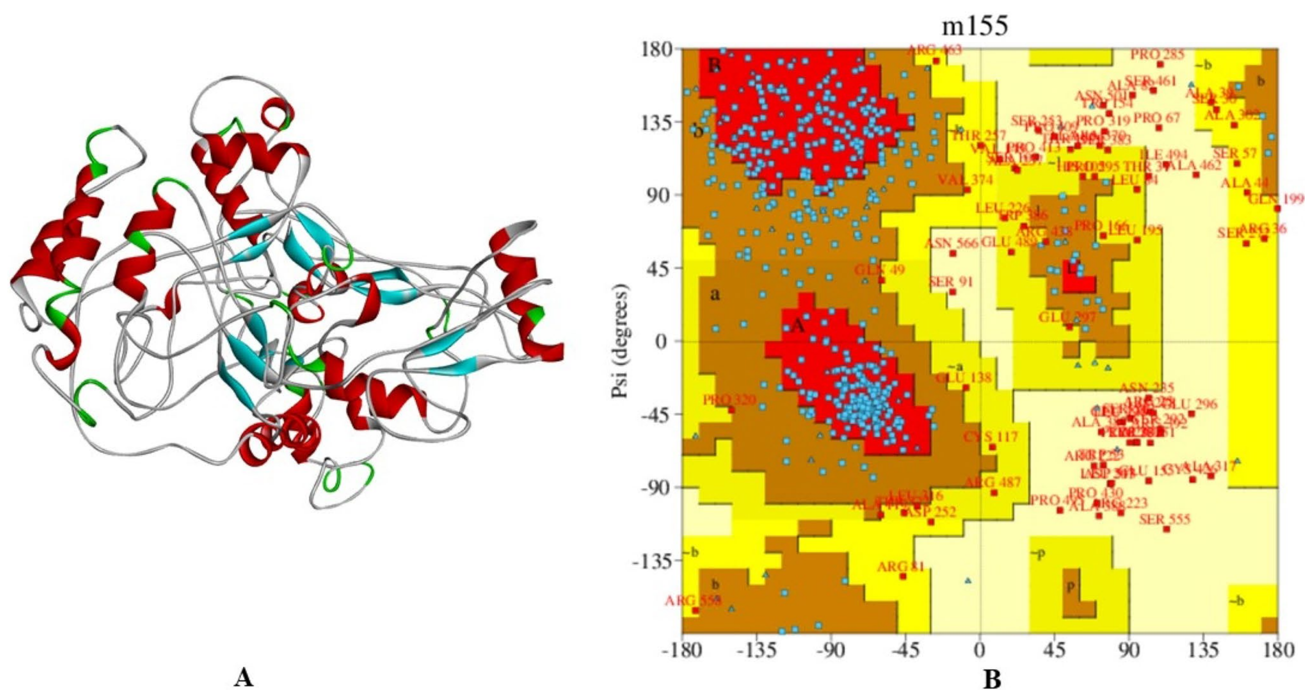

Fig. 3: 3D structure (**A**) and Ramachandran Plot (**B**) of bovine AMH receptor protein Mullerian inhibiting substance of *Bos taurus*

of peptide3 is making four H-bonds with Arg187, Gln186, Gln187 and Pro250 of AMH receptor. Pro1 of Peptide3 is interacting with His 91 of AMH via one H-bond. Arg4 of peptide3 with Glu13of AMH receptor, Phe8 of peptide3 with Ala183 of AMH receptor, Ser9 of peptide3 with Glu182 and Ala183 of AMH receptor and Ser11 of peptide3 with Pro174 and Gly176 AMH receptor are interacting via H-bonds.

A detailed interactions analysis of peptides with AMH receptor showing that there are total of 21 H-bond interactions, 18 H-bonds interactions and 15 H-bond interactions between AMH-peptide1, AMH-peptide2- and AMH-peptide3 respectively. In peptide1-AMH complex Glu1 of peptide1 is playing a key role in interaction via forming six H-bonds with Agr187 (Two H-bonds), Asp135 (two H-bonds), Asn140 (one H-bonds), and Thr185 (one H-bonds). In peptide2-AMH complex Arg1 is playing an important role in ppetide2-AMH complex along with other amino acid residues. In case of peptide3-AMH complex Glu5 and Glu6 are acting as an important interacting amino acids.

Determination of antibody titre and concentration of purified antibody

Antibodies were raised in the rabbits, and blood samples were obtained from all of them before immunisation and 10 days later. Serum was separated, and the titre of antisera was determined for each of the three groups. In the case of pre-immune sera, there was no titre in any of the groups. The titre was good after final immunisation for peptides 1 and 3, but no antibody titre was observed for peptide 2. In Fig. 5, the corresponding absorbance at 490 nm for various pre- and post-immune sera dilutions in all the groups is shown. Following the final blood draw, the antibodies were separated from the peptide 1 and peptide 3 groups, and the concentration of antibodies in various fractions is depicted in Figs. 6, 7, 8. For the peptide 1 group, the overall Ig G antibody concentration in serum was 8.005 mg/ml, whereas for the peptide 3 group, it was 10.7725 mg/ml (Figs. 9, 10).

Immunogenicity assessment of raised antibody by dot blot

The immunogenicity of the raised antibodies was assessed against the three designed peptides by dot blot. In the case of AMH 1 and AMH 3 peptides, antibodies showed typical dot blot in both 0.5 ng/ml and 5.0 ng/ml antigen concentration. A representative image of dot blot developed on X-ray film depicting the binding of AMH peptide and KLH (Keyhole limpet hemocyanin) as indicated by dark spots has been shown in Fig. 11. Antibodies were developed against KLH and bound with it as it was conjugated with all the peptides as a carrier protein. On other hand, the antibodies did not bind with BSA indicating absence of non-specific binding.

