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Original Article

Molecular typing of *Dichelobacter nodosus* in ovine footrot and expression of its fimbrial biogenesis protein PilQ as a candidate footrot vaccine

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Abstract

Background: Footrot is a highly contagious disease of the feet in sheep and goats. **Aims:** The study was conducted to determine the serogroup distribution of *Dichelobacter nodosus* in sheep affected by footrot on organized farms and flocks reared by farmers across four districts of the Kashmir Valley. **Methods:** Sheep from randomly selected organized farms and flocks raised by farmers and the Bakerwal tribes were screened for footrot lesions based on clinical signs, particularly lameness. Two hundred sixty swab samples were collected from sheep exhibiting footrot lesions, and *D. nodosus* infection was confirmed using 16S rRNA-specific PCR. For serogroup subtyping, multiplex PCR was performed. Furthermore, we utilized the pET28a expression vector to clone and express the outer membrane fimbrial protein antigen, which was confirmed using SDS-PAGE and Western blot analysis. **Results:** *D. nodosus* was detected in 149 samples by 16S rRNA-specific PCR. Serogroups B and E were detected in 85.9% and 10.74% of positive samples, respectively, while both serogroups were detected in 3.36% of the samples. The outer membrane domain containing the major antigenic epitopes of the *pilQ* gene of *D. nodosus* was successfully cloned into the pET28a expression vector. **Conclusion:** The prevalence of ovine footrot was found to be low during the study period. Serogroup B of *D. nodosus* was found to be predominant, while serogroup E was detected exclusively in some isolated flocks reared by farmers. The *pilQ* gene was successfully expressed in *Escherichia coli* and purified to near homogeneity, making it suitable for further exploitation as a serogroup-independent vaccine candidate against footrot.

Key words: *Dichelobacter nodosus*, Ovine footrot, *pilQ*, Serogrouping

Introduction

Footrot is a highly contagious disease that affects the feet of sheep and goats, and it has also been reported in cattle, horses, pigs, deer, and mouflon (Zanolari *et al*., 2021). The disease is caused by the synergistic action of several bacterial species, with *Dichelobacter nodosus* (*D. nodosus*) serving as the primary causative agent (Depiazzi and Richards, 1985; Farooq *et al*., 2021). This organism is a highly fastidious anaerobe that requires specialized media for growth and multiplication (Qureshi *et al*., 2021). Immunologically, *D. nodosus* is heterogeneous, with ten distinct serogroups (A to I and M) identified based on variations in the fimbrial antigen, which confers protective immunity (Ghimire *et al*., 1998). However, there is no cross-protection among the serogroups, and multivalent vaccines demonstrate reduced efficacy due to antigenic competition (Prosser *et al*., 2020). Furthermore, evidence suggests that transformation-mediated serogroup conversion of *D. nodosus* could occur on ovine hooves (Kennan *et al*., 2003). Therefore, continuous surveillance of *D. nodosus* serogroups is essential for effective, serogroup-specific vaccination programs to control ovine footrot in endemic areas.

Ovine footrot typically begins as interdigital dermatitis and progresses to destruction of the epidermal matrix and necrotic separation of the hoof from the underlying soft tissue, resulting in lameness and potentially causing substantial economic losses and animal welfare concerns. Clinical presentations vary and can be classified using a foot scoring system (Farooq *et al*., 2021). Although the disease exhibits a wide spectrum of severity, three forms are generally recognized: benign,

intermediate, and virulent footrot, caused by the respective strains of *D. nodosus* (Stewart *et al*., 1989). Farm management practices and favorable environmental factors influence the spread and progression of the disease (Wassink *et al*., 2003). Identification of serogroups of *D. nodosus* in footrot lesions using conventional methods is tedious and time-consuming. Polymerase chain reaction (PCR) with *16S rRNA* gene-specific primers for identification (La Fontaine *et al*., 1993; La Fontaine et al., 1999) and multiplex PCR (m-PCR) with serogroup-specific primers for serogrouping (Dhungyel *et al*., 2002) have been employed for the characterization of *D. nodosus* without the need for organism cultivation.

