

## Development of polyclonal antibodies against the recombinant protein of Barley yellow dwarf virus

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### Abstract

*Barley yellow dwarf virus* (BYDV) particle purification is challenging because of the limited phloem tissue and extremely low viral titers. The current study aimed to generate antibodies against the viral coat protein, without purification of the viral particles. To produce the recombinant coat protein, the genomic region of BYDV encoding the coat protein (CP), was cloned, and expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) strain. Physicochemical characteristics, subcellular localization, and immunogenicity of the BYDV CP (coat protein) were identified using an in-silico approach. The BYDV CP was synthesized by Synbio Technology, USA and cloned into the pET28a (+) expression vector, to produce recombinant fusion coat protein (rFCP-BYDV) in *Escherichia coli* (*E. coli*). The recombinant protein produced in inclusion bodies was denatured and purified with on-column refolding by affinity chromatography. Purified protein (rFCPBYDV) was used, as an antigen followed by four weekly intraperitoneal booster doses in mice to develop pAB antisera, which was collected by cardiac puncture, to raise polyclonal antibodies (pAB) in mice. The raised anti-BYDV CP immunoglobulins (IgGs) detected the recombinant BYDV CP even at 100 pg/mL and 1000-time diluted crude extract of proteins from BYDV-infected wheat plant leaves. Results from indirect ELISA titration showed that the anti-BYDVCP antiserum produced in mice had a titer of around 1:10,000. The findings offer a quick and simple immunodiagnostic technique for rapid detection of BYDV. To the best of our knowledge, this is the first report on the production of anti-BYDV CP pAB and their application for the diagnosis of BYDV disease in Pakistan.

**Keywords:** Barley yellow dwarf virus, Wheat, Disease diagnosis, Antibody, ELISA

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### Introduction

Each year, diseases and pests decimate over 42% of global agricultural production. The cost of yield losses caused by plant viruses is estimated to be more

than \$30 billion annually making it critically important to develop approaches to reduce the effects of plant virus diseases on crops (Hilaire et al., 2022; Jones, 2021; Jones and Naidu, 2019). Wheat is one of the world's most important cultivated staple food



crops, especially in Pakistan (Ali et al., 2016; Raza, 2011), affected by 100 different diseases (Savary et al., 2019), among which Barley yellow dwarf disease (BYD) is one of the most destructive and widely distributed diseases caused by the *Barley yellow dwarf virus* (BYDV); (Griesbach et al., 1990). BYDV-PAV (pavhordei) is the most prevalent of any other yellow dwarf strains of wheat in Pakistan (Siddiqui et al., 2012). According to estimates, this virus could result in up to 84% (1358 kg/ha) of grain yield losses in wheat (Nancarrow et al., 2021). BYDVs are classified into the family *Tombusviridae*'s genus *Luteovirus* (Walker et al., 2022). The most noticeable symptoms of BYDVs in infected wheat are decreased tillering, stunted plants, chlorosis of leaf blades (particularly tips), and vascular bundles (Choudhury et al., 2018; Miller and Rasochová, 1997). They are phloem-limited, and aphids are responsible for transmitting them in nature in a circulative and persistently manner (D'Arcy and Domier, 2000), but not mechanically or through seed (Miller and Rasochová, 1997). BYDV particles are icosahedral in shape, consist of a main ~22 kDa CP, and have no envelope with an average diameter ranging from 25-30 nm (Li et al., 2015; Miller et al., 2002). The BYDV (+ssRNA) genome is composed of linear genomic RNA that has seven open reading frames (ORFs) (1, 2, 3, 3a, 4, 5, and 6, respectively). The virus used noncanonical mechanisms to translate these ORFs (Miller and Lozier, 2022). ORF3 and ORF4 encode the (22-kDa) major coat protein (CP) and a (17-kDa) movement protein (MP), respectively, and the read-through proteins (RTP) are encoded by ORF5 along with ORF3, which are essential for aphid transmission of BYDV (Chay et al., 1996). The genomic RNA of these viruses is not polyadenylated and it lacks cap-structure and genome-linked protein (VPg) (Miller and Rasochová, 1997; Shams-bakhsh and Symons, 1997). It is particularly challenging to purify these viruses since they are primarily limited to the host plant phloem tissue in an extremely low concentration. This challenge is exemplified by the devastating impact of diseases on crops, such as wheat, where late diagnosis of diseases based on visual symptoms at mature stages has resulted in significant losses. To mitigate these losses, early and accurate diagnosis of plant diseases is crucial. Guidelines for diagnostic work in plant virology (Green, 1991) and research on disease management (Figueroa et al., 2018), provide valuable insights into identifying and combating these pathogens. By

implementing need-based treatments and identifying diseases early, the agricultural sector can not only reduce crop losses but also achieve economic and environmental benefits, as emphasized in various studies (Walls et al., 2019; Figueroa et al., 2018).

The traditional method of identifying plant pathogens is through visual examination or PCR-based detection. This is often possible only after major damage has already been done to the crop, so treatments will be of limited or no use. To save plants from irreparable damage by pathogens, farmers have to successfully identify an infection even before it becomes visible.

Since the 1970s, numerous variants of serological techniques have been widely employed by pathologists, greatly increasing plant pathologists' ability to detect and analyze plant viruses. Various serological and molecular approaches have been widely used to detect plant viruses throughout the last few decades. Plant viral serology can be used to examine virus relationships, identify the causative virus in plant diseases, detect viruses in plant foundation stocks, and identify symptomless virus infections. It can also be used to quantify viruses and locate viruses within cells or tissues (Agrios, 2004; Hull, 2013).

Despite the widespread availability of commercial diagnostic kits (CDKs) and the convenience of manual application, the ELISA approach remains the most popular diagnostic tool for detecting plant viruses, particularly when dealing with large numbers of samples. Most commercially available kits lack specific and comprehensive information about the epitope and antigen used to trigger an immune response and produce antibodies. Such data may be gathered during the developing process of antibodies, which starts with viral isolation, characterisation, antigen selection for immunization, and raising antibodies (Mrkvová et al., 2022).