Development of a direct ELISA

A direct ELISA was developed using the HRP conjugated antibodies. bAMH of different concentrations (0.005 ng/ml

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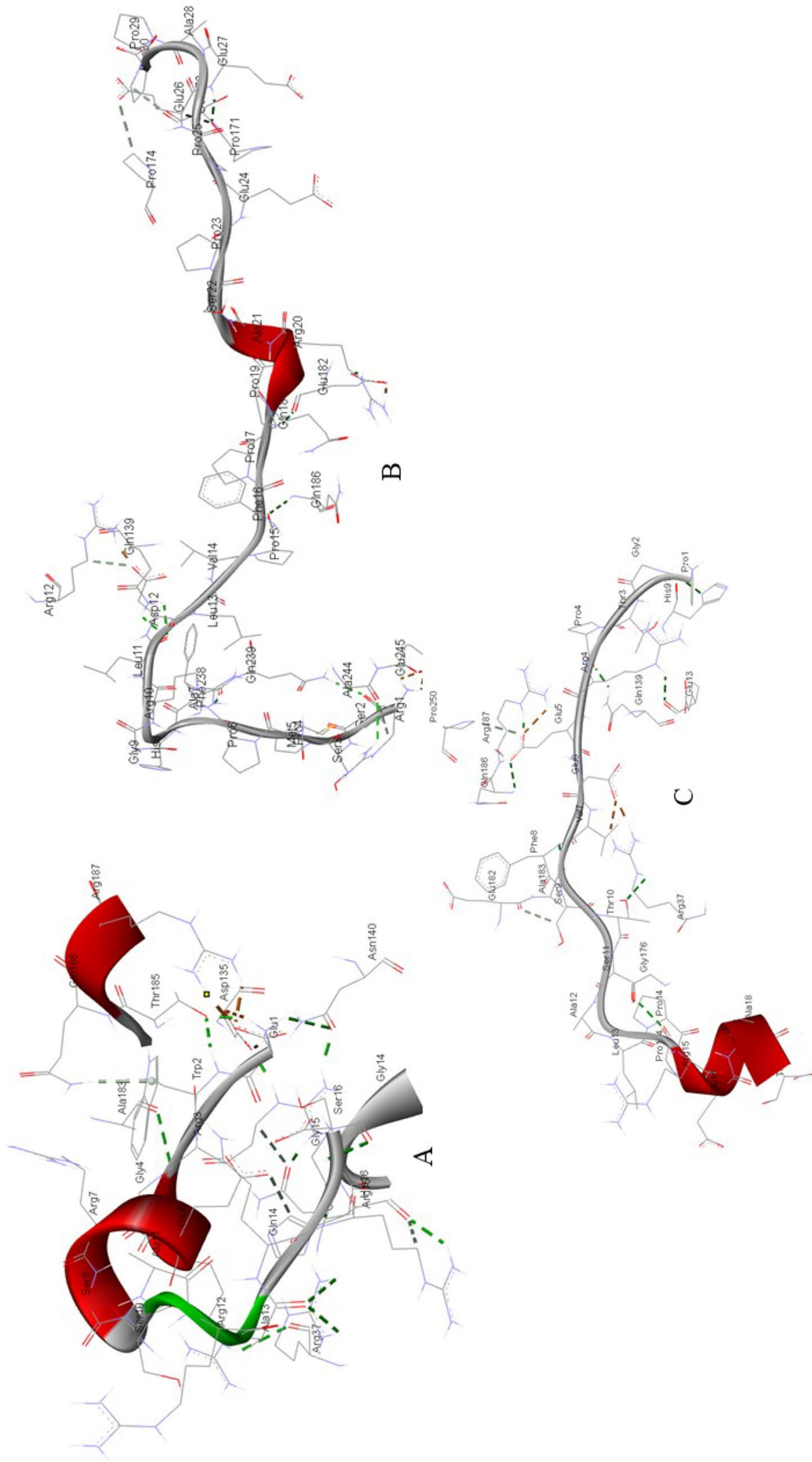

Fig. 4 Interaction of the individual peptides (**A**, **B** & **C**: Peptide-1, Peptide-2 & Peptide-3, respectively) with bovine AMH receptor

Journal : **Large 13205**	Article No : **3622**	Pages : **16**	MS Code : **3622**	Dispatch : **13-6-2023**

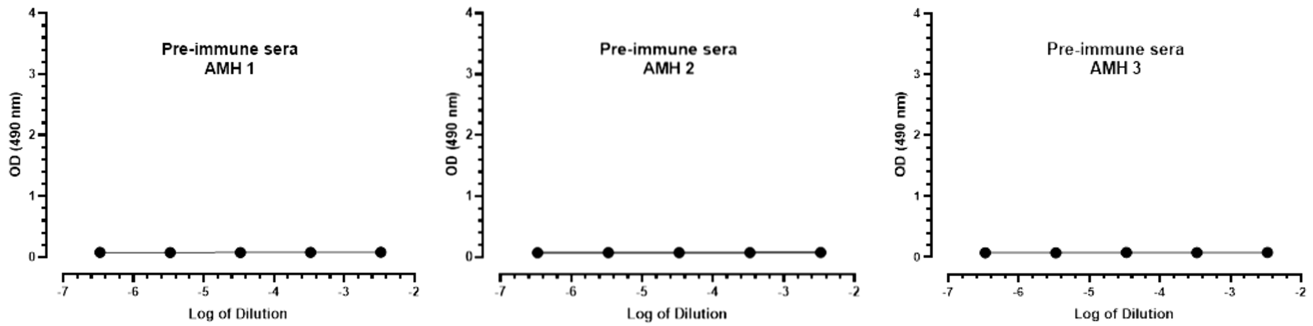

Fig. 5 Absorbance at 490 nm (Indirect ELISA) for different dilutions of pre-immune sera in AMH 1, 2 & 3 groups