In India, the disease has been reported as enzootic in the UT of Jammu and Kashmir for the past two decades (Wani *et al*., 2019). The disease has also been reported from other states, including Himachal Pradesh, Andhra Pradesh, Tamil Nadu, and Kerala (Sreenivasulu *et al*., 2013; Anto *et al*., 2014; Thomas *et al*., 2018). Epizootiological studies have been conducted, revealing the presence of serogroups B, C, E, and I, with a predominance of serogroup B, in the Kashmir valley (Wani *et al*., 2004; Wani *et al*., 2007; Hussain *et al*., 2009; Farooq *et al*., 2010; Rather *et al*., 2011; Kabli *et al*., 2014). Similarly, serogroups A, B, C, E, F, H, and I have been detected in the southern states of India (Sreenivasulu *et al*., 2013; Kumar *et al*., 2016). Our laboratory has identified immunogenic proteins for developing a universal vaccine against different serogroups of *D. nodosus*. Immunogenic outer membrane proteins (OMPs), including the fimbrial biogenesis protein PilQ, have been identified as potential universal serogroup vaccine candidates.

The *pilQ* gene encodes an outer membrane protein (OMP) that polymerizes into a secretin complex and plays an important role in fimbrial extrusion, natural transformation, and protease secretion (Averhoff *et al*., 2004; Hamilton *et al*., 2006). The surface component of the PilQ protein contains major antigenic epitopes and therefore could be exploited as a universal vaccine candidate against ovine footrot. The present investigation aimed to determine the serogroup distribution of *D. nodosus* from sheep affected with severe footrot, clone and express the *pilQ* gene of *D. nodosus* in *E. coli*, and purify recombinant PilQ protein for use as a potential vaccine candidate against footrot.

Materials and Methods

Clinical examination of sheep and sample collection

The Kashmir valley is home to a sheep population of 1.9 million, distributed among organized farms, sheep flocks managed by farmers, and migratory sheep flocks tended by nomadic Bakerwal tribes. Randomly selected organized farms and farmers reared flocks, and Bakerwal tribal flocks were inspected during the winter season spanning from December 2018 to April 2019. Animals exhibiting signs such as lameness were further examined for clinical indicators typical of footrot, including interdigital dermatitis and necrotizing inflammation with underrunning of part or all of the soft and hard horn of the hoof.

Exudates of footrot lesions with a lesion score ranging from 2 (interdigital dermatitis) to 4 (underrunning of the hard horn of the hoof) (Stewart *et al*., 1989) were collected from 260 naturally infected sheep. Samples were obtained using a cotton swab and immediately inoculated into a 1.5 ml microcentrifuge tube containing TASH broth. These samples were then transported to the laboratory on ice for subsequent processing.

Detection and serogrouping of *D. nodosus*

Swabs containing exudates were vortexed, thoroughly squeezed, and then removed from the tubes. The tubes were subsequently centrifuged at 8000 ×g for 10 min, and the resulting sediment was collected. The sediment was washed twice with distilled water and resuspended in 50 µL of nuclease-free water. This suspension was boiled for 5 min, cooled on ice for 10 min, and then centrifuged at 10,000 ×g for 10 min. Two microliters of the prepared samples were used as the template for PCR. All the samples underwent PCR for the detection of the *16S rRNA* gene of *D. nodosus*, as described previously by La Fontaine *et al*. (1993).

Samples confirmed positive for *D. nodosus* by *16S rRNA* gene-specific PCR were further subjected to serogrouping using multiplex PCR using A-I serogroup-specific primers (Dhungyel *et al*., 2002). DNA extracted from JK-07B and JKS25E strains of *D. nodosus* served as positive controls for serogroups B and E, respectively. Additionally, positive serogroup controls (A-I) were obtained from Dr. Om Dhungyel (University of Sydney). All the oligonucleotide primers utilized in this study were procured from GCC Biotech, Kolkata, India.