Certain plant viruses, especially those in the *Luteovirus* genus, present unique hurdles when purifying viral particles from infected plants for antibody generation. This procedure is frequently time-consuming and physically difficult (Shams-bakhsh and Symons, 1997). To overcome this problem, we investigated the use of polyclonal antibodies raised against the BYDV-PAV coat protein gene produced in bacteria. This method provides a promising solution for generating low-cost and simple immunological reagents for detecting BYDV in infected plants utilising ELISA (enzyme-linked immunosorbent assay) methods.



For the diagnosis of viral infections, Pakistan only utilizes phenotypic (symptoms) appearance or polymerase chain reaction-based approaches, which are time-consuming, labor-intensive, and expensive when employing a large number of samples. Since no antibodies are produced domestically, research labs in Pakistan import kits for the diagnosis of viral or other infections. It is imperative to study these viruses and expand the range of diagnostic tools so that we can deploy strategies to control these diseases. The main objective of the current study was, to develop polyclonal antibodies for the ELISA-based detection of *Barley yellow dwarf virus* (BYDV) by the overexpression of recombinant coat protein genes in the mouse model.

## Material and Methods

### Phylogenetic, physicochemical and immunogenic analysis

As previously described by Ali et al. (2013), sequence retrieval, alignments, and phylogenetic analysis procedures were used. The ExPASy ProtParam online program was used for determining the physicochemical properties of the BYDV CP protein “(<http://web.expasy.org/protparam>) (Gasteiger et al., 2005)”, and the Coat protein's antigenicity was examined using the Optimum Antigen<sup>TM</sup> design tool (GenScript, Piscataway, NJ, USA).

**Subcloning and sequencing of the BYDV CP gene**  
Synbio Technologies USA synthesized the BYDV CP gene and cloned it into pUC57-Amp. We sub-cloned it into pET-28a (+) via *Nde*I and *Hind*III digestion and transformed it into *E. coli* DH5 by the heat shock method using kanamycin selection. BYDV CP-pET-28a (+) was purified with the GeneJET Plasmid Miniprep Kit (Lot: 00729747) for DNA sequencing using T7 primers before being expressed in *E. coli* BL21 (DE3) for heterologous expression.

**Expression and localization of BYDV CP in *E. coli***  
To produce a starter culture, the *E. coli* strain BL21 (DE3) harbouring BYDV CP-pET28a (+) was grown overnight in Luria-Bertani (LB) medium supplemented with 50 g/ml kanamycin. The overnight starting culture was then diluted forty (40x) times in 25 ml of LB media and incubated at 37 °C and 200 rpm until the optical density (OD<sub>600</sub>) ranged between 0.4 and 0.8. Before inducing gene expression with isopropyl-D-thiogalactopyranoside

(IPTG) at 0.1, 0.5, or 1 mM concentrations, 1.5 ml of cell suspension was removed as an uninduced control. The cell suspensions, both induced and non-induced, were then cultured in an incubator under the previously specified conditions. At 3 and 6 h after induction, 1.5 ml of cell suspension from each incubated culture flask was collected and centrifuged in a chilled centrifuge (4°C) at 10,000 rpm for 7-10 minutes. SDS-PAGE was used to analyse the samples that resulted (Laemmli, 1970).

### Recombinant BYDV CP Purification

Following large-scale gene expression, the cells were lysed using sonication before being centrifuged at 13,000 rpm for 15 minutes at 4°C to separate the soluble fraction from the insoluble fraction. The insoluble fraction of recombinant BYDV CP was solubilized and purified using affinity chromatography with NTA resin (nickel-nitrilotriacetic acid) columns (for details of the process and buffer composition (Table-S1), see supplementary materials). Overnight at 4°C, the eluted refolded protein was dialyzed against several changes of 1X PBS buffer (pH 7.3) with continuous stirring. This procedure was used to eliminate excess imidazole and salts. The pure protein concentration was measured using Nanodrop (Thermo Fisher Scientific) and Bradford's method (Bradford, 1976).

### Antibody preparation and antiserum titration

Polyclonal antisera were raised according to the protocol outlined by Salem et al. (2018). Swiss Webster white (albino) mice (4-6 weeks old) were employed in the research at the animal house located in the School of Biological Sciences (SBS), University of the Punjab, Lahore, Pakistan. The animals were housed in a controlled environment with regulated temperature and lighting, following a 12-hour light/dark cycle. They were provided with unrestricted access to food and water.

In the first step, 100 µg of the recombinant BYDV CP protein in 1X PBS buffer emulsified with CFA (complete Freund's adjuvant, Sigma, USA) in a 1:1 volume ratio before injecting it subcutaneously into six-week-old Swiss Webster white (albino) mice. Following that, four weekly booster injections (200 µg) were given, in 1X PBS buffer and an equal volume of IFA (incomplete Freund's adjuvant, Sigma, USA) in a 1:1 volume ratio. Antiserum was collected a week after the last booster injection through the cardiac puncture, prior to blood



collection, the mice were given deep anaesthesia containing ketamine hydrochloride and xylazine (50 mg/kg and 5 mg/kg of animal body weight, respectively) through intraperitoneal administration, followed by incubation at 37 °C for 1 h, centrifugation at 4000 rpm. Antisera were stored at -20°C for downstream analysis.

### Calibration of antibodies and antigens

The calibration of the primary antibody and antigen coating is critical to the performance of an ELISA. Different dilutions of raised antisera (50X, 100X, 1000X, and 10,000X) and different antigen concentrations from 10 pg to 2 µg were employed for this purpose. The antigen was immobilised in microtiter plate wells using 50 mM KCl-borate buffer (pH 8.0; 100 ng/well), then sealed and incubated overnight at 4 °C. Wells were washed three times with 1X PBST buffer after the coating solution was removed. A blocking buffer (5% W/V skim milk in TBS-T) was added and incubated for 2 hours at 37 °C. Antisera diluted in blocking solution (100 ul/well) was added and incubated for 1 hour at 37 °C. After washing, an Anti-mouse HRP-conjugated IgG antibody (50,000X) (200 ul/well) was applied and incubated at 37 °C for 1 hour. Following washing, TMB solution (100 ul/well) was added and incubated for 15 minutes. 2 M H<sub>2</sub>SO<sub>4</sub> (100 ul/well) was used to halt the reaction. A microplate reader (OD450/630) (HUMAREADER plus, human GMBH) was used to quantify absorbance at 405 nm, and immunoreactivity was determined using three replicate readings.

