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RESEARCH PAPER

Agrobacterium mediated callus based transformation protocol for transferring *DREB1A* gene in wheat (*Triticum aestivum* L.)

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Key Message: Four wheat cultivars were evaluated for transferring of *DREB1A* gene through callus based *Agrobacterium* mediated transformation protocol. The Lasani-08 was selected to be the best one and the transgenic plants were developed using its calli.

ABSTRACT: The present study was designed to optimize protocol for transferring *DREB1A* gene in wheat and selection of one suitable cultivar among Lasani-08, Inqlab-91, Chakwal-97 and GA-02 for the development of transgenic plants. The already optimized callus induction and regeneration media was used for tissue culture. Different treatments of optical density (O.D.), hygromycin, cefotaxime and acetosyringone concentrations were optimized. After optimization of these parameters, the four wheat cultivars were compared with different treatments of infection and co-cultivation time. The Lasani-08 was found to be one of the best cultivars among four cultivars based on the maximum hygromycin resistant calli (18.36%). Its calli were shifted on regeneration media for the development of transgenic plants. The transformed gene was confirmed using gene specific primers through conventional PCR. A lethal dose (50 mg/l) of hygromycin for selection of transformed calli, 300 μM of acetosyringone to enhance transformation process and 500 mg/l cefotaxime to eliminate *Agrobacterium* after co-cultivation, were optimized. The infection time (5 min) and co-cultivation time (48 h) were found to be the most suitable parameters for the maximum transformation efficiency. PCR confirmed that 0.38% of regenerated plants were transgenic. This study will provide a way for transferring other agronomically important genes in wheat cultivars for their improvement.

Keywords: Acetosyringone, *Agrobacterium*, Cefotaxime, Genetic transformation, Hygromycin, Wheat

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is the famous staple food among the peoples of the world including Pakistan. The current report of 2018 indicated that population growth rate in the world is around 1.09% per year (//www.worldometers.info/world-population/). But the cultivation of wheat crop and its yield are not significantly increasing to meet the needs of the growing population. As a result, a gap has been created between its demand and supply. The main reason of this gap is abiotic factors especially drought that affects its yield and decreases the cultivated land due to urbanization. The gap can be filled by improving different traits of wheat that may increase its yield.

Different approaches are being used to develop new varieties of wheat or to improve its existing cultivars. One of them is *Agrobacterium* mediated transformation. The transformation technology is the latest and most effective tool for crop improvement. The early reports of transformation in wheat were published in 1992, 1993 and 1997 (Vasil et al., 1992; Vasil et al., 1993; Weeks et al., 1993; Cheng et al., 1997). Rashid et al. (2010); Raja et al. (2010)

reported the transformation protocol using *Xa21* gene for bacterial blight in wheat. Dehydration responsive element binding protein (*DREB1A*) enhanced tolerance against the drought stress. This transcription factor has been used to improve the rice for abiotic stress tolerance (Datta et al., 2012; Ravikumar et al., 2014). However, Pellegrineschi et al. (2004) stated that *DREB1A* showed a significant improvement in wheat for osmotic stress tolerance. Though, these protocols were not reproducible. These reports indicated that wheat is recalcitrant in transformation due to different factors such as cultivars, cefotaxim, acetosyringone, hygromycin concentration, optical density (O.D) of *Agrobacterium*, infection time and co-cultivation period etc. These factors vary from crop to crop and even with in the same species having different cultivars.

Keeping in view these facts, the present study was planned for optimization of various transformation factors. After optimization, four best tissue culture responsive wheat cultivars (Mehmood et al., 2013) were compared for their transformation efficiency response based on hygromycin resistant calli. The best one i.e. Lasani-08 was further used to develop transgenic plants incorporating *DREB1A* gene under the control of 35S promoter through *Agrobacterium* strain EHA101 using callus based transformation protocol.

MATERIALS AND METHODS

Callus induction

This work was done in Genetic Transformation Laboratory, NIGAB, NARC, Islamabad, Pakistan. Seeds of selected wheat cultivars (Chakwal-97, Lasani-08, Inqalab-91 and GA-02) were used as explants for callus induction. The optimized protocols of callus induction for each cultivar (Mehmood et al., 2013) were used for callus formation. MS media with 8 g/l agar supplemented with 2, 4-D (3 mg/l) for Lasani-08 and Inqlab-91, while 2.5 mg/l for Chakwal-97 and 3.5 mg/l for GA-02 were used for callus formation. While in regeneration media, 3 mg/l BAP was used to regenerate the hygromycin resistant (transformed) calli of Lasani-08.