Cloning and expression of the *pilQ* gene

PCR amplification of the pilQ gene

A pair of primers Pil-Q Ntr-Forward-5´GTC GCG GAT CCG TTG CAG GAG GAA ATA AT3´ and Pil-Q Ntr-Reverse-5´GGT GCT CGA GTT AGT TCA TTC TTT CAT CAA TAG T3´ incorporating *Bam*HI and *Xho*I restriction sites (underlined), respectively, were designed based on the complete coding sequence of the *pilQ* gene of *D. nodosus* (GenBank accession no. MN067549). Initially, the amino acid sequence of the PilQ outer membrane protein (OMP) was analysed using SignalP 5.0 and TMHMM 2.0 online tools to detect any signal peptide (SP) and transmembrane domains. Antigenic index analysis of the PilQ OMP was subsequently performed using the Protean program of DNASTAR Lasergene software. The primers amplified a 1242 bp N-terminal outer membrane domain (from nucleotide no. 79 to 1320) of the 2205 bp *pilQ* gene, which contains the major antigenic epitopes.

The gene segment was amplified from DNA template extracted from the JK-07B strain of *D. nodosus* using high-fidelity DNA polymerase, Phusion *Taq* (Thermo

Fisher Scientific, Waltham, MA, USA). he amplification cycles consisted of 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min. The PCR product was visualized on a 1.5% agarose gel and purified using the MinElute PCR Purification Kit (Qiagen, Germany).

Cloning the pilQ gene into the cloning vector

The gel-purified PCR products were digested with *Bam*HI and *Xho*I restriction enzymes (Fermentas, USA). Specifically, 10 µL of purified PCR product was double-digested using 1.0 µL each enzyme along with 2.5 µL of enzyme-specific buffer; the final volume was adjusted to 20 µL with nuclease-free water. The mixture was then incubated at 37°C for 15 min. The digested products were analysed on a 2% agarose gel and eluted using the QIAquick Gel Extraction Kit (Qiagen, Germany). Simultaneously, the pET28a plasmid was also digested with the same enzymes under similar conditions. The eluted PCR products of the *pilQ* gene from *D. nodosus* and the digested plasmid were ligated using T4DNA ligase. The resulting recombinant plasmid was subsequently transformed into *E. coli* DH5α competent cells via heat-shock treatment and plated onto LB agar containing kanamycin (30 µg/ml). Recombinant clones were screened for the presence of the *pilQ* gene insert by colony PCR and restriction digestion, followed by outsourcing for sequencing using the Sanger sequencing method.

Expression of recombinant PilQ protein

Following confirmation of the insert, the recombinant plasmid was transformed into *E. coli* BL21 cells. Recombinant clones were induced for protein expression using isopropyl β-D-1-thiogalactopyranoside (IPTG) in LB broth containing kanamycin. Aliquots of 1 ml from IPTG-induced cultures were collected hourly from 2 to 8 h of incubation at 37°C and subsequently pelleted by centrifugation at 8000 ×g. The pellets were resuspended in 50 µL of 1X Laemmli buffer in Eppendorf tubes and then boiled for 10 min. After cooling, the samples were subjected to SDS-PAGE using a 12% acrylamide gel to analyze the expression pattern of the recombinant protein (Laemmli, 1970). Upon completion of electrophoresis, the gel was stained overnight with Coomassie Brilliant Blue R-250, followed by destaining with a solution containing 10% acetic acid, 45% methanol, and 45% water. Finally, the gel was visualized and photographed.