### Indirect ELISA

Indirect ELISA was carried out as described by Darsono et al. (2018). One gram of healthy and infected wheat leaf tissue was crushed using liquid nitrogen. Protein extraction was accomplished by grinding the tissue at 4°C in an extraction buffer (50 mM Mops-NaOH, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 2% PVP, pH 7.5). After centrifugation (8000 rpm, 4°C, 10 min), supernatant (100 L) was applied to an immunoplate with an additional coating solution (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, 2% PVP, pH 9.6) and incubated overnight at 4°C.

Wells were washed three times with TBS-T, blocked with TBS-T blocking solution (1% skim milk, 0.5% BSA), and then incubated for 60 min at room temperature with a 1:1000 dilution of raised polyclonal antibody. After washing five times with TBS-T, anti-mouse HRP-conjugated IgG (diluted

50,000X) was added (200 ul/well) and incubated for 1 hour at 37°C. Following TBS-T washes, TMB solution (100 ul/well) acquired colour at room temperature and was halted with 2 M H<sub>2</sub>SO<sub>4</sub> (100 L/well) for yellow colour upon acidification.

### RT-PCR

BYDV infection in wheat plants was established by RT-PCR. TRI REAGENT® (Catalog #TR118) was used to get total RNA from 100 mg of leaf samples from field-grown healthy and infected plants that showed typical signs of BYDV. Based on the sequence in the GenBank, accession # HE985229, the following coat protein-targeted primers were made with Primer3Plus, details are given in **Table-1**. According to manufacturer instructions, total extracted RNA was used in the QIAGEN® One-Step RT-PCR method at concentrations ranging from 1 pg to 2 g per reaction.

### Statistical analysis

Statistical analysis was conducted using GraphPad Prism software. ANOVA followed by Tukey posttest (at p <0.05) was employed to assess significant differences between treatments. Data were presented as mean standard error based on three independent experiments.

**Table-1. The set of primers used to amplify BYDV CP by polymerase chain reaction.**

S. No	Name	Sequence	Length	Restriction Site
1	BNd-CP-F	5'- <u>CATATGAATTCAGTAGG</u> CCGTAGG-3'	24 (nt)	NdeI CA^TATG
2	BHd-CP-R	5'- <u>AAGCTTCTATTTGGGAG</u> TTAGCATAC-3'	26 (nt)	HindIII A^AGCTT

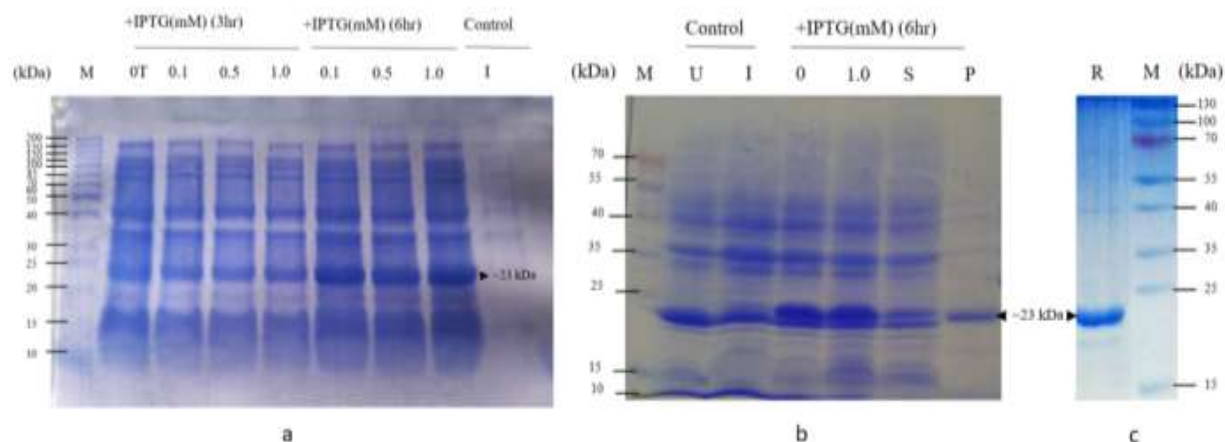
## Results

### Physicochemical and immunogenic analysis

The physicochemical characteristics (amino acid composition, half-life estimation, aliphatic index, molecular weight, instability index, composition of atoms, grand average of hydropathy (GRAVY), theoretical *pI*, and extinction coefficient) of the BYDV CP protein sequence were predicted using ExPASy ProtParam. For BYDV CP without the tag, the predicted molecular weight is 2202.07 Da, the theoretical *pI* is 11.49, and the aliphatic index value is 71.25, indicating that this protein is thermally stable and contains a high amount of hydrophobic amino acids.







**Figure-1. Overexpression, purification, and refolding analysis of the expressed Barley yellow dwarf virus coat protein gene in *Escherichia coli*.**

(A) Effect of inducer concentration (IPTG) on the BYDVCP expression level; (B) localization of expressed BYDVCP and (C) on column refolding and elution of the BYDV CP protein using Ni-NTA. 0T is the protein fraction collected before the IPTG induction. The pET28 (a+) without the insert was used as a control (I= induced; U= un-induced). Non-induced (0), induced (1.0 mM), the soluble fraction (S), the insoluble fraction (P), on column refolded protein elution (R) and protein marker (M)

The instability index was 44.37, which means that it is an unstable protein, and the number of negatively charged residues was lower as compared to positively charged residues (-ve = 11, +ve = 34). The GRAVY score of protein was found to be negative (-0.516), indicating that protein is non-polar. Furthermore, the antigenicity of the BYDV CP protein was located in four distinct stretches, which were RAGPRRRNGRRRTGRC, VGRRGPRRANQNGPC, CRAEAINGKEFQUEST, and RRRRRRAIRPVVVVQC (Table-S2). The *in-silico* analysis of BYDV CP revealed that the N-terminal is more immunogenic as compared to the C-terminal, which has only one epitope out of four (Table S2).