Optimization of lethal dosage of hygromycin

Construct had a plant selectable marker gene (*hpt*) which is resistant to hygromycin, therefore a lethal dosage of hygromycin was optimized using its various concentrations (0, 10, 30, 50, 70, 90 and 100 mg/l) for non-transformed calli. These treatments were applied in MS media accompanied with 0.8% agar and 30% sucrose. The 21 days old calli were used in this experiment for the lethal dose optimization. The 12 replication of each treatment were used. Data was recorded after 2 to 3 weeks.

Bacterial strain and plasmid

Transformation was done by using *Agrobacterium tumefaciens* strain EHA-101 having plasmid containing *DREB1A* gene (drought resistant), hygromycine resistance gene (plant selectable marker gene) and kanamycin resistant gene (bacterial selectable marker gene) (Fig. 1).

Agrobacterium culture condition

To culture the *Agrobacterium* strain EHA101 Yeast Extract Peptone (YEP) media accompanied with kanamycine (50 mg/l) was used. This culture was placed on rotary shaker for 24 hour in dark at 28 ^{0}C. The speed of rotary shaker adjusted 105-110 rpm.

Optimization of acetosyringone concentration and optical density of *Agrobacterium*

Different levels (0.5, 0.8, 1.1 and 1.4) of O. D. (Optical Density) for *Agrobacterium* culture was measured at 600 nm by photonanometer, then the culture was centrifuged for 10 min at 3000 rpm speed. The pellet was re-suspended in liquid media (callus induction media without agar) containing various levels (100, 200, 300 and 400 μM) of acetosyringone for optimization. These treatments of acetosyringone were also used in co-cultivation media.

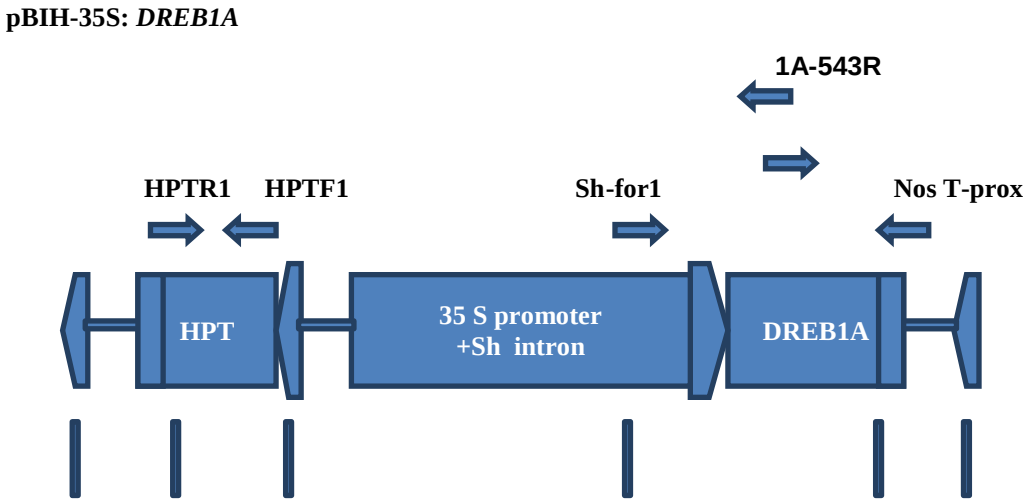

Fig. 1 Construct diagram (Source: NIGAB, NARC Islamabad, Pakistan)

Co-cultivation and selection

After the infection of calli by *Agrobacterium*, the calli were placed on co-cultivation media for 48 h. The cocultivated calli were washed with autoclaved distilled water and callus induction media containing cefotaxime (500 mg/l). After washing, these were shifted to selection media containing hygromycine (50 mg/l). The optimum level of acetosyringone and O.D. were recorded based on percentage number of hygromycine resistant calli after placing on the selection media.

