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Expression and characterization of the Mx2 protein in a heterologous expression system as a biomarker for early pregnancy detection in buffalo

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ABSTRACT

The world's largest producer of milk is India. This significant accomplishment is solely made possible by the existence of the *Bubalus bubalis* (Buffalo) species. The primary concern with buffaloes is the lack of early pregnancy detection, which is directly related to their high milk production. Thus, in the current investigation one of the conceptus-induced proteins, myxovirus resistance 2 (Mx2), which is produced between days 18 and 21 of pregnancy, was expressed and characterized in a heterologous protein production system. Partial Mx2 was markedly amplified in pregnant animals by conventional PCR. The suitability of the protein as a possible molecule for early pregnancy detection kits was determined using *in silico* analysis. The nature of the Mx2 protein under *in vitro* conditions was anticipated on the basis of its physiochemical properties, which are necessary for the formulation of any assay. Mx2 is highly antigenic in nature and can be exploited to produce antibodies, according to immunogenic characteristics. Using coding area primers, the partial Mx2 protein was extracted from a pregnant buffalo (n=30), amplified, and subsequently cloned and inserted into *Escherichia coli* BL21 (DE3). The 21 kDa band observed by western blotting showed that the protein is antigenic in nature. In conclusion, the expression and molecular characterization of partial Mx2 protein can be done in heterologous expression system. This Mx2 protein in buffalo may serve as a reliable marker of conceptus implantation, and can be incorporated into diagnostic tests for early pregnancy.

Key words: buffalo; pregnancy detection; Mx2 protein; expression; *E coli.*

Introduction

In India the buffalo population contributes significantly to the country's economy, providing 50% of the buffalo milk produced worldwide (SUBBANNA et al., 2021). However, buffaloes' reproductive efficiency is significantly reduced despite high production due to several inherent reproductive issues, including silent heat, delayed puberty, a protracted postpartum anestrous period, and a protracted intercalving period. Consequently, early gestational

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pregnancy identification may improve reproductive efficiency. In buffaloes, almost 30–40% of early embryonic mortality (EEM) occurs 15–18 days after conception (CAMPANILE et al., 2016), leading to reproductive failure and substantial financial losses. Therefore, a reliable technique to diagnose early pregnancy is needed for identifying animals that are losing embryos. Although several diagnostic techniques, such as transrectal ultrasonography and perrectal palpation, have been developed to date, none can be considered the gold standard for reliably diagnosing pregnancy in the field. Hence, the identification of signaling molecules that play a role in conceptus-maternal communication in the early stages of pregnancy is crucial.

The initial events that occur during the blastocyst implantation phase are vital for the establishment of pregnancy. The growth of blastocysts is dependent on the histotrophic secretion of the uterus during the peri-implantation stage (SÁNCHEZ et al., 2018). These secretions consist of a variety of nutrients, enzymes, growth factors, hormones, and transport proteins that are regulated by embryo–maternal cross-talk (SPENCER and BAZER, 2004).

In early studies aimed at identifying pregnancy in animals, two proteins: pregnancy-specific protein A (PSP-A) and pregnancy-specific protein B (PSP-B) were extracted from bovine placental tissue using immunoelectrophoresis, marking the first identification of detectable pregnancy-specific (PSP) or pregnancy-associated glycoproteins (PAGs) in maternal circulation (BUTTLER et al., 1982). PSP-A, an α-fetoprotein not exclusively associated with pregnancy, has a molecular weight of 65–70 kDa and an isoelectric point (pI) ranging from 4.6 to 4.8. In contrast, PSP-B, a more pregnancy-specific protein, has a molecular weight of 47–53 kDa and a pI of 4.0–4.4. Subsequently, a distinct pregnancy-associated glycoprotein with a molecular weight of 67 kDa and four isoelectric variants (pI=4.4; 4.6; 5.2 and 5.4) was isolated. This protein differed from other placental proteins by its sialic acid content (SASSER et al., 1986) and was later identified as bPAG-1, the predominant immunoreactive form. PAGs have been found in the maternal blood from days 28 to 30 following artificial insemination in cows (ZOLI et al., 1991). In pregnant animals, PAG levels gradually increase from weeks six to thirty-five and then continue to rise even faster from weeks thirty-five until the final weeks of gestation.

In addition to these molecules, Interferon tau (IFN-t) plays a crucial role in maternal recognition of pregnancy. IFN-t is one of the earliest molecules implicated in the ruminant early maternal recognition process (KOWALCZYK et al., 2021). At approximately 16–25 days in buffalo, the trophectodermal cells of the blastocysts release this protein, and its concentration increases with conceptus elongation. Due to its antiviral, antiproliferative, and immunomodulatory characteristics, this interferon regulates both the luteotropic and immune system activity essential for successful implantation of the embryo (KOWALCZYK et al., 2021). Moreover, IFN-τ induces temporal changes in peripheral and local tissues during its release (BAZER, 2013; RUHMANN et al., 2017). By suppressing the release of prostaglandin F2α release in the uterus, it prevents luteolysis and preserves corpus luteum function. Indeed, in various cell types, including endometrial, luteal, and peripheral blood cells, IFNt stimulates the expression of interferon-stimulated genes (ISGs), such as interferon-stimulated gene 15 ubiquitin-like modifier (ISG 15), Myxovirus resistance 2 (Mx2), and 2′,5′-oligoadenylate synthetase 1 (OAS1).