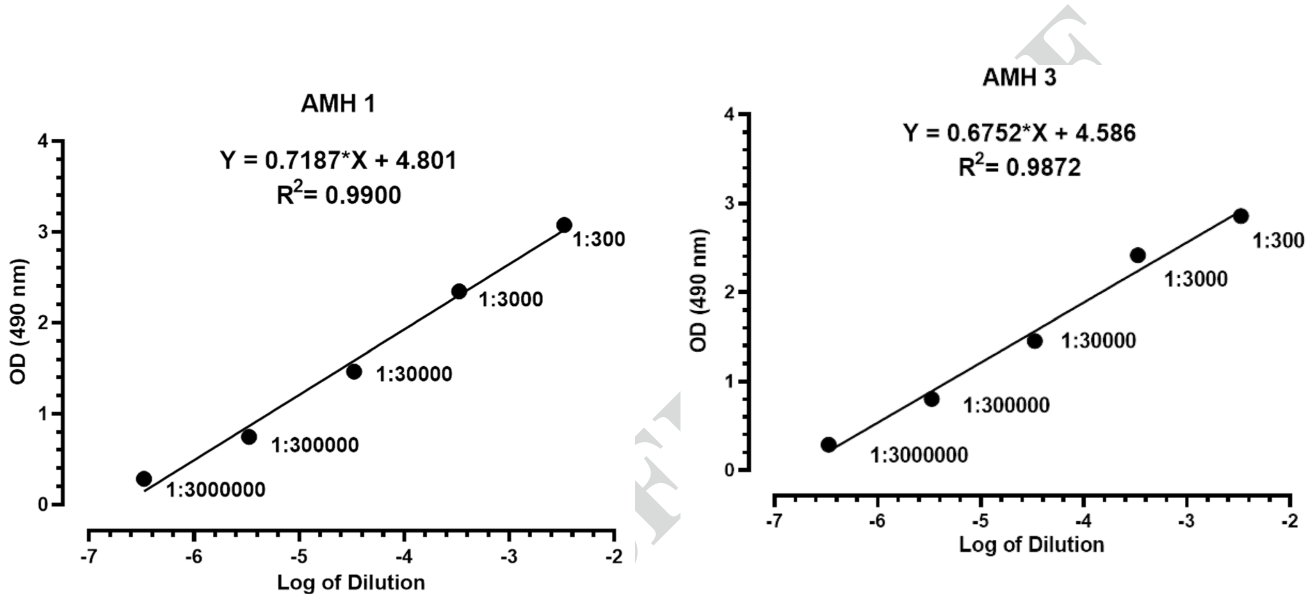

Fig. 6 Absorbance at 490 nm (Indirect ELISA) for different dilutions of sera after the final booster dose developed against AMH 1 peptide

Fig. 8 Absorbance at 490 nm (Indirect ELISA) for different dilutions of sera after the final booster dose developed against AMH 3 peptide

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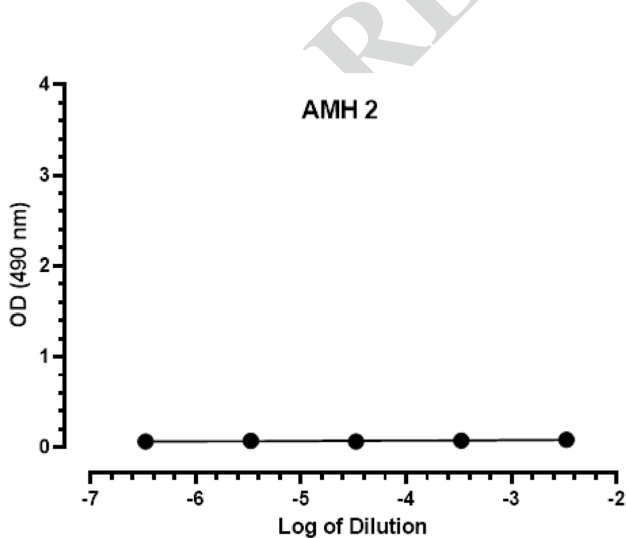

Fig. 7 Absorbance at 490 nm (Indirect ELISA) for different dilutions of sera after the final booster dose developed against AMH 2 peptide

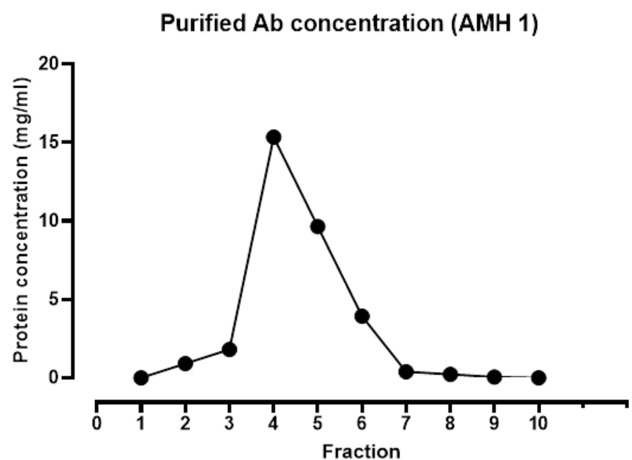

Fig. 9 Concentration of AMH 1 antibody in different fractions following purification of 3 ml sera by Protein A Antibody Purification Kit. Maximum amount of antibody was recovered in fraction 4 (15.345 mg/ml) and the overall antibody concentration in sera was 10.772 mg/ml