Optimization of cefotaxime

After co-cultivation step, the infected calli were washed with three different concentrations of cefotaxime i.e. 250, 500 and 750 mg/l in different media. Each treatment was replicated four times. The data was recorded using the following formula:

$$\text{Disinfection rate of cefotaxime (\%)} = \frac{\text{No. of calli showing no } \textit{Agrobacterium} \text{ growth}}{\text{Total no. of calli used}} \times 100$$

Optimization of infection and co-cultivation time

The proliferated and healthy calli of four wheat cultivars (Lasani-08, Chakwal-97, Inqlab-91 and GA-02) were submerged for 1, 5 and 10 min (infection time) in *Agrobacterium* suspension 0.8 O.D. containing acetosyringone (300 µM). These infected calli were cocultivated on cocultivation media [MS salts plus vitamins (4.3 g/l), Myo-inositol (0.1 g/l), Sucrose (30 g/l), agar (6 g/l), pH (5.75-5.8) and acetosyringone (300 µM)] for 48 and 72 h at 28 ºC.

Selection of suitable wheat cultivar for transformation and plantlet formation

The data for each cultivar was recorded after three weeks as percentage of hygromycin resistant calli. One wheat cultivar showing best result was selected for additional transformation experiments.

Development of transgenic plants

The *DREB1A* gene was transformed by callus based method of *Agrobacterium* mediated transformation. The seeds of wheat cultivar (Lasani-08) were used as explants. Optimized callus induction and regeneration media (Mehmood et al., 2013) was used in callus based transformation. The 21 days old calli were co-cultivated with *Agrobacterium* for 48 h, then washed with cefotaxim (500 mg/l) before transferring on selection media having 50 mg/l hygromycin. The selected calli were transferred on regeneration media for regeneration of plantlets from the transformed calli. After shoot development, they were shifted on half strength MS media without any growth regulator for roots development. Later on, they were shifted to hydroponic medium for acclimatization and roots elongation. They were shifted to pots in a glass house after acclimatization and roots development.

Confirmation of transgenic plants

The genomic DNA was isolated from all putative transgenic plants through CTAB protocol (Murray and Thompson, 1980). *DREB1A* gene specific primers were used for PCR amplification purpose.
F 5'- TGAACTCATTTTCTGCTTT-3'
R 5'-TAATAACTCCATAACGATA-3'
The PCR product was run on gel electrophoresis for half an hour at 100 V current. Agarose gel (1%) was used and it was envisioned by gel documentation.

Transformation efficiency

Transformation efficiency was calculated on the basis of PCR positive plants using the following formula:

$$\text{Transformation efficiency (\%)} = \frac{\text{Total number of hygromycin resistant calli used on regeneration media}}{\text{Total number of PCR positive plants}} \times 100$$

Statistical analysis

For each individual treatment, their data was recorded in percentage and analyzed using ANOVA. The significant difference of means was compared at 5% alpha level.

RESULTS AND DISCUSSION

Lethal dose of hygromycin

The number of browning calli increased along with the increasing amount of hygromycin from 10 to 50 mg/l, while necrosis of calli started at 30 mg/l hygromycin to onward. The growth of calli reduced by increasing the amount of hygromycin. The data showed that 50.83% calli became necrotic and showed no growth when 50 mg/l hygromycin was used (Table 1). So, 50 mg/l was considered as a lethal dose of hygromycin for selection of the transformed calli. Beyond this concentration of hygromycin, the complete necrosis of calli was observed after two to three weeks therefore, no growth was noticed. The lethal dose of hygromycin (50 mg/l) optimized in this study has been supported by many previous reports (Rashid et al., 2001; Kumar et al., 2004; Ignacimuthu et al., 2006; Raja et al., 2010; Rashid et al., 2010) and opposed to other studies like Mihaljevic et al. (2001); Chen and Punja (2002); Reddy et al. (2016) who reported 15 mg/l, 2.0 mg/l and 75 mg/l, respectively. These dissimilarities in the hygromycin concentration may be due to difference in genotypes.