Interferon tau (IFN-τ) interacts with type I interferon receptors (IFNAR1 and IFNAR2) located on uterine epithelial cells and peripheral immune cells, initiating the JAK-STAT signaling cascade. This pathway leads to the activation of STAT1 and STAT2, which subsequently form a complex with IRF9, known as interferon-stimulated gene factor 3 (ISGF3) (IVASHKIV and DONLIN, 2014). Once formed, ISGF3 translocates to the nucleus, where it binds to interferon-stimulated response elements (ISREs) in the promoter regions of various interferon-stimulated genes (ISGs), including Mx2, thereby enhancing their transcription. The Mx2 gene not only contributes to pregnancy maintenance by protecting against viral infections but also counters luteolytic signals. The Mx2 protein is particularly important for pregnancy detection because it is significantly upregulated during the early stages of pregnancy, between days 18 and 21 (THAKUR et al., 2017, BATRA et al., 2019). This gene is significantly up-

regulated during pregnancy in related species, such as cows, gilts, and mares, suggesting its potential use in accurate pregnancy testing (KHAN et al., 2023). Intrauterine injections of interferon tau (IFN-t) have been shown in a number of trials to stimulate the expression of the Mx2 gene in cow peripheral blood leucocytes (MATSUYAMA et al., 2012). According to microarray and qPCR analyses, Mx2 is among 72 genes in peripheral blood leukocytes expressed at significantly higher levels on days 14 and 21 of gestation compared to day zero (KIZAKI et al., 2013). Now a days, numerous miRNAs and urinary metabolites have been discovered that can easily enter the peripheral circulation of dams during the early stages of pregnancy (SARANGI et al., 2022; BATRA et al., 2024). However, their role in early pregnancy remains unclear. Therefore, to develop Mx2 protein based assays for early pregnancy diagnosis, the present study aimed to produce a partial recombinant protein using a heterologous expression system. These assays may aid in detecting pregnancy during the early stages of conceptus implantation.

Materials and methods

Sample collection. A total of 50 healthy buffaloes of varying ages and parity from the Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana farm were included in this study. The animals were divided into two groups: Group I consisted of 45 artificially inseminated buffaloes, while Group II comprised 5 non-inseminated animals that served as controls. Blood samples were collected from both groups on days 18 post-artificial insemination (AI) by jugular venipuncture. On day 35 following AI, all the animals were checked by Ultrasound (USG) for pregnancy using an ultrasonography machine (Toshiba, Japan).

RNA isolation. The blood samples from the pregnant animals were treated with TRIzol Reagent (Thermoscientific, Waltham, Massachusetts, USA) to extract total RNA. Briefly, 600 µl of TRIzol reagent was combined with 400 µl of blood and vortexed homogeneously. To separate the aqueous phase from the organic phase, chloroform was used. The upper aqueous phase was precipitated by adding 500 µl of isopropanol and incubating overnight at -20°C. The RNA was pelleted at 12000 rpm for 20 minutes, followed by washing with 70% ethanol. The pellet was once again suspended in 20 µl of water free of nucleases. The RNA was immediately reverse transcribed to cDNA.

Reverse transcription. Total RNA was reverse transcribed using a Revert aid cDNA Synthesis Kit (Thermoscientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The reverse transcriptase PCR cycling conditions were as follows: initial incubation at 25°C for 5 min, reverse transcription at 42°C for 1 hour, and deactivation at 70°C for 5 min.

Primer design and selection of partial proteins. The primers used were designed with the gene sequence of accession no. NM_001291322.1 (buffalo). These primers were mutated at the 5' end for specific recognition by the *Nhe*I restriction enzyme and at the 3' end for specific recognition by the H*ind*III restriction enzyme. (F-5' CAGGCTAGCATGTCGCTGTCCTTCAGGCCTTTAAAG-3' and R-5' CCAAAGCTTGCTATGATGTTCTGGGCTCTAC-3').

In silico prediction of partial Mx2. The main structure of the partial Mx2 protein was predicted using the Swiss Prot modeler, which provides a list of the QMEAN scores of several models as well as the global model quality estimate. The Self-Optimized Prediction Method with Alignment (SOPMA) tool was utilized to predict the secondary structure of the Mx2 protein (SAPAY et al., 2006). Protein Homology Recognition Engine V 2.0 (PHYRE) (KELLEY et al., 2015) was used to predict the secondary structure graphically after PSI-BLAST was used to compare the Mx2 protein sequence to a sizable sequence database. By comparing the sequences with the regular expressions specified in ELM, the Eukaryotic linear Motif (ELM) prediction tool was used to predict motifs in the Mx2 protein sequence. This software distinguishes between matches that correspond to hypothetical motifs on the basis of the sequence and matches that are related to experimentally validated motifs in the ELM database. Using the Prot-Param (SAHAY et al., 2020) prediction server of ExPASy, the physicochemical properties were computed. Several characteristics of the Mx2 protein, including its molecular weight, theoretical isoelectric point

(pI), total number of positive and negative residues, extinction coefficient, instability index, aliphatic index, and grand average hydropathy (GRAVY), were predicted. The NetPhos server application was used to forecast several phosphorylation locations in Mx2 that have the ability to bind phosphate groups (JIA et al., 2021). There are three possible types of glycosylation: c-linked, which primarily occurs at tryptophan residues; O-linked, which occurs at serine or threonine residues; and N-linked, which occurs at asparagine residues. To forecast disulfide bridges, the CYS_REC tool (http://www.softberry.com/cgi-bin/programs/propt/cys_rec) was used. This tool identified the locations of cysteines and calculated the most likely S–S bond pattern of the pairings in the Mx2 protein sequence. The existing epitopes of the Mx2 protein were assessed via databases of B-cell epitopes. The antigenicity of the protein due to its linear and conformational epitopes was predicted using IEDB analysis software (LIU et al., 2020) by providing the Mx2 sequence.

Conventional PCR and gel purification. PCR was carried out in a 25 µl reaction using a high-fidelity Phusion Master Mix (2x concentration) with HF buffer (NEB, Ipswich, MA, USA), 5 µl of template cDNA, and 0.4 µM forward and reverse primers in a Veriti Applied Biosystems thermal cycler. The PCR cycle consisted of three minutes of initial denaturation at 94°C, forty cycles of denaturation at 94°C for thirty seconds, annealing at 55°C for forty-five seconds, two minutes of elongation at 72°C, and finally ten minutes of final elongation at 72°C. Using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) the PCR product was purified and then eluted in elution buffer.