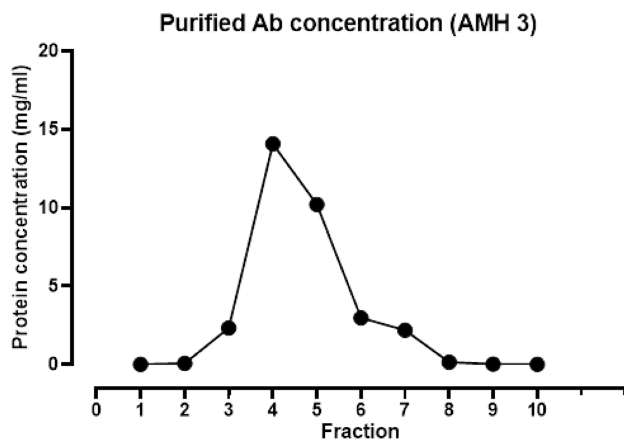

Fig. 10 Concentration of AMH 3 antibody in different fractions following purification of 4 ml sera by Protein A Antibody Purification Kit. Maximum amount of antibody was recovered in fraction 4 (14.08 mg/ml) and the overall antibody concentration in sera was 8.005 mg/ml

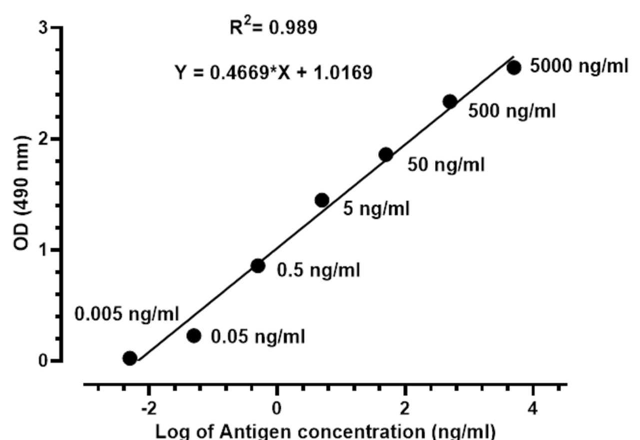

Fig. 12 Standard curve of direct ELISA depicting the OD values of different bAMH concentration (0.005 ng/ml to 5 µg/ml) when purified bAMH was used for coating and 100 ng/ml of HRP conjugated AMH 1 antibody was applied for signal generation

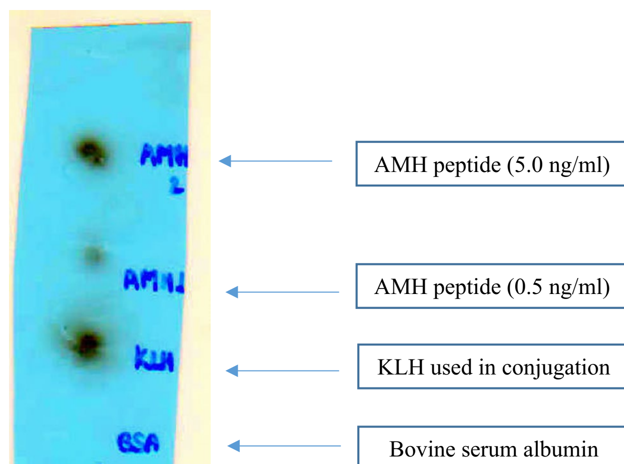

Fig. 11 Representative image of Dot ELISA developed on X-ray film depicting the binding of AMH peptide (AMH 1: 0.5 ng/ml, AMH 2: 5 ng/ml) and KLH (Keyhole limpet hemocyanin, used in conjugation of peptides) as indicated by dark spots. Developed antibody did not bind with the BSA (bovine serum albumin) indicating no non-specific binding

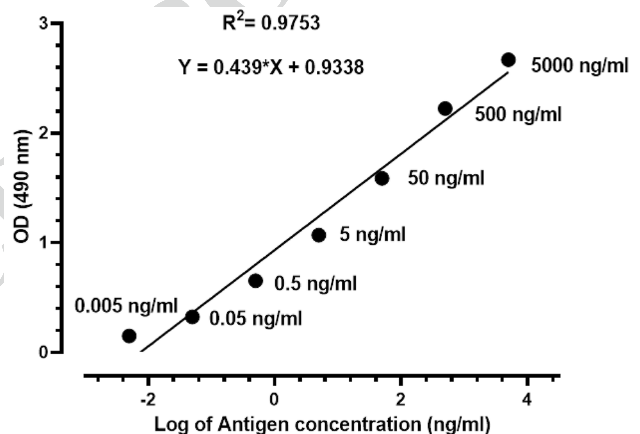

Fig. 13 Standard curve of direct ELISA depicting the OD values of different bAMH concentration (0.005 ng/ml to 5 µg/ml) when purified bAMH was used for coating and 100 ng/ml of HRP conjugated AMH 3 antibody was applied for signal generation

463 to 5 µg/ml) were used as standard and coated on the standard 464 wells. Unknown plasma samples were coated on the samples 465 wells for the detection of the level of the hormone. Conju- 466 gated antibodies were applied for signal generation. Standard 467 curve of direct ELISA depicting the OD values of differ- 468 ent bAMH concentration for AMH 1 and AMH 3 has been 469 shown in Figs. 12, 13. For both the antibodies, the absorb- 470 ance for 0.05 ng/ml AMH level was significantly different 471 from the blank wells. So, this model of ELISA can precisely 472 detect purified bAMH precisely for a large range. But, sev- 473 eral problems were recorded in direct ELISA. The bovine 474 plasma generally contains very less amount of bAMH (in ng 475 quantity) and thus coating the well with plasma is not a good 476 way for its detection. Plasma contains several other proteins 477 like albumin, globulin etc. Hence, there is always a chance 478 that a minute amount of AMH can bind with the plate and 479 it can also be covered by other proteins. Further, immobili- 480 sation of the target protein cannot yield satisfactory result. 481 Hence, we dropped the idea of direct ELISA to be used in 482 further experiments.