Western blotting

Whole cell antigens separated on polyacrylamide gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond LFP, UK) following the method of Towbin *et al*. (1979). Blotting was performed at 40 V and 250 mA current for 2 h and 50 min in a semidry western blot machine (TE 70 ECL, Amersham Biosciences, UK). The membrane with transferred proteins was then blocked with 5% skimmed milk in tris-buffered saline (TBS) for 1 h at room temperature with continuous shaking, followed by washing with 0.05% TBS-Tween 20 (TBS-T). Subsequently, the PVDF membrane was incubated with HRP-linked Anti-His monoclonal antibody (Cloud Clone Corporation, USA) for 1.5 h at room temperature and then washed with TBS-T. Finally, color development was initiated by adding 10 ml of 0.05% DAB in TBS mixed with 3 µL of 30% hydrogen peroxide, and incubation was carried out in the dark for 5-10 min. The reaction was stopped by washing with distilled water, after which the membrane was air-dried and visualized.

Purification of recombinant PilQ protein

The poly-histidine (6X His) tagged fusion protein was purified under denaturing conditions using Ni-NTA affinity chromatography (Qiagen, USA). The induced culture was pelleted by centrifugation at 8000 ×g for 15 min, and the pellet was resuspended in lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH 8.0) at 1:20 of the original culture volume. This mixture was incubated at 37°C with shaking for 3 h to ensure complete lysis. The cell suspension was subjected to sonication at 15 Hz pulse for 20 s with a 15 s pause (15 cycles) on ice. The sonicated bacterial culture was centrifuged at 10000 ×g for 15 min at 4°C. The supernatant was carefully collected without disturbing the cell debris, and protein concentration was measured by Bradford assay. Subsequently, it was mixed with Ni-NTA agarose column resin (Qiagen, Germany) in proportions recommended by the manufacturer and incubated overnight at 4°C. The lysate and resin mixture were then carefully loaded into an empty column in a standing position, and the flow-through was collected. The column was washed thrice with wash buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH 6.5). After washing, elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris base, pH 4.5) was overlaid onto the column in 1 ml volumes and collected as aliquots. Finally, a 12% SDS-PAGE gel was run with the eluted protein aliquots to confirm protein purification.

Results

Prevalence, detection, and serogrouping of *D. nodosus*

A total of 260 swab samples from animals exhibiting signs of lameness and typical footrot lesions. These samples originated from organized sheep farms, sheep flocks of the four districts (Ganderbal, Pulwama, Srinagar, and Budgam), and sheep belonging to the nomadic Bakerwal tribe. 16S rRNA *D. nodosus*-specific PCR was performed, and out of 37 samples collected from organized farms, 22 (59.45%) were positive for *D. nodosus* by 16S rRNA PCR (Fig. 1A). Further, multiplex PCR for serogrouping revealed that, among these 22 positive samples, 21 (95.45%) belonged to serogroup B, while one sample (4.5%) showed mixed infection with both serogroups B and E (Figs. 1B and D, and Table 1). Similarly, from the 190 samples collected from sheep flocks in the four districts, 107 (56.3%) were positive for

D. nodosus by 16S rRNA PCR. Serogrouping by multiplex PCR indicated that 89 (83.17%) were serogroup B (Fig. 1B), 14 (13.08%) were serogroup E, and 4 (3.7%) exhibited mixed infection of serogroups B and E (Fig. 1C and Table 3). Likewise, of the 33 samples (3.36%) collected from the Bakerwal tribal flocks, 20 (60.6%) were *D. nodosus* positive, with 18 (90%) belonging to serogroup B and 2 (10%) to serogroup E (Table 2). Overall, 149 (57.3%) samples were found positive for *D. nodosus*, and serogroups B and E were detected in 128 (85.9%) and 16 (10.74%) samples, respectively, while both serogroups were detected in 5

Table 1: Serogroup distribution of *D. nodosus* in five organized farms of Kashmir

Govt sheep breeding farms (district)	Sheep screened	Sheep affected with footrot	Samples positive for *D. nodosus*	Serogroups		
				B	E	B+E
Gowbal, (Ganderbal)	560	2	2	1	0	1
Daksum, (Anantnag)	1500	27	13	13	0	0
Kralpathri, (Budgam)	800	3	3	3	0	0
Fakir Gujri, (Srinagar)	2159	3	2	2	0	0
FVSc & AH, Shuhama, (Ganderbal)	456	2	2	2	0	0
Total	**5475(100%)**	**37(0.67%)**	**22(59.45%)**	**21(95.45%)**	**0**	**1(4.5%)**