Cloning and transformation. The pET28a vector and the Mx2 PCR product were subjected to two fold digestion with *Nhe*I restriction enzyme and *Hind*III restriction enzyme followed by ligation with T4 Dna Ligase. First, the ligated product was transformed into the *E. coli* DH5alpha strain for cloning, and then it was transformed into the BL21(DE3) strain for further expression. In brief, 5 µl (5 ng) of plasmid DNA was added, and each vial of BL21 Star (DE3) cells was incubated on ice for 30 minutes. Furthermore, a 2-minute heat shock without shaking at 42°C was given. The cells were immediately placed on ice, and then 250 µl of room temperature Luria broth was added. The mixture was shaken for thirty minutes at 200 rpm at 37°C. A full transformation reaction mixture was added to a Luria agar plate that contained kanamycin. Several colonies were multiplied and kept in an incubator shaker at 200 rpm and 37°C. Recombinant clones were chosen via the kanamycin resistance gene. Following the manufacturer's directions, the plasmids were separated from positive clones using a plasmid isolation kit (Invitrogen, Waltham, Massachusetts, USA).

*Expression of MX2.*To express clones with a 6xHis tag, 5 mL of LB/Amp (100 µg/mL) broth was inoculated with 50 µl (1:100) of each positive LB broth in two 50 mL sterile tubes. After that, the tubes were incubated overnight at 37°C in a shaking incubator. The next day, 100 mL of LB/Amp broth was mixed with 2 mL (1:50) of the culture that had grown overnight. The culture was cultivated for a total of 0.4–0.6 optical density values A_{600} at 37°C while being shaken. Following induction, samples were collected at 0, 2, 4, 6, 12, 24, and 36 hours. Various IPTG concentrations (0.25; 0.50; 0.75; 1.0 and 1.5 mM) were used for induction. SDS–PAGE was performed to determine the expression kinetics and purification of the expressed recombinant protein.

Preparation and purification of samples under conditions. Urea and guanidinium buffers were combined to standardize the process of separating the proteins under the denaturing conditions. The cell pellet was resuspended at a rate of 2–3 mL per gram wet weight in guanidinium lysis buffer, pH 7.8 (6 M guanidine hydrochloride, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl, and 10 mM 2-mercaptoethanol). The pellet was incubated in a shaker incubator for 30 minutes at 37°C to ensure complete cell lysis. The cell lysate was subjected to seven cycles of strong sonications for 30 seconds while it was kept on ice (80A).The lysate was centrifuged again for 15 minutes at 3000×g to eliminate the cell debris. To remove the cell debris, the lysate was centrifuged once more for 15 minutes at 3000×g. Simultaneously, 0.5 mL of the bed volume of the Ni-NTA column (Qiagen Hilden, Germany) was equilibrated with 10 mL of urea lysis buffer pH 7.8 (8 M urea; 20 mM sodium phosphate pH 7.8; 500 mM NaCl) buffer, and the supernatant was discarded. The lysate was also added

to a nickel-NTA column, and the flow-through was collected. Two different urea wash buffer volumes (five bed volumes or 2.5 milliliters) were used to wash the column: pH 5.5 and pH 6.4 (8 M urea, 20 mM sodium phosphate, and 500 mM NaCl). The eluate was collected with buffer pH 4.0 and analyzed by SDS–PAGE.

Western blotting. The extracted Mx2 protein was electroblotted using a mouse anti-His horse radish peroxidase (HRP)-labeled antibody (Invitrogen, Waltham, Massachusetts, USA).In brief, the isolated protein was blotted onto a PVDF membrane after being subjected to SDS–PAGE on a 15% resolving gel. This mixture was blocked for one hour at room temperature in a shaking incubator with 3% bovine serum albumin (BSA)-PBS-Tween 20 blocking solution, after being washed for fifteen minutes three times in PBS-Tween 20 wash buffer. The membrane was subjected to incubation with an anti-His HRP-labeled antibody (1:2000) for one hour. After cleaning, the membrane was developed in a 30% DAB/H_2O_2 substrate solution.

Statistical analysis. Data obtained from PCR for expression value of the Mx2 transcript and ultrasound results were analyzed using the Chi square test in graph pad prism software. Differences among means were analyzed using the Least Significant Difference (LSD) method, with statistical significance set at ($P<0.05$).

Result

Amplification and cloning of partial MX2. The partial Mx2 gene produced an amplicon size of 599 bp [along the initial flanking ends of 18 bp containing sites for restriction enzymes (*Nhe*I and *Hin*dIII restriction)] via RT–PCR. The amplification was seen in thirty animals. This amplified product was successfully cloned, inserted into the pET28a vector and maintained in the *E.coli*-DH5alpha host system**.** The inserted plasmids corresponded to the expected size of 599 bp with a gene-specific primer for partial Mx2and 599+297 bp with a vector-specific primer in a 1.5% agarose gel. (Fig. 1).

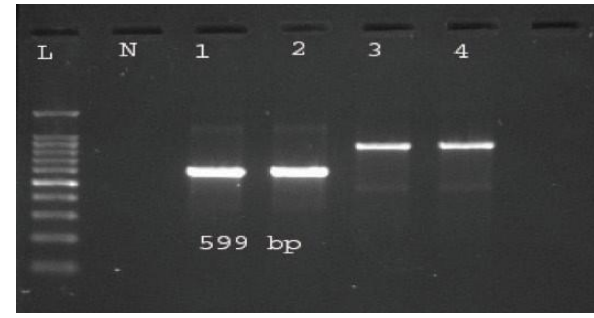


Fig. 1. Agarose gel electrophoresis of PCR products generated using the partial MX2 ORF primer pair

[Lane 1: Negative control Lane 2, 3: Positive Plasmid with Gene F, R (599 bp). Lanes 4--5: Positive plasmids with vectors T7 F and T7R (599+297 bp)