Development of a competitive ELISA 483

484 A competitive ELISA was successfully developed 485 using the epitope specific antibodies. A standard curve 486 with OD value (490 nm) in Y axis and $\log_{10}$ of antigen

Journal : Large 13205	Article No : 3622	Pages : 16	MS Code : 3622	Dispatch : 13-6-2023

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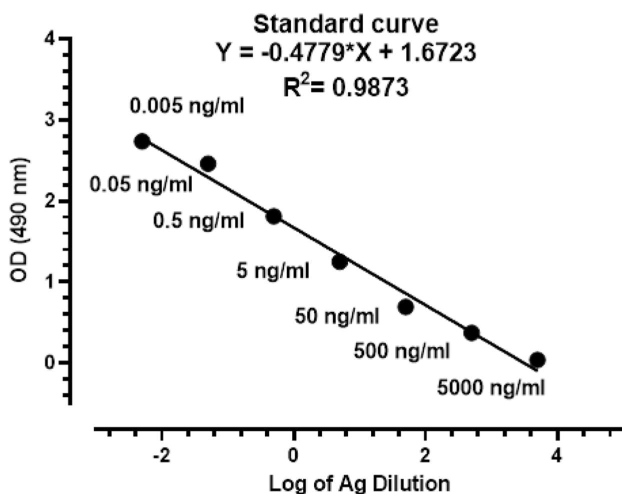

Fig. 14 Standard curve of competitive ELISA depicting the OD values of different bAMH concentration (0.005 ng/ml to 5 µg/ml) when a concentration of 5000 ng/ml bAMH was used for coating and 100 ng/ml of AMH 1 antibody was applied to bind with both antigens

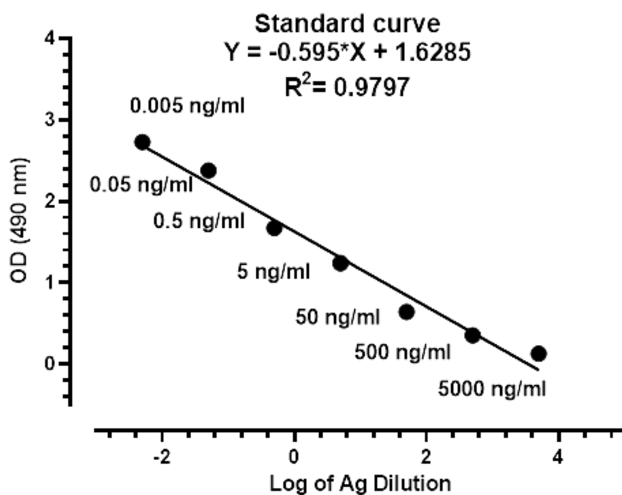

Fig. 15 Standard curve of competitive ELISA depicting the OD values of different bAMH concentration (0.005 ng/ml to 5 µg/ml) when a concentration of 5000 ng/ml bAMH was used for coating and 100 ng/ml of AMH 3 antibody was applied to bind with both antigens

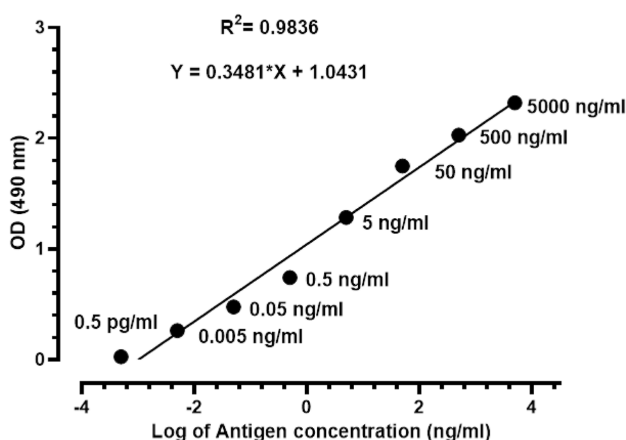

Fig. 16 Standard curve of Sandwich ELISA depicting the OD values of different bAMH concentration (0.5 pg/ml to 5 µg/ml) when 100 ng/ml unconjugated AMH 1 antibody was used as coating antibody and 100 ng/ml of HRP conjugated AMH 3 antibody was used as detection antibody