### Heterologous expression of BYDV CP gene

After cloning of the CP gene, the DNA sequencing result confirmed proper in-frame insertion of the entire CP gene with a 6 × His- tag at the N-terminal in the expression vector (Fig. S1, S2). The expression construct (BYDVCP-pET-28a (+)) was used to transform the *E. coli* BL21 (DE3) strain for heterologous gene expression. The expression result showed that there was no clear difference in the level of expression regarding different inducer (IPTG) concentrations (0.1, 0.5, and 1 mM). However, 6 h post-induction duration yielded a higher amount of recombinant BYDV CP compared to 3 h (Fig. 1a). Therefore, large-scale expression culture was harvested at 6 h. Production of recombinant BYDV

CP in *E. coli* was indicated by an intense band on 12% SDS-PAGE with an approximate size of about 24 kDa, which roughly matched the predicted size of the entire BYDV CP plus the fused 6 × His-tag amino acid. The gel showed a protein band in both the induced and non-induced cultures at a position equivalent to the recombinant protein's molecular weight but not in vector control (Fig.1b). This finding suggests that lactose and galactose presence, even at trace levels, in complex LB media could serve as inducers in non-induced cells of recombinant protein production (Studier, 2014). The anticipated band was only seen in the insoluble fraction (Fig. 1b).

### Refolding and purification of recombinant BYDV CP

The BYDV CP protein was primarily produced as inclusion bodies in *E. coli* (Fig. 1b). Thus, using the on-column refolding, the solubilized protein was purified in its folded state as demonstrated in (Fig. 1c). After refolding and purification, the overall yield of the BYDV CP protein ranged from 3-5 mg per liter of the culture, making enough of the protein available for conducting mouse immunization trials. It was also observed that the addition of 10% glycerol and 0.8% sarkosyl stabilized the purified refolded protein for longer-term storage similar to a previous report (Tufail et al., 2021).



### Mice Immunization

Following clearance from the Ethics Committee, School of Biological Sciences, University of the Punjab, Lahore, Pakistan, antiserum was created by immunizing Swiss Webster white (albino) mice with expressed 6×his-tagged rFCP-BYDV. Six-week-old mice were given a 100 µg injection of expressed coat protein, followed by four weekly intraperitoneal doses of 200 µg, without the 6×his-tag being removed. Because of the projected low immunogenicity, the N-terminal fusion protein cleavage was considered unnecessary (Mutasa-Gottgens et al., 2000). The mice were emulsified with an identical volume of Freund's complete adjuvant for the initial injection, followed by four consecutive intramuscular injections at weekly intervals using incomplete adjuvants. Blood was taken after five injections, and the blood was kept at 37°C for one hour before being centrifuged at 4000 rpm.

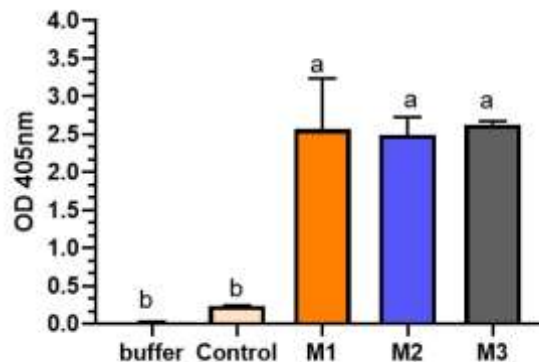
### Development and evaluation of polyclonal antibodies

#### Establishment and optimization of indirect-ELISA

By using mice antisera, the titer of the polyclonal antibodies in each animal was assessed. The threshold value (TV) = mean negative control plus twice the standard deviation of the negative control was used to analyze the data quantitatively by comparing the means of their measured absorbance values to a calculated TV (Koh et al., 2020). If the samples' mean absorption readings were higher than the TV, the samples were considered positive.

However, the titer of the final bleed antisera in all three groups of immunized mice was higher compared to buffer and the control animals, and a significant difference was observed between buffer, control, and immunized mice absorbance values (Fig 2). The ELISA values were uniform from the antisera obtained from all immunized animals. The alphabet letters (a, b, etc.) show statistically significant differences between variables like buffer, control, and immunized mice. Significant differences between variables were computed using a one-way ANOVA (one-way analysis of variance) followed by a Tukey posttest, significance threshold set at  $p <$

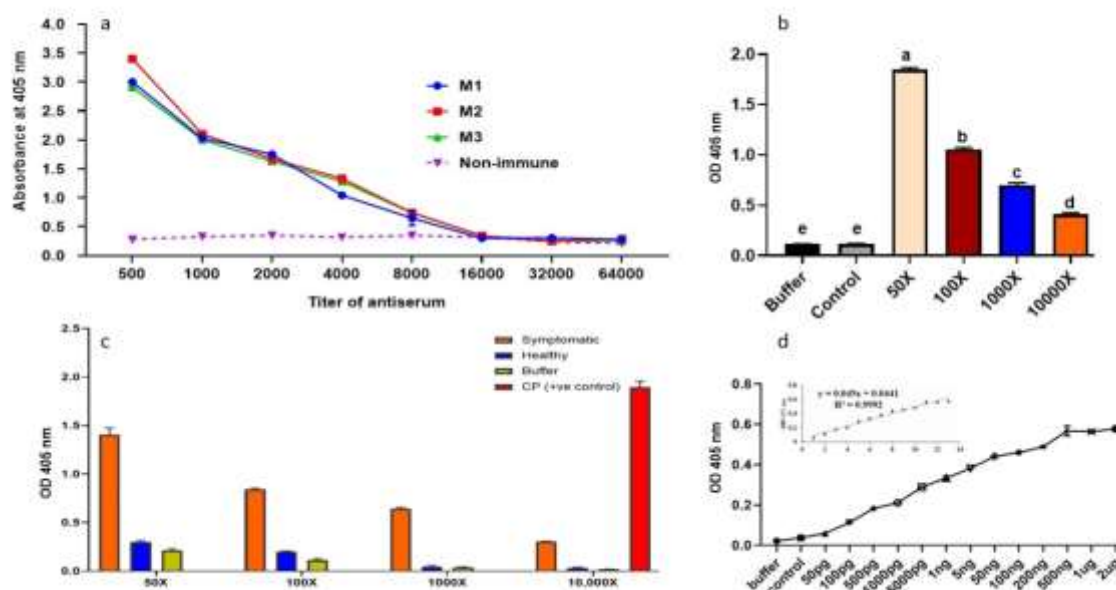
0.05 (Zhang et al., 2021). Results from indirect ELISA titration showed that the anti-BYDVCP antiserum produced in mice had a titer of around 1:10,000 (Fig. 3a).