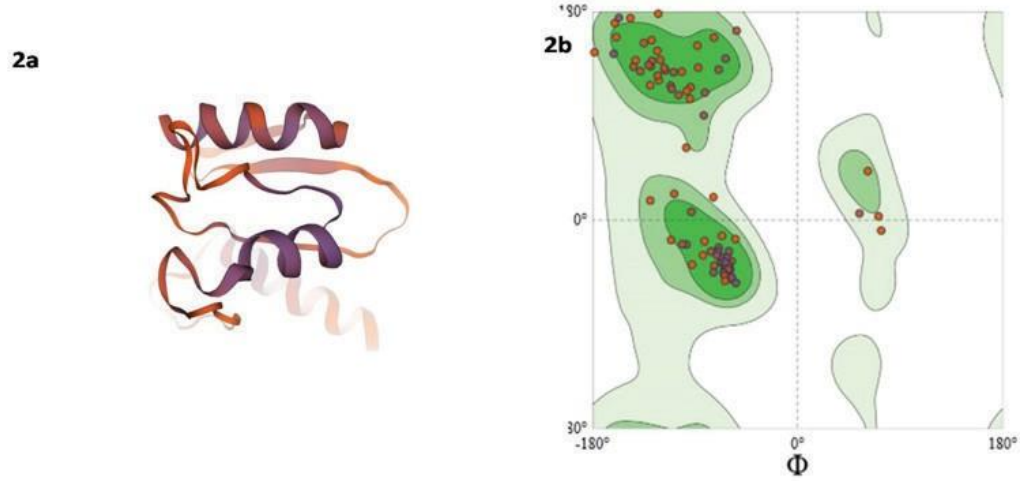
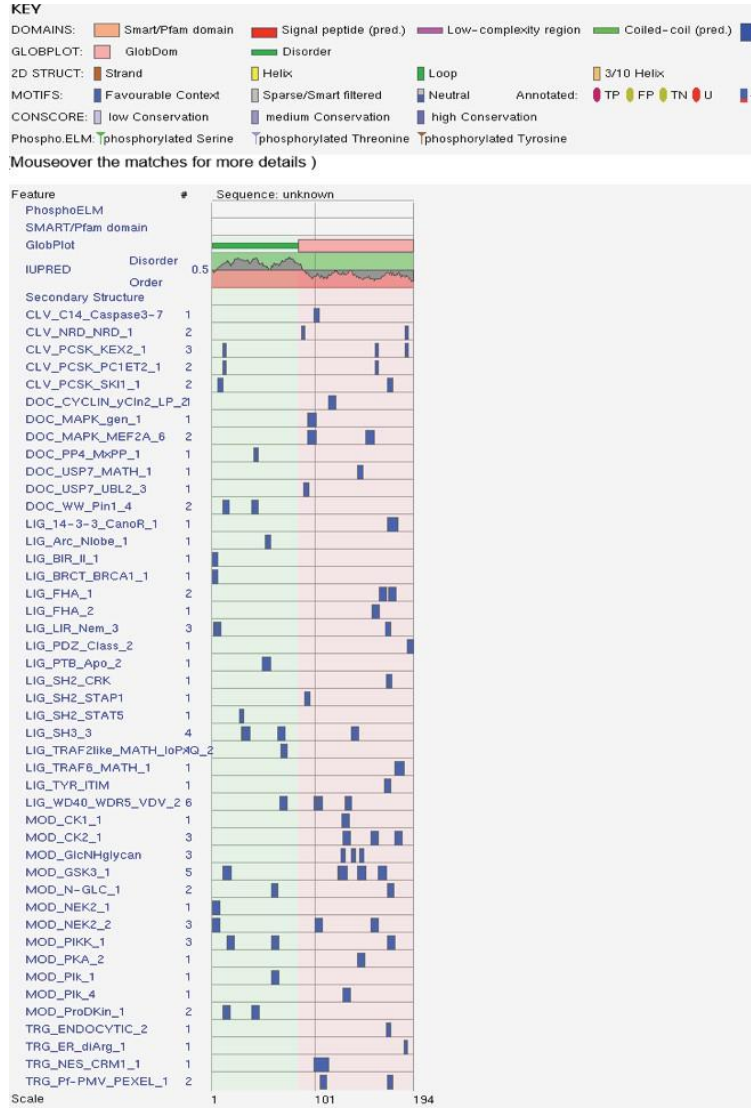


Fig 2. a) Swiss pdb view of the Mx2 model generated after energy minimization; b) Ramachandran plot of Mx2 generated using the SwissProt model

In silico characterization of partial MX2. The Swiss Prot Modeler produced only three models, each with a GMQE value of 0.28 and a Q mean value of 0.53±0.08. Fig. 2a shows the graphical sequence that was created by the Swiss Modeler. A Ramachandran plot was created from the Rampage site to ascertain the stereochemical features of the Mx2 protein (Fig. 2b). Procheck and Verify 3D, a well-known protein structure checking program, was then used to examine the structure. A total of 93% of the residues had phi/psi angles that fell in the most preferred regions, 2.97% had phi/psi angles that fell in additional authorized regions, 2.25% had phi/psi angles that fell in generously allowed regions, and 1% had phi/psi angles that fell in banned conformations.

For the homology-modeled structure, the total Verify 3D value was -0.02. This score suggests that the modeled structure of the Mx2 protein is appropriate. In the self-optimized prediction approach, the secondary structure characteristics revealed that random coils (73.20%) dominated the secondary structure elements, followed by alpha helices (21.65%) and extended strands (5.15%). ELM (Eukaryotic linear Motif) revealed that the protein included a motif that could be identified by a distinct amino acid sequence (Supplementary fig.1). The