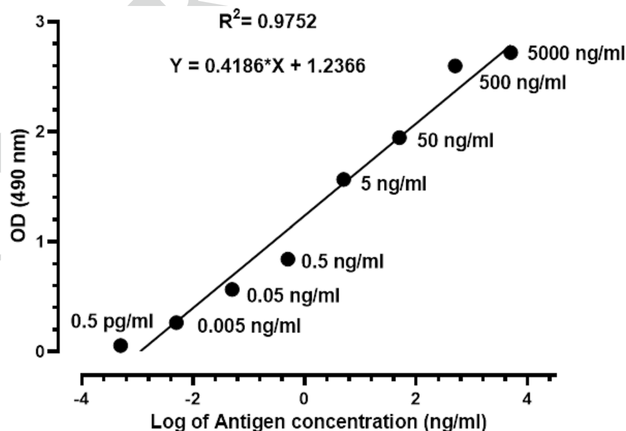

Fig. 17 Standard curve of Sandwich ELISA depicting the OD values of different bAMH concentration (0.5 pg/ml to 5 µg/ml) when 100 ng/ml unconjugated AMH 3 antibody was used as coating antibody and 100 ng/ml of HRP conjugated AMH 1 antibody was used as detection antibody

concentration (0.005 ng/ml to 5 µg/ml) in X axis was plotted (Figs. 14, 15). With the help of this standard curve the concentration of bAMH in unknown plasma samples were also calculated. The standard curve showed linear relationship between OD value and $\log_{10}$ value of antigen concentration. The equation and correlation coefficient has been displayed in the figures. The OD of 0.005 ng/ml was close to antigen blank well. It can be said that the assay can detect up to 0.05 ng/ml of bAMH from plasma samples of bovine. One of the disadvantageous points of this model is that every time we need to add a fixed amount of bAMH in all the wells. As, the hormone is costly it makes the assay expensive. Secondly, the competition between known antigen and unknown sample is happening in two different phases. Antigen with known concentration was coated on the bottom of the well whereas antigen present in unknown sample was in liquid phase. So, we preferred to use Sandwich ELISA for further analysis.

Development of a sandwich ELISA

Two sandwich ELISAs have been developed utilising four antibodies: unconjugated AMH 1, HRP conjugated AMH 1, unconjugated AMH 3 antibody, and HRP conjugated AMH

3 antibody. Unconjugated AMH 1 antibody was utilised as the coating antibody in one ELISA, and conjugated AMH 3 antibody was employed as the detection antibody. In the other, unconjugated AMH 3 antibody was utilised as the coating antibody, while conjugated AMH 3 antibody was employed as the detection antibody. Figures 16, 17 show a standard curve with OD (490 nm) at the Y axis and log10 of antigen concentration (0.5 pg/ml to 5 g/ml) at the X axis. A linear equation guided the curve. The figures show the equation and the correlation coefficient. Both ELISAs detected bAMH at concentrations of up to 0.5 pg/ml. Sandwich ELISA was the most sensitive and specific ELISA type. As a result, it was employed for further studies including the detection of bAMH from unknown materials. Both sandwich ELISA models performed equally well in terms of detection. Given that both antibodies were epitope specific, we used two distinct antibodies developed against two distinct epitopes. Monoclonal antibodies are similar to epitope specific antibodies. They will bind to the protein in two separate places. Polyclonal antibodies usually originate in two species, one in rabbit and one in rat or mouse. One can be employed as a capture antibody, the other as a detection antibody, and a secondary conjugated antibody is utilised to generate signal. We cannot employ a secondary antibody in this circumstance because it will connect with both the capture and detection antibodies because they are from the same species (rabbit). As a result, conjugating one of the antibodies, as we did with HRP, is clearly required. Kumar et al. (2010) developed a sandwich type ELISA for human AMH using two monoclonal antibodies, one of which was biotinylated for signal production. Groome et al. (2011) employed two antibodies against two epitopes and tagged the detection antibody with an enzyme such as HRP that produced colour with a substrate such as OPD, TMB, or ABTS.

Validation of assay

Specificity test

Specificity was tested by assaying samples containing known supra-physiological concentrations of the AMH-related members of growth and differentiation factors (LH and FSH) and with non-related members (BSA, progesterone). No cross-reactivity was observed in any of the conditions. Both of our antibodies were also found to be non-reactive with human AMH.

Sensitivity test

The sensitivity for both direct and competitive ELISAs was found to be nearly 0.05 ng/ml. Sandwich ELISA produced the greatest results, with a sensitivity of 5.0 pg/ml (Table 2). Sensitivity of the commercial bAMH ELISA kit was found to be 1.52 pg/ml (Bioassay Technology Laboratory, catalogue no. E0241Bo). The human AMH ELISA developed by Long et al. 2000, exhibited a sensitivity of 98 pg/ml. A sensitivity of 1960 pg/ml had previously been reported by Rey et al. (1994, 1996). The sensitivity was 0.08 ng/ml in a second-generation AMH ELISA created by Kumar et al. (2010). This illustrates how incredibly sensitive our assay is in comparison to assays that are readily available on the market.

Inter-and intra-assay coefficient of variation (precision)

Inter-assay CV denotes variation on distinct plates, whereas intra-assay CV denotes variation between various recorded quantities of a sample inside an experiment. Five samples with varying bAMH levels that included both low and high

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Table 2 Comparison of sensitivity, Intra-assay precision and Inter-assay precision among our assay and other AMH assays

AMH assay	Current assay		Long et al. (2000)		Rey et al. (1994, 1996)		Commercial kit	
Sensitivity	5.0 pg/ml		98 pg/ml		1960 pg/ml		1.52 pg/ml	
	Precision (%)	AMH (pg/ml)	Precision (%)	AMH (ng/ml)	Precision (%)	AMH (ng/ml)	Precision (%)	AMH (pg/ml)
Intra-assay precision	5.67	72.44	5.3	5.04	5.1	5.88	< 8.00	3–700
	3.12	183.11	5.1	34.3	7	44.8		
	4.94	368.24						
	3.61	522.24						
	4.27	732.25						
Inter-assay precision	8.77	79.30	8.7	4.34	9.9	5.88	< 10.00	3–700
	7.87	161.27	6.6	35.98	13.8	44.8		
	4.53	356.30						
	5.76	569.33						
	6.70	798.19						