**Figure-2.** Polyclonal antibodies titer check in all experimental animals (M1, M2, and M3) immunized with rFCP-BYDV compared with buffer and negative control (immunized with 1XPBS buffer).

#### Optimization of primary antibody dilutions

The optimal coating concentration of raised primary antibody (pAb) for the range of reactivity was determined by the serial dilution method, using the determined concentration of pure BYDV CP fusion protein antigen (100 ng/well). Antisera against BYDV CP fusion protein allowed detection of purified BYDV CP fusion protein antigen from 50 X dilution to 10,000X dilution. The mean absorbance at 405 nm of BYDV CP fusion protein antigen decreased from 1.583 (50 X) to 0.461 (10,000 X). The readings of the buffer and the healthy control were lower from an absorbance mean value of 0.122 and 0.137, respectively. Up to 10,000 X dilution, the antibody was readily distinct, with an absorbance mean value of 0.41 exceeding the threshold value (TV) of 0.203. Sample values were considered significant if their mean absorbance values exceeded the TV value. So, the optimal dilution range of the pAb was considered up to 1:10,000 (Fig. 3b). The statistically significant variations between the variables are indicated by the letters a, b, c, and d.



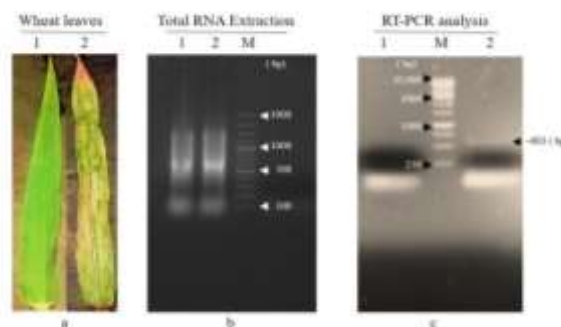
**Figure-3. Optimization of indirect ELISA test for the detection of BYDV:** (A) polyclonal antibodies titer check in all experimental animals (M1, M2, and M3) immunized with 6×His-BYDV CP purified protein compared with buffer and negative control (immunized with 1XPBS buffer); (B) optimization of primary antibody (serum) coating concentration using serial dilution method; (C) sensitivity of the raised antibodies by the ELISA assay in different dilutions of protein lysates from BYDV-infected wheat plant; (D) Standard Curve plotted for optimization of antigen by serial dilution method, and linear regression shows a working range from 100 pg/mL to 500 ng/mL ( $R^2 = 0.9992$ )s on the virus's pathogenicity

### Applicability of the developed Indirect-ELISA

To figure out the reliability of the indirect ELISA, the antisera's sensitivity was further examined via indirect ELISA analysis (Darsono et al., 2018) using healthy and infected crude extracts of wheat plants from 50 X dilution to 10,000 X dilution. These samples were first confirmed by RT-PCR analysis for the presence of a BYDV-PAV virus using CP-specific primers (Fig. S3). The infected crude extract dilution gradually reduced the  $OD_{405\text{ nm}}$  from 50 X (1.4) to 10,000 X (0.306) dilution. Purified recombinant BYDV CP was used as a positive control. No significant reaction was observed with the buffer and the crude extracts from the healthy plants (Table 2, Fig. 3c).

**Table-2. Indirect ELISA test result for sensitivity range of the raised antiserum against different dilutions of sap**

Dilution	Symptomatic	Healthy	Buffer	CP (+ve control)
50X	1.405	0.299	0.213	0.000
100X	0.842	0.200	0.112	0.000
1000X	0.642	0.045	0.036	0.000
10,000X	0.306	0.031	0.018	1.90



**Figure-4. RT-PCR-based detection of BYDV infection in wheat leaves.** (A) Photograph of wheat leaves with and without symptoms (1= non-symptomatic; 2= symptomatic); (B) Total RNA ex-traction from sugarcane leaves (1= non-symptomatic; 2= symptomatic); (C) RT-PCR amplification of BYDV CP gene (1= healthy leaves, 2= infected leaves), and M= 100 bp and 1kb DNA ladder

### The standard curve

For the quantification of BYDV in plant tissues with an indirect ELISA using raised antisera, the standard curve was settled by serially diluted concentrations of standard BYDV CP fusion coat protein (10 pg to 2 µg) (Zhang et al., 2021). The standard curve for the



BYDV CP fusion coat protein and OD<sub>405</sub> nm was found as follows:  $y = 0.045x + 0.0441$  and  $R^2 = 0.9992$ , and BYDV CP fusion protein's lowest detection limit was approximately 100 pg/mL. According to the results, linear regression has a workable range of 100 pg/mL to 500 ng/mL (Fig. 3d). The plant samples that were used in ELISA, were first confirmed by RT-PCR analysis for the presence of a BYDV-PAV virus using CP-specific primers (Fig. 4a, b, c).