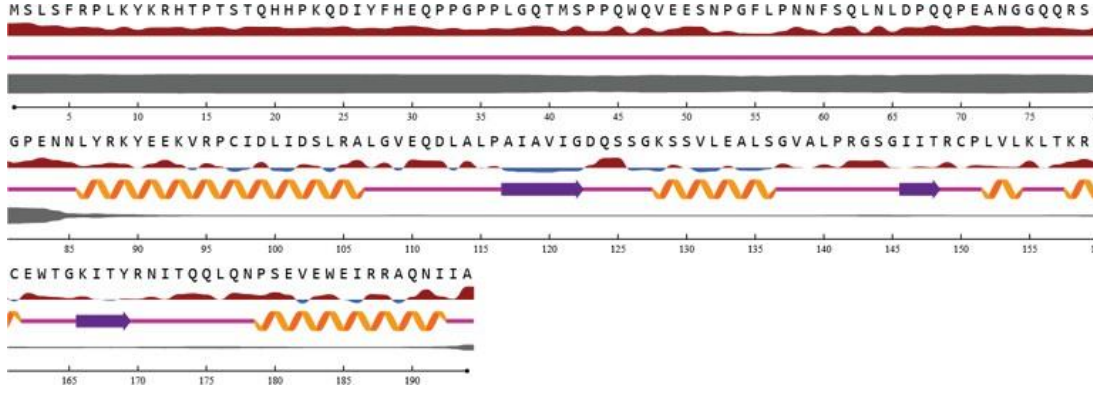


Supplementary Fig 1. Graph depicting various ELM motifs present in the partial Mx2 sequence

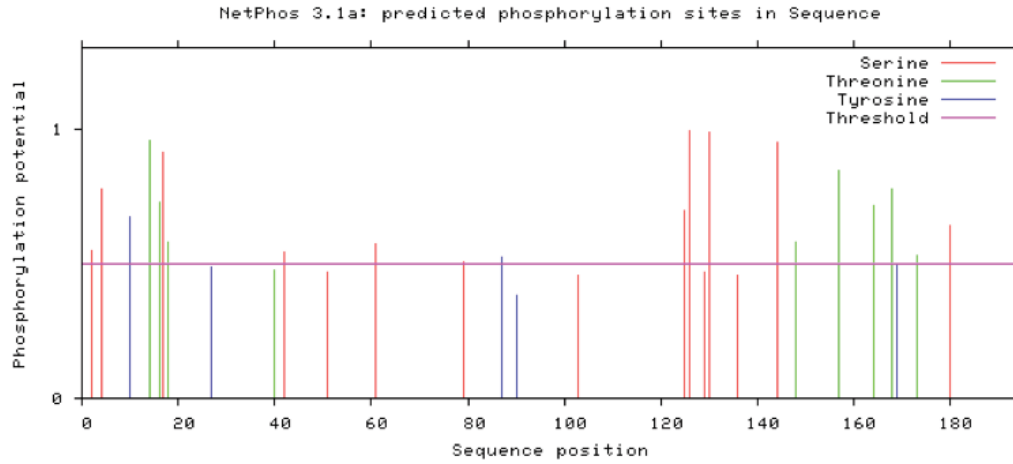
majority of proteins that are GTPase-binding proteins are functionally characterized by these motifs. According to NetSurfP, the Mx2 protein contains both exposed and buried amino acid residues, indicating that transmembrane regions are present in this protein (Supplementary fig.2). The RSA (relative surface accessibility) value ranges from 0.012 to 0.994. The detailed output of this prediction is not shown here (available from the authors).

Physiochemical properties of the protein. The Mx2 protein was predicted to have an average molecular weight of 21747.65 daltons. Mx2's calculated isoelectric point (pI) value of 8.44 indicated that it was naturally basic. This shows that at pH 8.44, Mx2 is compact and stable, and the protein surface charge is zero. Asp + Glu accounted for 20 of the total negatively charged residues in Mx2, whereas Arg + Lys made up the remaining 22 positively charged residues. The Mx2 molecule consists of 3057 atoms with the atomic composition $C_{960}H_{1525}N_{277}O_{290}S_5$. The extinction coefficient (EC) of Mx2 for a wavelength of 280 nm in water was calculated using ExPASy's ProtParam. With all pairs of Cys residues becoming cystines, the EC value ranges from 24075 Abs 0.1% (=1 g/l) 1.107 to 23950 Abs 0.1% (=1 g/l) 1.101, assuming that all Cys residues are reduced. Cys, Trp, and Tyr were present in high concentrations, as shown by the high EC value in Mx2. The Mx2 protein in question had an instability score of 61.15, indicating that it was unstable under *in vitro* conditions. For Mx2, the aliphatic index was 78.81. By summing the hydropathy values of each amino acid residue and dividing by the total number of residues or sequence length, the GRAVY value of the Mx2 protein was determined. Mx2 has a negative value of -0.708, which suggests that it is more hydrophilic. The estimated half-life of the Mx2 protein was 30 hours (mammalian reticulocytes, *in vitro*), >20 hours (yeast, *in vivo*) and >10 hours (*E. coli, in vivo*). This predicted that the time taken by the Mx2 protein would decrease after its synthesis in the cell.

Prediction of phosphorylation sites, glycosylation sites and immunogenic sites. The Mx2 protein phosphorylation sites in the threonine, serine, and tyrosine residues were predicted by the NetPhos server. There are a total of 21 sites that can be phosphorylated, including 11 serine residues, 8 threonine residues, and 2 tyrosine residues (Supplementary fig.3). Net Glyco anticipated that the Mx2 protein might be glycosylated at several locations. Three possible N-linked glycosylation sites and ten possible O-linked glycosylation sites were discovered. Three cysteine residues were identified by the CYS_REC method in the Mx2 molecule at positions 97, 150, and 161. However, no residue of this kind can create a disulfide bond. The molecular understanding of the biorecognition and antigen–antibody complex building process by the Mx2 protein is enhanced through B-cell



Supplementary Fig 2. Graphical representation of 194 residue predictions across 1 sequence using Net Surf P
Relative surface accessibility: Red is exposed and blue is buried, thresholded at 25%
Secondary structure: Helix, Strand, Coil
Disorder: Thickness of line equals probability of disordered residue



Supplementary Fig 3. Graph showing different sites of phosphorylation in partial Mx2

and T-cell epitope mapping and prediction. B-cell epitope prediction via IEDB analysis revealed 7 potential immunogenic sites in Mx2 that can act as antigens, and seven sites can act as T-cell epitopes. Additionally, many sites can act as conformational epitopes for B cells.