Optimization of cefotaxime

Results in Table 2 demonstrate that cefotaxime (500 to 750 mg/l) used in media 3 produced 100% calli that were safe from contamination and no growth of *Agrobacterium* was seen due to its complete elimination. There was toxic effect on cells of calli using 750 mg/l cefotaxime. The results revealed that 500 mg/l cefotaxime was suitable to get rid of bacterial contamination. The significant difference was seen in different levels of cefotaxime concentration. It has also been reported that *Agrobacterium* was observed on the calli using 250 mg/l concentration of cefotaxime. The growth of calli slowed down and ultimately the rate of transformation dropped due to the contamination. In this experiment, 750 mg/l cefotaxime was applied only in washing or pre-selection media for controlling the growth of *Agrobacterium*. It means that antibiotic cefotaxime (500 mg/l) is optimized concentration and it should be used in

pre-selection and washing media for complete elimination of *Agrobacterium*. The previous study reported the same concentration (500 mg/l) of cefotaxime to kill and remove the *Agrobacterium* (Khoudi et al., 2009; Reddy et al., 2016). Some other concentrations of cefotaxime were also reported such as 200 mg/l (Xing et al., 2008), 400 mg/l (Yenchon and Te-chato et al., 2012). In another report, carbencillin (250 mg/l) along with cefotaxime (500 mg/l) was reported to kill the *Agrobacterium* (Raja et al., 2010). These differences might be due to the use of different *Agrobacterium* strains, crops, and explants.

Optimization of optical density of *Agrobacterium* and acetosyringone

The data in Table 3 indicate that all treatments of acetosyringone were statistically different. When 300 µM acetosyringone was used in co-cultivation and inoculation media, then survival rate of calli on selection media was 9.84%. The survival rate of calli (hygromycin resistant calli) on other concentration of acetosyringone was low. Similarly, in case of optical density, all treatments were significantly different to each other. The maximum hygromycine resistant calli (8.75%) was observed when O.D of 0.8 for *Agrobacterium* was used. All other showing less number of hygromycine resistant calli so it is concluded that 0.8 O.D. was found to be optimum for maximum transformation of gene into the calli. Data also indicated that when 300 µM acetosyringone and 0.8 O.D. of *Agrobacterium* was used then maximum (13.12%) hygromycine resistant calli were recorded. The optimized concentration of acetosyringone (300 µM) in this study was slightly same to its optimum concentration in previous studies such as 200-400 µM (Raja et al., 2010) and 350 µM (Tripathi et al., 2010). The greater variation was seen in optimized concentration (100 µM, 200 µM and 400 µM) of acetosyringone in previous studies (Amoah et al., 2001; Rashid et al., 2010; He et al., 2010; Rashid et al., 2012). The previous studies i.e. Khanna and Daggard (2003); Jones et al. (2005) reported the same O.D. (0.8 to 1.0). Similarly, the optimized O.D. 1-1.5 have been reported by the previous research studies by Cheng et al. (1997), O.D. 1.3 by Amoah et al. (2001); O.D. 0.5-0.6 by Cheng et al. (2003) and Bi et al. (2006) and O.D. 0.5 by Rashid et al. (2010). These differences in levels of O.D. might be due to the variation in promoters, *Agrobacterium* strain, genotype and types of explant as well. Similar to our findings, Rashid et al. (2010) proclaimed that when *Agrobacterium* O.D. was increased from 0.5 up to 0.75 and 1, then their growth on calli could not be controlled.

Optimization of co-cultivation and infection time

The results showed that significant differences ($P<0.05$) were observed among co-cultivation, infection time and wheat lines. The 48 h of co-cultivation time was the most suitable for all infection time. The lasani-08 showed maximum hygromycin resistant calli (18.33%) when *Agrobacterium* was infected to calli for 5 min and co-cultivated for 48 h. While Inqlab-91 showed minimum hygromycin resistant calli (2%) when infection time was 10 min and co-cultivation time was 72 h. The low percentage of hygromycin resistant calli was observed when infection and co-cultivation time were increased. The earlier study also reported that infection time is an important factor which affects the transformation efficiency of *Agrobacterium* (Nan et al., 2006; Qiu et al., 2007). Amoah et al. (2001) reported in their article that inoculation, co-cultivation time and cell density affect the rate of transformation of foreign gene in wheat. The present study shows that when co-cultivation time increased beyond 48 h, the percentage of hygromycin resistant calli (transformation efficiency) became less due to the overgrowth of *Agrobacterium* on calli. The co-infection time (5 min) reported in this study was contradicted to infection time (3 min) that was reported by Roy et al. (2006) who achieved the maximum transformation efficiency in tomato. While 10 min infection time was reported for tomato by Wu et al. (2006). These differences might be due to the selection of different explants and crops in their studies.