## Discussion

Detection and accurate identification of plant viruses are critical for effective virus management and control (Lima et al., 2012; Marqués et al., 2022; Mehetre et al., 2021; Rubio et al., 2020; Valenzuela et al., 2022; Varma and Singh, 2020; Wang et al., 2022). Various laboratory-based approaches have been developed to achieve reliable virus detection, including cytological, serological, molecular, physical, and biological characterization of viruses (Nickel et al., 2004). Among these, serological methods have proven to be specific and reliable for rapid virus identification (Noorani et al., 2015; Astier et al., 2001; Lima et al., 2012; Naidu and Hughes, 2003). This study focused on developing a robust detection method for Barley yellow dwarf virus (BYDV) using polyclonal antibodies (pAbs) raised against the recombinant fusion coat protein.

The *in silico* analysis of the BYDV coat protein (CP) sequence provided valuable insights into its immunogenic regions, emphasizing the importance of the N-terminal region in eliciting an immune response. Bioinformatics techniques, such as *in silico* analysis, play a crucial role in understanding protein structure and function, thereby aiding in the design and construction of fusion constructs. These methods offer advantages over traditional approaches, reducing time, and cost, and improving the safety and efficacy of predicted immunogenic epitopes (Haghroosta et al., 2020; Kaur et al., 2020; Madhi et al., 2021). This analysis was complemented by physicochemical characterization of the BYDV CP, revealing its stability and antigenic regions, which were further verified experimentally.

The successful expression of the BYDV CP gene in *Escherichia coli* (*E. coli*) highlighted the potential of heterologous gene expression for producing viral proteins. The insertion of a 6 × His-tag at the N-terminal facilitated efficient protein purification and

subsequent refolding. Despite the protein being initially produced as inclusion bodies, the on-column refolding approach proved effective in obtaining folded and functional recombinant protein (Singh et al., 2014). By gradually lowering the concentration of GndCl, the on-column refolding approach successfully refolded the rCP, producing enough rCP for conducting mouse immunisation trials (Fig. 1c). Similar reports in the literature support the efficacy of this approach, emphasizing the challenges associated with recombinant protein production and the need for innovative strategies (Astuti et al., 2019; Darsono et al., 2018; Garcia et al., 1997; Gulati-Sakhuja et al., 2009; Hema et al., 2003; Jain et al., 2005; Khatabi et al., 2012).

The development of polyclonal antibodies against the recombinant BYDV CP was a crucial step in the establishment of an effective detection method. As predicted previously (Mutasa-Gottgens et al., 2000; Kumari et al., 2001; Gulati-Sakhuja et al., 2009), the 6×His-tag fused with the recombinant protein was not expected to have immunogenic activity. A fusion tag also has the added benefit of boosting protein synthesis, solubility, and stability, all of which accelerate the purification procedure (Esposito and Chatterjee, 2006; Sørensen and Mortensen, 2005). Furthermore, the heterologous clone can be safely stored before being used to create an antigen in order to supply a consistent flow of pure coat protein for the long-term production of pAbs (Salem et al., 2018).

The raised antisera exhibited high sensitivity and specificity, enabling the reliable detection of the virus in both pure fusion protein and infected plant extracts. The optimization of antibody dilutions and the development of a standard curve provided a robust framework for quantifying BYDV levels in plant tissues. The generated polyclonal antibodies demonstrated their potential as valuable tools for plant virus research, including investigations into virus movement and interactions within host plants and vectors (Brault et al., 2001).

The success of this study in producing high-titer polyclonal antibodies against the recombinant BYDV CP highlights the feasibility of using bacterial expression systems for the large-scale production of viral antigens. This approach offers advantages in terms of efficiency, speed, and cost-effectiveness compared to traditional methods (Schwartz et al., 2011; Hartley, 2006; Nolasco et al., 2006; Petrovic et al., 2003; Saijo et al., 2005; Shi et al., 2003). The





availability of these antibodies enhances the capability to detect and monitor plant viruses, contributing to improved disease management strategies.

## Conclusion

The development of polyclonal antibodies against the recombinant fusion coat protein of BYDV using an *E. coli* expression system represents a significant advancement in plant virus detection. The high sensitivity and specificity of the generated antibodies offer promising applications in plant material sanitation and research endeavors. The integration of recombinant proteins and polyclonal antibodies into diagnostic assays provides a powerful tool for the accurate and early detection of plant viruses, ultimately supporting effective virus control and management strategies.

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## Contribution of Authors

Khalid M & Rashid N: Performed experiments, analyzed the data, contributed in write up of article and approved final draft.

Zaidi NSS: Performed the data analysis

Tahir M: Conceived idea, developed research methodology, supervised the experiments, wrote manuscript and approved final draft.

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## Supplementary Material

### I. Supplementary Methods

#### Purification and refolding of expressed protein

The cell pellets were resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 4 mM  $\beta$ -mercaptoethanol (ME), and Roche Complete™ Protease Inhibitor Cocktail EDTA-free tablets (Roche, Cat # 30307800)). The resuspended cells were lysed by up an ultrasonic sonicator on ice for 30 minutes in cycles of 20 seconds on and 40 seconds off. The cell lysate was then centrifuged at 13,000 rpm for 30 min at 4 °C to harvest inclusion bodies. The pelleted inclusion bodies were solubilized in 40 mL of resuspension buffer (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 4 mM  $\beta$ -ME, 5 mM imidazole, and 6 M guanidine hydrochloride (GndCl)). After solubilization of the inclusion bodies, the solution was centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was filtered using a 0.22- $\mu$ m syringe filter. For refolding and purification of the target protein, the solution was passed through a nickel-nitrilotriacetic acid (Ni-NTA) column pre-equilibrated with the resuspension buffer. To achieve maximal binding of the target protein, the collected flow-through was passed two to three times through the column. To remove nonspecifically bound proteins, the column was washed with 30 column volumes (CV) of wash buffer 1 (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 4 mM  $\beta$ -ME, 30 mM imidazole, and 6 M GndCl). To refold the target protein, the column was washed with refolding buffers such that the concentration of GndCl was gradually decreased (from 6 M to 0 M) in five steps (Table S1). The refolded protein was eluted from the column with 10 CV of elution buffer (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 4 mM  $\beta$ -ME, 0.8% sarkosyl, 10% glycerol, and 250 mM imidazole). The purity of the protein was analyzed through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions representing the purified protein were pooled. The purified protein was then dialyzed against dialysis buffer (1X PBS buffer, pH 7.3) overnight at 4 °C to remove excess salts and imidazole.