SDS–PAGE of expressed protein. The 21 kDa dark band generated by various partial Mx2 clones indicates the partial overexpression of Mx2. The best conditions for achieving the highest level of protein expression were 1 mM IPTG and 12 hours of incubation following induction (Fig 3a). After expression, the location of the expressed protein within the cell was determined by repeatedly freezing and thawing the protein. Even after freeze–thaw cycles, SDS–PAGE revealed that there was more protein in the pellet than in the supernatant, indicating that the protein remained inside the cells and in their inclusion bodies. Denaturing conditions were therefore used to purify the protein. The protein was isolated via sonication and denaturing procedures, which included lysis with 6M guanidinium hydrochloride. Protein was released from the inclusion bodies following seven sonication cycles in

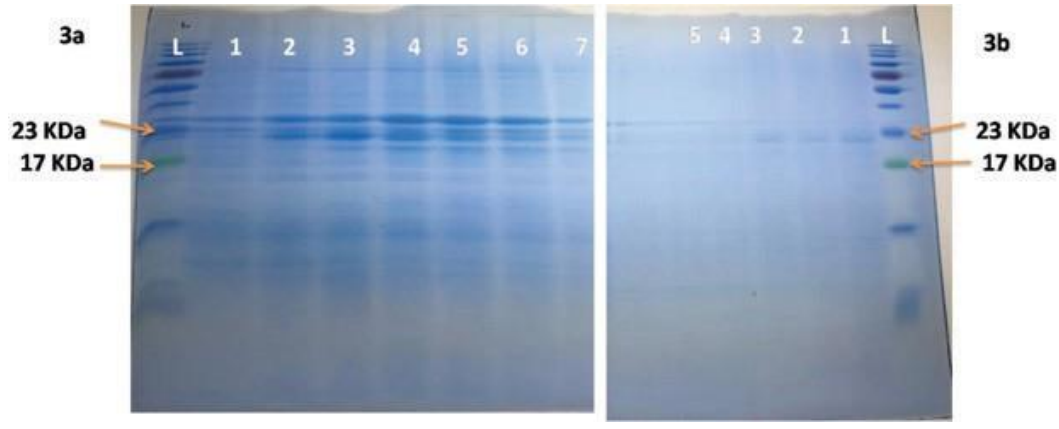


Fig. 3. a) SDS–PAGE analysis of MX2 at different time intervals at 37°C.
(Lane 0, N:Uninduced culture. Lane 1: 2 h after induction. Lane 2: 4 h after induction. Lane 3: 12 h after induction. Lane 4: 24 hrs after induction. Lane 5: 36 hrs after induction); b) SDS–PAGE analysis of purified MX2 protein after dialysis and staining with Coomassie R250 blue (Lanes 1--4: Protein after dialysis)

a 6M guanidine hydrochloride solution. Since the protein was expressed with an N-terminal His tag, it was purified by passing it through a nickel NTA column. The various eluates from the column were then checked by SDS–PAGE. Every stage was completed under denaturing circumstances. SDS–PAGE revealed a single band of approximately 21 kDa, indicating its purity (Fig 3b).

Immunoblotting with Mx2 anti-His antibody. The protein that had been purified was still denatured; therefore, it was dialyzed against increasing urea concentrations before being dissolved in PBS buffer at pH 6.0, which was significantly greater than its isoelectric point of pH 8.4. The protein would not be charged and would remain insoluble if the pH of the solvent was the same as the pI value. Therefore, a pH of 6.0 was appropriate for preserving protein solubility during renaturation. Using an anti-His antibody, the reactivity of the resolved partial Mx2 protein on the PVDF membrane was examined. The Mx2 protein produced a response that corresponded to a color band of 21 kDa, which was an immunogenic protein (Fig. 4). This demonstrated the antigenic nature of the incomplete Mx2 protein.

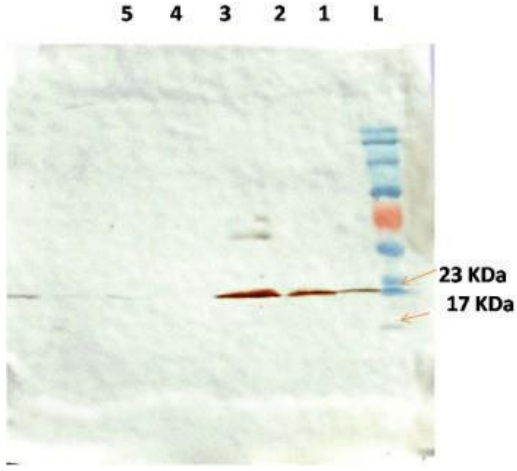


Fig. 4. Detection of the Mx2 protein (immunoblotted on a PVDF membrane)

Reaction of the Mx2 protein with the anti-His HRPO antibody with DAB as the substrate (Lanes 1--4: purified Mx2 protein (21 kDa) blotted after SDS–PAGE. Lane 5: Negative uninduced culture)

Statistical analysis. On the basis of the results of the Mx2 PCR, 60% of the animals were found to be pregnant (n=30/50), while 40% of the animals were found to be non-pregnant (n=20/50). In contrast to these findings, USG results showed that 28.0% (n=14/50) of the animals were pregnant and 72% (n=36/50) were not pregnant. However, the actual proportion of animals that gave birth after the gestation period ended was just 20% (n=10/50). According to Mx2 amplification results, the proportion of animals that were mistakenly diagnosed as pregnant (false-positive pregnancy) was 36% (n=18/50), while the proportion of animals that were pregnant but mistakenly diagnosed as non-pregnant (false negative animals) was 4% (n=2/50). On the basis of these results, the Chi square test showed that there was a significant ($P<0.5$) difference between the numbers of pregnant and non-pregnant animals for amplification.

Discussion

Early pregnancy diagnosis is essential for maximizing the lifetime productivity of dairy animals. Accurate and timely pregnancy detection plays a crucial role in effective reproductive management. By identifying non-pregnant animals early, initiating prompt treatment, and rebreeding them, the calving interval can be reduced, keeping the postpartum barren period close to 60 days. However, the lack of reliable early pregnancy diagnostic techniques exacerbates the problem. Therefore, the potential of the Mx2 protein to serve as a biomarker with the desired properties for early pregnancy diagnosis was investigated in this study.