Table 1 Effect of hygomycine on wheat calli

Hygromycin (mg/l)	Calli showing browning (%)	Calli showing necrosis (%)	Calli showing growth (%)
0	0^{e}	0^{e}	100^{a}
10	22.08^{d}	0^{e}	77.5^{b}
30	38.75^{b}	31.25^{d}	27.91^{c}
50	49.16^{a}	50.83^{c}	0^{d}
70	31.25^{c}	68.75^{b}	0^{d}
90	0^{e}	100^{a}	0^{d}
100	0^{e}	100^{a}	0^{d}

Alpha = 0.05; Browning of calli = Critical value for comparison = 2.8958; Necrosis of calli = Critical value for comparison = 3.2808; Growth of calli = Critical value for comparison = 2.9265

Table 2 Effects of cefotaxime in different media to control the growth of *Agrobacterium*

Cefotaxime (mg/l)	Media 1	Media 2	Media 3	Mean	Remarks
250	$*5.83^{ef}$	4.15^{f}	45^{b}	18.32^{c}	Healthy calli
500	8.32^{de}	7.50^{def}	$\mathbf{100^{a}}$	38.61^{b}	Healthy calli
750	12.50^{c}	11.65^{cd}	100^{a}	41.83^{a}	Necrosis start
Mean	8.88^{b}	7.76^{b}	81.66^{a}		

Note: Media 1= Cefotaxime in washing media; Media 2 = Cefotaxime in pre-selection media; Media 3 = Cefotaxime in washing and pre-selection media both; * Uncontaminated calli %
Alpha = 0.05; Critical value for cefotaxime comparison = 2.4089; Critical value for media comparison = 2.4089; Critical value for interaction between media and cefotaxime comparison = 4.1724

Table 3 Optimization of acetosyringone concentration and the optical density of *Agrobacterium* for transformation

Acetosyringone (μM)	Transformation efficiency on the basis of hygromycine resistant calli (%)				Mean
	Bacterial optical density				
	0.5	0.8	1.1	1.4	
100	2.5^{fg}	3.75^{ef}	2.5^{fg}	1.25^{g}	2.5^{d}
200	4.37^{e}	7.5^{c}	6.25^{cd}	4.38^{e}	5.63^{c}
300	9.37^{b}	13.12^{a}	9.37^{b}	7.5^{c}	9.84^{a}
400	6.87^{c}	10.62^{b}	7.5^{c}	5.0^{de}	7.50^{b}
Mean	5.78^{b}	8.75^{a}	6.41^{b}	4.53^{c}	

Alpha = 0.05; Acetosyringone = Critical value for comparison = 0.8978; Optical density = Critical value for comparison = 0.8978; Interaction of acetosyringone and O.D = Critical value for comparison = 1.7956

Table 4 Effects of co-cultivation period and infection time on the rate of transformation efficiency

Treatments (infection + co-cultivation time)	Co-cultivation time (h)	Infection time (min)	Lasani-08	Ga-02	Chakwal-97	Inqlab-91	Mean
1	48	1	8.97^{fg}	8.67^{fg}	5.33^{ijk}	4^{jklm}	6.74^{d}
2		5	18.36^{a}	14.66^{b}	11.33^{cde}	8.67^{fg}	13.25^{a}
3		10	15.33^{b}	13.33^{bc}	9.33^{efg}	8^{fgh}	11.5^{b}
4	72	1	4.66^{jkl}	4.67^{jkl}	4.67^{jkl}	2.67^{lm}	4.16^{f}
5		5	11.33^{cde}	12^{cd}	10^{def}	7.33^{ghi}	10.16^{c}
6		10	9.33^{efg}	6^{hij}	3.33^{klm}	2.0^{m}	5.16^{e}
	Mean		11.33^{a}	9.88^{b}	7.33^{c}	5.4^{d}	

Critical value for comparison of treatment mean = 1.0291; Critical value for comparison of cultivar means = 0.8402; Critical value for comparison of interaction = 2.0581