### II. Supplementary Tables

**Table S1. Composition of refolding and elution buffers used for on-column refolding and purification of BYDV CP-fused hexon epitopes.**

Buffer	Composition	Column volumes (CV)
Refolding buffer 1	50 mM Tris-HCl, pH 7.3, 500 mM NaCl, 4 mM $\beta$ -ME, 4 M GndCl, 0.8% sarkosyl, and 10% glycerol	30
Refolding buffer 2	50 mM Tris-HCl, pH 7.3, 400 mM NaCl, 4 mM $\beta$ -ME, 2 M GndCl, 0.8% sarkosyl, and 10% glycerol	30
Refolding buffer 3	50 mM Tris-HCl, pH 7.3, 300 mM NaCl, 4 mM $\beta$ -ME, 0.5 M GndCl, 0.8% sarkosyl, and 10% glycerol	30
Refolding buffer 4	50 mM Tris-HCl, pH 7.3, 200 mM NaCl, 4 mM $\beta$ -ME, 0.25 M GndCl, 0.8% sarkosyl, and 10% glycerol	30
Refolding buffer 5	50 mM Tris-HCl, pH 7.3, 200 mM NaCl, 4 mM $\beta$ -ME, 0.8% sarkosyl, and 10% glycerol	60
Elution buffer	50 mM Tris-HCl, pH 7.3, 500 mM NaCl, 4 mM $\beta$ -ME, 0.8% sarkosyl, 10% glycerol, and 250 mM imidazole	10



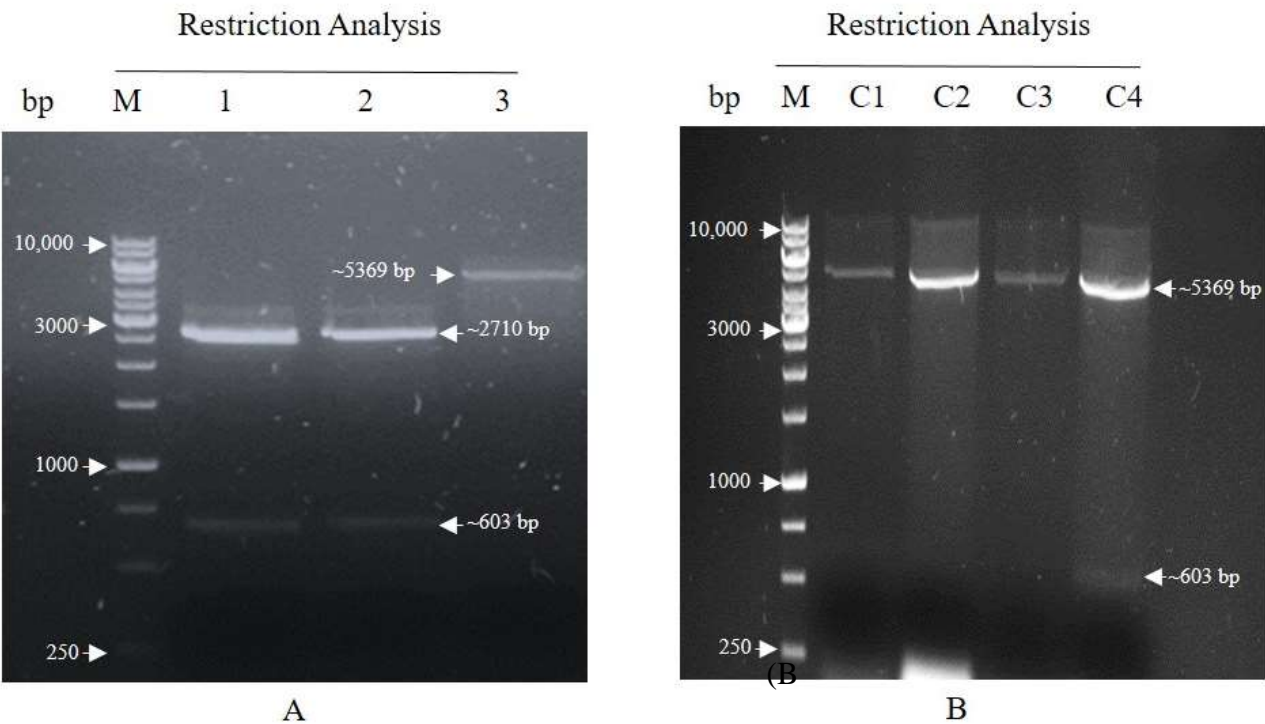
Table S2. Predicted epitopes of the BYDV CP by GenScript OptimumAntigen™ design tool

No	Start	Antigenic Determinant	Length	Antigenicity/Surface / Hydrophilicity	Disordered Score	Synthesis	Mus_musculus   Rattus_norvegicus   Oryctolagus_cuniculus blast
1	35	RAGP <u>RRR</u> NGRR <u>TGR</u> C	14	3.28/1.00/1.38	0.3691	Easy	64%   78%   63%
2	4	V <u>GRR</u> G <u>PRR</u> ANQNG <u>P</u> C	14	3.18/0.93/0.86	0.3349	Easy	63%   50%   50%
3	154	<u>C</u> RAE <u>A</u> ING <u>K</u> EFQ <u>E</u> ST	14	2.24/0.86/0.55	NONE	Easy	49%   42%   42%
4	19	<u>RRRRR</u> AI <u>R</u> PVVVV <u>VQ</u> C	14	1.30/0.64/0.60	0.3045	Easy	56%   56%   70%

**Note:**

1. An extra "C" (high-lighted as green) is added to the C-terminus (or N-terminus) to facilitate conjugation.
2. Positive charged residues (K,R,H) are in blue.
3. Negative charged residues (D,E) are in red.