Since the open reading frame (ORF) of Mx2 spans 2133 base pairs, expressing the full-length protein can be challenging (FERRER-MIRALLES and GARCIA-FRUITÓS, 2024). Hence, identifying a suitable peptide with high immunogenicity is a key requirement for expression in various systems. There is a critical need for antibody-based detection kits for early pregnancy diagnosis in buffaloes, and immunological characterization is vital for this process. In this investigation, seven immunogenic B-cell epitopes and many conformational epitopes were identified within the Mx2 protein. The distinct

antigenicity peaks suggest that these protein regions are highly antigenic. Recombinant antibodies targeting these linear and conformational epitope sites can potentially detect Mx2 in the serum of pregnant buffaloes as early as 18–21 days post-artificial insemination.

Although Mx2 is expressed under the influence of interferon tau between days 16–18 of pregnancy, its release mechanism in response to viral infection and interaction with other molecules remains unclear. Recent studies have explored the structural and functional properties of the bovine Mx2 gene and its association with the buffalo ISRE promoter region (BABIKER et al., 2016). Structural modeling can help assess cross-species conformational differences, determine cross-reactivity, and predict protein function during processes such as viral infection or conceptus-induced signaling (BATRA et al., 2018). Thus, for the incomplete protein of Mx2, three-dimensional structures were created in the current work using Swiss Modeler 9.18.

For the protein structure, a Ramachandran plot was created to determine the energetically permissible regions for the backbone dihedral ψ angles against the φ of the amino acid residue (PARK et al., 2023). Ramachandran plot analysis showed that 93.1% of the amino acids were in the most favorable regions, 2.97% in additionally allowed regions, and 2.25% in generously allowed regions, indicating an overall favorable structure. Overall, 3D verification of the homology-modeled structure revealed that the modeled structure of the Mx2 protein was satisfactory.

All proteins need to fold correctly to stabilize in three dimensions, and this folding is dictated by the secondary structures that are present in the protein (JOSHI et al., 2012). The results of the Mx2 study revealed that among the secondary structural elements, random coils dominated (73.20%). According to this analysis of the secondary structure percentage, random coils indicate the flexibility and dynamicity of the generated molecule. It has previously been revealed that homology modeling of interferon tau can reveal its interaction with other molecules involved in pregnancy detection (QING et al., 2022).

Previous modeling of interferon tau has also demonstrated its interactions with other pregnancy-related molecules (QING et al., 2022). To investigate its interaction with interferon tau and other proteins, the Mx2 structure was developed. The NetSurfP analysis of Mx2 indicated both buried and exposed residues, confirming the presence of transmembrane regions.

When a protein's stability *in vivo* is estimated from its main sequence, there is a strong association between the protein's physiochemical properties and stability (WANG et al., 2021). The average molecular weight of Mx2, negative charge, and positive charge residue of the protein were predicted. The relative volume of the protein occupied by aliphatic side chains and other predisposing factors that contribute to the increased thermal stability of the protein is determined by the protein's aliphatic index (AI), which for Mx2 is 78.81.For the Mx2 protein, the instability index was so high that it was >40 (61.15), and so unsuitable for *in vitro* investigations, further reducing the likelihood of any heterologous expression. The examination of Mx2 produced a negative GRAVY value of -0.478, indicating that the compound has a relatively high proportion of hydrophilic residues, and that it is easily accessible to charged ions and has a secretary nature. The pH at which a protein has no net charge is known as its isoelectric point (pI). This suggested that the protein would not dissolve at these pI values and that the buffer pH needed to make the protein soluble would need to be somewhat remote. The theoretical isoelectric point (pI) of 8.44 categorizes Mx2 as a basic protein, which poses challenges for purification under standard chromatographic conditions due to its limited solubility near its pI.

Mammalian cells differ from other production platforms used to manufacture recombinant proteins, primarily in their capacity to undergo complicated post translational modifications. The prediction of certain post translational modifications (PTMs), including phosphorylation, glycosylation, methylation, and disulfide bond formation, considerably reduces the amenability of proteins to soluble expression and correct folding (RAMAZI and ZAHIRI, 2021). Crucially, for many eukaryotic proteins to attain a native, physiologically active shape,

numerous PTMs are needed. PTMs drastically alter a protein's essential properties, such as charge, hydrophobicity, and solvent accessibility, which have an impact on protein stability and solubility. PTMs should therefore be considered key factors influencing effective protein production.

Protein phosphorylation can change a protein's function and location. It is involved in the operation of numerous signaling cascades. There are a total of 21 sites that can be phosphorylated in Mx2, with 11 serine residues, 08 threonine residues, and 2 tyrosines having a high threshold for binding to the phosphate group. Glycosylation is an essential factor in controlling the stability, function, and structure of proteins (SOLÁ and GRIEBENOW, 2009). Mx2 contains many asparagine, threonine, and tryptophan residues, but only three possible N-linked and ten O-linked glycosylation sites were found. Three cysteine residues were identified by the CYS_REC tool as being present in the Mx2 molecule at positions 97, 150 and 161. However, none of these residues were discovered to form a disulfide bond, which might be detrimental if it were to occur during the Mx2 molecule renaturation process.

Different heterologous proteins have been shown on the surfaces of bacterial cells using bacterial surface expression methods. Passenger proteins can be expressed on the surfaces of bacteria by fusing them between the membrane-spanning domain and the signal peptide. Numerous recombinant proteins have been generated from diverse strains of the host *E. coli* by purifying them using different expression vectors, such as pMAL, pET, and pGEX (ROSANO and CECCARELLI, 2014). Improving the solubility and production of the target recombinant protein is the main goal when various expression vectors and host cells are used. Since the targeted partial Mx2 fragment lacks disulfide bonds, correct folding is less critical for its biological activity. Disulfide bonds can interfere with folding in the cytoplasm due to its reducing environment. Furthermore, although Mx2 is normally glycosylated, the prokaryotic host lacks the machinery for these modifications, so it was expressed in deglycosylated form. Notably, antigenic activity has been demonstrated in deglycosylated proteins in prior human and murine studies (ROSANO and CECCARELLI, 2014). Glycosylation of Mx2 can be pursued in future studies to enhance its *in vivo* half-life, as deglycosylated forms are known to degrade more rapidly.