Table 5 Callus based transformation and development of the transgenic plants

Details	Number	% age	Description of % age
Total calli used	2880		
Hygromycin resistant calli	518	17.98	(b/a)x100
Number of calli regenerated	10	1.93	(c/b)x100
Survival in hydroponic	6	1.15	(d/b)x100
Survival in pots	2	0.38	(e/b)x100
PCR positive	2	0.38	(f/b)x100
Fertile	1		
Sterile	1		

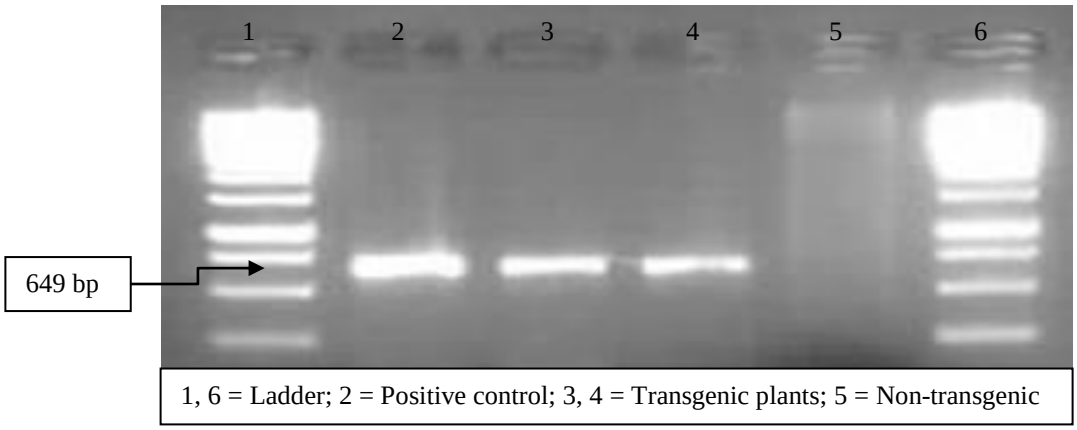

Fig. 2 PCR amplification of *DREB1A* gene

Development and confirmation of transgenic plants from calli of Lasani-08

Table 5 indicated that 2880 calli of Lasani-08 were co-cultivated with *Agrobacterium*. The hygromycine resistant calli were transferred on regeneration media for regeneration out of which only 1.93% calli were regenerated. After the elongation of roots in hydroponic, the six plantlets were shifted to glasshouse condition for proper acclimatization and growth. Later, only two plants were survived and remained positive after PCR (Fig. 2). The transformation efficiency (0.38%) was recorded (Table 5). The earlier studies reported that the wheat varieties i.e. Millewa, Cadenza, Baldus, Vesna, *T. durum, T. dicoccum and T. turgidum* showed better response for transformation which were transformed by different genes (Mooney et al., 1991; Amoah et al., 2001; Chugh & Khurana, 2003; Wu et al., 2003; Mitic et al., 2004; Patnaik et al., 2006; He et al., 2010). In present study, Lasani-08 showed the best response for transformation of *DREB1A* gene as compared to other cultivars used. These differences are due to their different capability for callus formation, regeneration and transformation. A spring wheat "Bobwhite" was reported that has high regeneration, callus formation and transformation ability (Janakiraman et al., 2002).

CONCLUSION

The 50 mg/l hygromycin and 500 mg/l cefotaxime were optimized for selection of transformed calli and to kill the excessive *Agrobacterium*, respectively. Moreover; bacterial O.D. (0.8) at 600 nm, acetosyringone (300 μM), infection time (5 min) and co-cultivation time (48 h) were optimized to enhance the transformation of *DREB1A* gene in wheat. The wheat cultivar Lasani-08 exhibited the supreme response for transformation as compared to others. The transformation efficiency of *DREB1A* was calculated as 0.38% confirmed by PCR. In this study, it was noted that transgenic lines developed by this method were very sensitive in nature, so serious care should be undertaken from initial step to the final stage. These findings will be helpful in future for transformation of other genes of interest in this cultivar for its improvement.

Author Contribution Statement: Khalid Mehmood conducted experiments and wrote his PhD Thesis. Muhammad Arshad and Ghulam Muhammad Ali planned and designed the research study. Abdul Razzaq and Rehmatullah Qureshi contributed in writing the research article. All the authors read and approved the manuscript.

Conflict of Interest: The authors clarify that they have no conflict of interest.

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