**III. Supplementary Figures**



**Fig. S1 Subcloning of BYDVCP-pUC57 to the pET-28a (+) expression vector. Double digestion analysis of synthetic BYDVCP-pUC57-Amp, and pET-28 (a+) using *Nde*I, *Hind*III restriction sites (A), (lane 1, 2 = BYDVCP-pUC57 and lane 3 = pET-28a (+)); BYDVCP-pET-28a (+) clone confirmation by double digestion analysis (B), (C1-C4 = *E. coli* strain DH5 $\alpha$  transformants carrying BYDVCP-pET-28a (+), M = 1kb DNA ladder. BYDVCP ( $\pm$  603 bp), pUC57-Amp (~2710bp), and pET-28a (+) (~5369bp)**



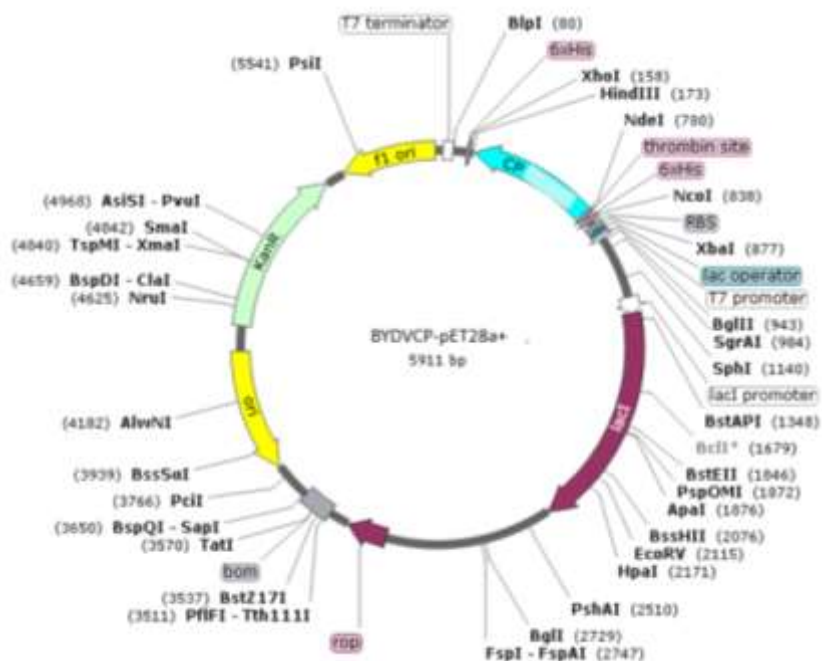


Fig. S2 Snap Gene map showing in-frame insertion of the entire BYDVCP gene for the generation of expression construct for *E. coli*

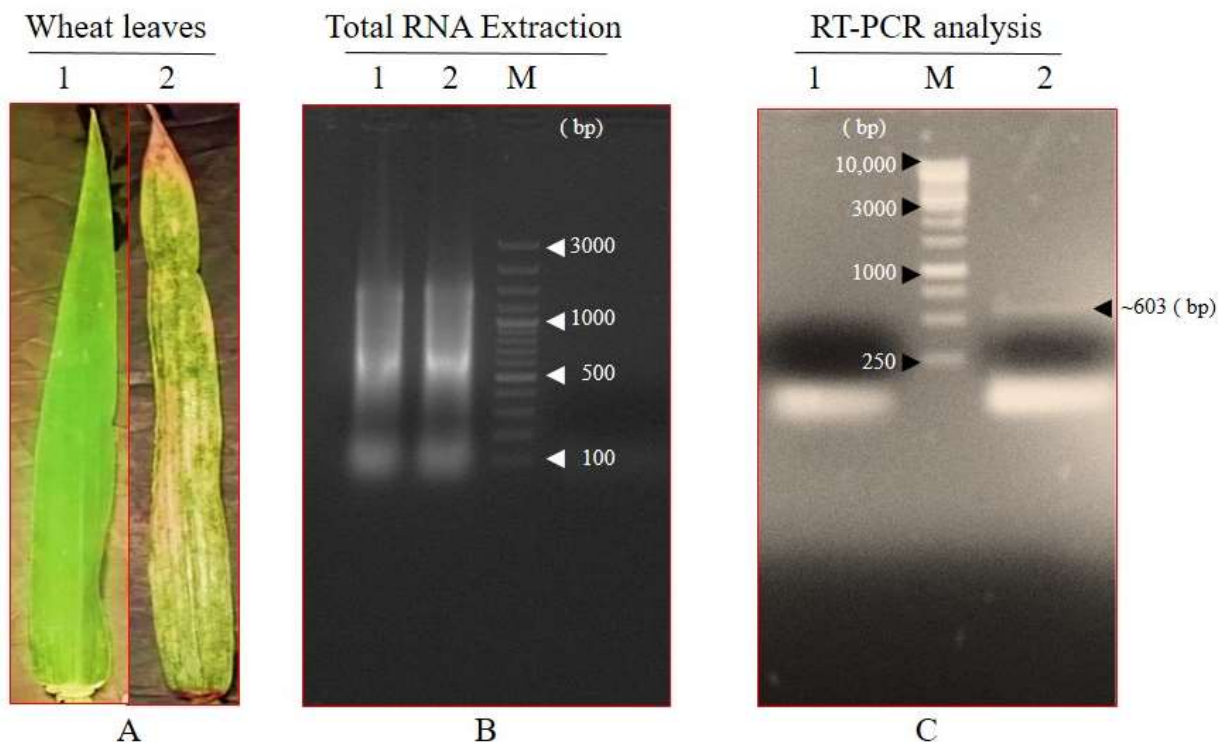


Fig. S3 RT-PCR-based detection of BYDV infection in wheat leaves. (A) Photograph of wheat leaves with and without symptoms (1= non-symptomatic; 2= symptomatic); (B) Total RNA extraction from sugarcane leaves (1= non-symptomatic; 2= symptomatic); (C) RT-PCR amplification of BYDV CP gene (1= healthy leaves, 2= infected leaves), and M= 100 bp and 1kb DNA ladder