One analytical antigen detection technique that can be used to find recombinant proteins is Western blotting or immunoblotting. It uses an anti-His monoclonal antibody for both capture and detection, and is a specific and sensitive method for detecting heterologous proteins, although the cost of the capture antibodies is extra. Additionally, our findings show that blotting using the Mx2 protein produced an antigenic band with a size of 21 kDa, indicating that the protein has strong antigenic potential. Furthermore, the Mx2 protein produced from BL-21 can be used to generate monoclonal and polyclonal antibodies, both of which have extensive uses in ELISA. Antibodies raised against this protein are expected to serve as reliable early pregnancy markers in buffalo.

Commercially available assays based on Pregnancy-Associated Glycoproteins (PAGs) have been developed for pregnancy detection in cattle. However, a limitation of PAG-based testing is that measurable concentrations typically begin to appear around day 28 of gestation. Furthermore, PAGs persist in circulation postpartum, due to their high concentration at calving and have a relatively long half-life, ranging from 7.4 to 9 days in European cattle and approximately 9.2 to 10.1 days in African Azawak Zebu cows (SOUSA et al., 2002, HAUGEJORDEN et al., 2006). An alternative approach involves the use of Early Pregnancy Factor (EPF) that has been commercialized as the Early Conception Factor (ECF) test, which claims to detect pregnancy within 48 hours of fertilization. However, the ECF test has shown high unreliability (CORDOBA et al., 2001), primarily because EPF is not exclusively pregnancy-specific and it may also be secreted by non-placental tissues, such as certain tumors, leading to false-positive results. Mx2-based proteins may aid in the detection of pregnancy in the early phases of conceptus implantation. An Mx2-based pregnancy detection test is expected to be cost-effective, specifically upregulated during pregnancy, minimally affected by non-pregnancy factors, consistently expressed over a meaningful duration, and free of residual presence after pregnancy.

Conclusions

In summary, this study aimed to clone, express, and characterize a partial Mx2 protein from buffalo serum using a prokaryotic expression system. Mx2 expression was found to be upregulated during days 18–21 of pregnancy, making it a promising candidate for early pregnancy detection. The optimized expression protocol enables the production of a biologically active protein, with a size of 21 kDa for antibody development. This could lead to the creation of a cost-effective, strip-based diagnostic tool. In addition to detecting pregnancy-related responses to the embryo, partial Mx2 may also be studied to explore the role of UBE1L in embryonic viability, placentation, and implantation.

Ethics statement

The samples collected in these studies from buffaloes used in this study were approved by the Institutional Animal Ethics Committee (IAEC), registered as 1669/GO/ReBiBt/S/12/CPCSEA, at the 27th meeting held on 12th June 2023.

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Authorship contribution statement

Conceptualization, K.B.; methodology, K.B.; software, KB; validation, K.B., M.S. and N.S.; formal analysis, K.B.; investigation, K.B. and N.S.; resources, K.B.; data curation, KB; writing—original draft preparation, K.B. and S.M.; writing—review and editing, K.B., N.S. and S.M.; visualization, S.M.; supervision, K.B.; project administration, K.B.; funding acquisition, K.B. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Data availability statement

The DNA and protein sequence data generated during this study are deposited in the NCBI repository with accession no.PQ619851.

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BATRA, K., N. SHARMA, M. SINGH, S. MAAN: Protein Mx2 kao biomarker za rano otkrivanje gravidnosti u bivola - svojstva i ekspresija u heterolognom sustavu. Vet. arhiv 96, 113-127, 2026.

SAŽETAK

Indija je najveći proizvođač mlijeka u svijetu. Ovo je važno postignuće moguće ponajviše zbog postojanja vrste *Bubalus bubalis* (bivoli). Povezano s proizvodnjom mlijeka, od velike je važnosti učinkovita reprodukcija odnosno rano otkrivanje gravidnosti. Stoga je istražen jedan od proteina čija se proizvodnja potiče koncepcijom, protein 2 rezistentan na miksovirus (Mx2), koji se proizvodi između 18. i 21. dana gravidnosti. Za ciljeve ovog istraživanja, analizirana su svojstva navedenog proteina iz sustava heterologne ekspresije. Klasičnim PCR-om ustanovljeno je znakovito povećanje parcijalnog Mx2 u gravidnih životinja. Analizom *in silico* provjereno je može li ovaj protein poslužiti kao molekula u kompletima za rano otkrivanje gravidnosti. Svojstva proteina Mx2, u *in vitro* uvjetima, procijenjena su na osnovi njegovih fizikalno-kemijskih svojstava, što je nužno za izradu bilo kojega testa. Sudeći prema dosadašnjim istraživanjima imunogenosti, Mx2 ima prirodno visoka antigena svojstva i može poslužiti za proizvodnju protutijela. Upotrebom početnica za određivanje kodirajuće regije, parcijalni protein Mx2 ekstrahiran je iz gravidnih ženki bivola (n=30), amplificiran, kloniran i unesen u bakteriju *Escherichia coli* BL21 (DE3). Proteinska vrpca od 21 kDa analizirana metodom *Western blotting*, pokazala je da je ovaj protein prirodni antigen. Zaključeno je da se ekspresija i molekularna karakterizacija parcijalnog proteina Mx2 mogu provesti u sustavu heterologne ekspresije. Protein Mx2 u bivola može poslužiti kao pouzdan marker implantacije pri začeću i može se uvrstiti u dijagnostičke testove za rano otkrivanje gravidnosti.

Ključne riječi: bivol; detekcija gravidnosti; protein Mx2; ekspresija; *E coli*