571 values were used to compute the CV. For the concentrations
572 of 72.44, 183.11, 368.24, 522.24, and 732.25 pg/ml AMH,
573 respectively, the intra-assay CV was 5.67%, 3.12%, 4.94%,
574 3.61%, and 4.27% (Table 2). All the values combined were
575 under 6%. For levels of 79.30, 161.27, 356.30, 569.33, and
576 798.19 pg/ml AMH, respectively, the inter-assay CV was
577 8.77%, 7.87%, 4.53%, 5.76%, and 6.70%, and the overall value
578 was 9% (Table 2). For the 5.88 and 44.8 ng/ml AMH level,
579 Rey et al. (1994, 1996) previously reported 5.1% and 7.0%
580 intra-assay precision and 9.9% and 13.8% inter-assay preci-
581 sion. Long et al. (2000), detected 5.3% and 5.1% intra-assay
582 precision for 5.04 and 34.3 ng/ml AMH level and 8.7% and
583 6.6% inter-assay precision for 4.34 and 35.98 ng/ml AMH
584 level. These precisions are 8% and 10% in commercially avail-
585 able kits as well. In terms of assay precisions, it can be argued
586 that our test is either better or on par with others.

587 Recovery test

588 We combined 100 µl of plasma samples with known concen-
589 trations of bAMH (98, 188, 228, 356, and 546) with known
590 concentrations of bAMH in order to calculate the recovery
591 percentage (Table 3). Calculating the recovery % involved
592 comparing the expected and observed data. The average

Table 3 Recovery test of sandwich ELISA

Sample	AMH concentration (pg/ml)	Recovery test 1[a]			Recovery test 4[d]		
		AMH (pg/ml)		Recovery (%)	AMH (pg/ml)		Recovery (%)
		Expected	Observed		Expected	Observed	
A	98	140.9524	138.36	98.16	543.6364	540.96	99.51
B	188	226.6667	220.63	97.34	625.4545	612.31	97.90
C	228	264.7619	270.12	102.02	661.8182	646.94	97.75
D	356	386.6667	382.79	99.00	778.1818	762.85	98.03
E	546	567.619	575.36	101.36	950.9091	910.21	95.72
Mean ± SEM		99.58 ± 0.91			97.78 ± 0.60		
Sample	AMH concentration (pg/ml)	Recovery test 2[b]			Recovery test 5[e]		
		AMH (pg/ml)		Recovery (%)	AMH (pg/ml)		Recovery (%)
		Expected	Observed		Expected	Observed	
A	98	180	187.52	104.18	2474.286	2389.21	96.56
B	188	261.8182	255.32	97.52	2560	2357.4	92.09
C	228	298.1818	290.6	97.46	2598.095	2543.27	97.89
D	356	414.5455	411.97	99.38	2720	2622.98	96.43
E	546	587.2727	590.32	100.52	2900.952	2657.69	91.61
Mean ± SEM		99.81 ± 1.24			94.92 ± 1.28		
Sample	AMH concentration (pg/ml)	Recovery test 3[c]			Recovery test 6[f]		
		AMH (pg/ml)		Recovery (%)	AMH (pg/ml)		Recovery (%)
		Expected	Observed		Expected	Observed	
A	98	331.4286	330.25	99.64	4634.545	4212.99	90.90
B	188	417.1429	420.32	100.76	4716.364	4387.9	93.04
C	228	455.2381	439.25	96.49	4752.727	4121.25	86.71
D	356	577.1429	570.6	98.87	4869.091	4357.64	89.50
E	546	758.0952	730.25	96.33	5041.818	4111.22	81.54
Mean ± SEM		98.42 ± 0.87			88.34 ± 1.99		

[a] Five µl of 1000 pg/ml of bAMH were added to 100 µl of sample
[b] Ten µl of 1000 pg/ml of bAMH were added to 100 µl of sample
[c] Five µl of 5000 pg/ml of bAMH were added to 100 µl of sample
[d] Ten µl of 5000 pg/ml of bAMH were added to 100 µl of sample
[e] Five µl of 50,000 pg/ml of bAMH were added to 100 µl of sample
[f] Ten µl of 50,000 pg/ml of bAMH were added to 100 µl of sample

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recovery percentages (Mean + SEM) were determined to be 99.58 + 0.91, 99.81 + 1.24, 98.42 + 0.87, 97.78 + 0.60, 94.92 + 1.28, and 88.34 + 1.99, respectively (Table 3). As opposed to greater concentrations, recovery was evidently improved at lower values. Long et al. (2000) also noted that adding 10 µl of 140 pmol/l rhAMH, 5 µl of 1400 pmol/l rhAMH, and 10 µl of 1400 pmol/l rhAMH to known plasma samples of 14.7–25.6 pmol/l resulted in recovery percentages of 99.6–2.6, 88.2–6.3, and 86.6–6.6, respectively. In the second generation AMH assay created by Kumar et al. (2010), the recovery was 102, 106, 104, and 102%, respectively, when endogenous AMH concentrations of 0.67, 1.16, 2.21, and 1.47 ng/ml, respectively, were spiked with exogenous AMH dosages of 1.30, 2.49, and 3.57 ng/ml. We can draw the conclusion that the recovery percentage in our test was fairly comparable to other published AMH tests.

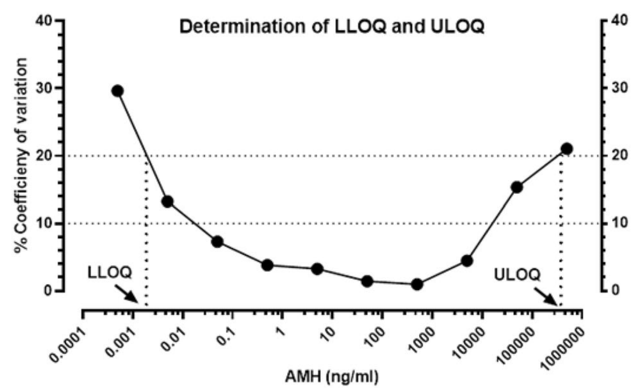

Fig. 18 Determination of LLOQ and ULOQ of sandwich ELISA plotting different concentrations of bAMH (0.5 pg-500 µg/ml) in X axis and percentage of coefficient of variation in Y axis

Lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ)

LLOQ and ULOQ was calculated to define true acceptable limit of standard curve. Differentiable OD values can be obtained for a wide range. But it is important to know the "reportable range" within which the analyte can be measured with acceptable value of precision, accuracy and error (Lee et al. 2016). Extrapolated standard curve points below or above the LLOQ and ULOQ, respectively, cannot be utilised to report sample concentrations. LLOQ and ULOQ refer to the lowest and highest concentrations of the standard curve that may be measured with acceptable precision and accuracy ("Bioanalytical Method Validation," 2018). We took samples with AMH concentration ranging from 0.5 pg/ml to 500 µg/ml. For detection of LLOQ two conditions should be met i.e. firstly, the analyte response should be five times higher compared to the blank response; secondly, the response should be reproducible and identifiable with 20% precision 80–120% of accuracy (O'Kennedy and Murphy 2017). In our study both the conditions were fulfilled at 0.005 ng/ml bAMH level (13.25% CV with more than five times OD value compared to blank) (Table 4, Fig. 18). For ULOQ, the CV exceeded 20% at 500 µg/ml. So, the true acceptable range of standard curve can be considered from 0.005 ng/ml to 50 µg/ml. For more perfection (10% precision and 90–110% accuracy) the range will be 0.05 ng/ml to 50 µg/ml (Fig. 18). The standard curve range is 0.003 ng/ml to 700 ng/ml when compared to the commercially available bAMH ELISA kit (Bioassay Technology Laboratory, catalogue no. E0241Bo). The LLOQ in assay developed by Kumar et al. (2010) was 1.16 ng/ml with 20% CV. Our assay has either similar or better range compared to other assays and can detect the bAMH very precisely within that range.

Table 4 Determination of LLOQ and ULOQ of sandwich ELISA through calculating the CV value for diferent AMH concntration (0.5 pg to 500 µg)

AMH Conc	OD1	OD2	OD3	OD4	OD5	OD6	Mean	SE	CV
500 µg	3.25	2.2047	2.368	3.647	2.57	3.48	2.91995	0.250991	21.05511
50 µg	2.357	2.7821	3.129	3.25	2.415	2.29	2.70385	0.169329	15.33994
5 µg	2.3254	2.4869	2.3758	2.281	2.4681	2.568	2.417533	0.044302	4.48878
500 ng	2.0892	2.0884	2.0768	2.1025	2.1147	2.056	2.087933	0.008315	0.975481
50 ng	1.8024	1.8047	1.81	1.7652	1.821	1.8452	1.808083	0.010694	1.448771
5 ng	1.305	1.3425	1.3758	1.2906	1.3204	1.405	1.339883	0.017853	3.263847
0.5 ng	0.7857	0.7752	0.8368	0.7548	0.8067	0.8214	0.796767	0.012459	3.830309
0.05 ng	0.5068	0.5536	0.4978	0.5421	0.523	0.4487	0.512	0.015268	7.304343
0.005 ng	0.3561	0.2785	0.3256	0.3896	0.2877	0.3025	0.323333	0.017497	13.25492
0.5 pg	0.122	0.0625	0.0625	0.092	0.0625	0.0954	0.082817	0.010027	29.65754

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Journal : Large 13205 | Article No : 3622 | Pages : 16 | MS Code : 3622 | Dispatch : 13-6-2023

644 ## Conclusion

645 AMH has the potential to be employed as a biomarker of
646 fertility for all domestic animals, including cattle in particu-
647 lar. The production of antibodies against this protein can be
648 employed for both diagnostics and the selection of animals
649 depending on their AMH levels. However, the likelihood of
650 cross-reactivity is increased by polyclonal antibodies, and
651 monoclonal antibodies are expensive and challenging to
652 produce. The production of epitope-specific antibodies can
653 resolve both issues. In order to combat bAMH, we first cre-
654 ated epitope-specific antibodies. As indirect ELISA and Dot
655 blot demonstrated, it could bind with the peptides. The same
656 was used to create ELISA models, and Sandwich ELISA was
657 discovered to be the most sensitive and effective of all the
658 available kinds. This can be applied to the selection of farm
659 animals based on their capacity for reproduction at any point
660 of their lifespan and the detection of bAMH from unidenti-
661 fied samples.

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666 port and the necessary facilities for this study. Graphical abstract was
667 created in BioRender.com.

668 **Data availability** Data will be provided on reasonable basis.

669 ## Declarations

670 **Conflict of interest** There are no conflicts to declare. The authors de-
671 clare that they have no conflict of interest and no competing finan-
672 cial interest. An Indian patent entitled "Peptide sequences and epitope
673 specific antibodies for detection of bovine Anti-Mullerian hormone
674 (bAMH)" has been filed with application number 202111038528.

675 **Ethical approval** Ethical approval was taken from Institutional Animal
676 Ethics Committee (IAEC), National Dairy Research Institute, Karnal,
677 Haryana, India during its 45th meeting on 21/12/2019 having serial no
678 45-IAEC-19–23. All the methods were performed following necessary
679 guidelines and regulations.

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