Table 2: Serogroup distribution of *D. nodosus* in private sheep flock owners and migratory Bakarwal sheep flocks in four districts of Kashmir

District	Place	Flocks	Sheep screened	Sheep affected with footrot	Samples positive for *D. nodosus*	Serogroup distribution		
						B	E	B+E
Ganderbal	Tullmulla	5	175	21	14	14	0	0
	Wayul	4	121	17	12	0	12	0
	Kangan	3	72	7	4	2	1	1
	Shalbugh	3	97	7	5	5	0	0
	Saloora	4	129	8	7	7	0	0
	Wahidpoora	5	212	11	4	4	0	0
	Alusteng	1	147	8	5	1	1	3
Budgam	Sholipora	5	315	21	11	11	0	0
	Yadipora	1	25	2	0	0	0	0
	Qumero	2	133	12	5	5	0	0
	Wasoo	3	112	7	4	4	0	0
	Ringzabal	3	117	9	5	5	0	0
Srinagar	Fakir Gujri	14	600	22	12	12	0	0
	Khimber	6	322	11	4	4	0	0
Pulwama	Awantipora	2	230	27	15	15	0	0
Total		**61**	**2807 (100%)**	**190 (6.70%)**	**107 (56.30%)**	**89 (83.17%)**	**14 (13.08%)**	**4 (3.70%)**
Ganderbal (Bakerwal sheep)	Kangan and Sonamarg	14	980	33 (3.36%)	20 (60.6%)	18 (90%)	2 (10%)	0
Total		**75**	**3787 (100%)**	**223 (5.89%)**	**127 (56.95%)**	**107 (84.25%)**	**16 (12.60%)**	**4 (3.15%)**

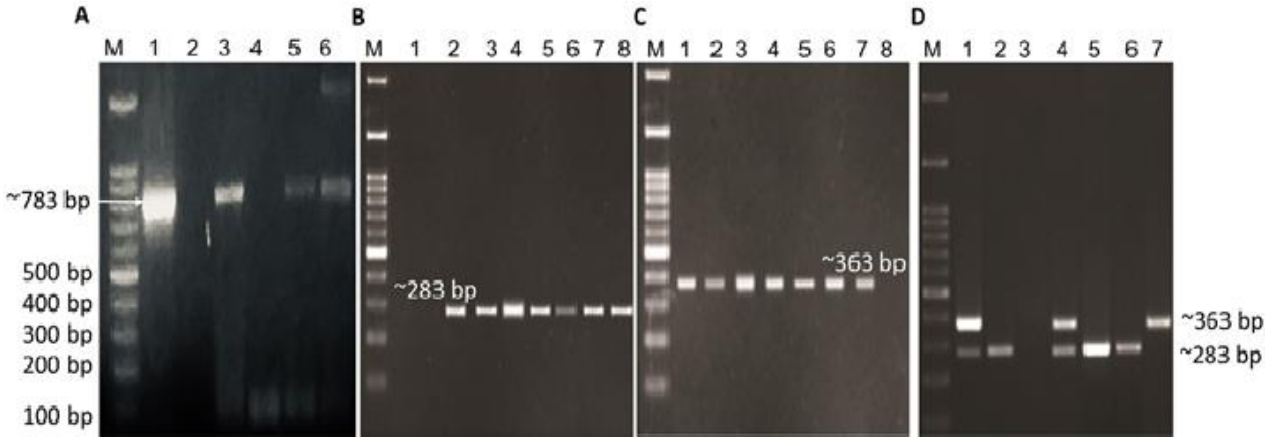

Fig. 1: (**A**) AGE of PCR product for *16S rRNA* gene of *D. nodosus*. Lane M: 100 bp DNA ladder. Lane 1: Positive control (~783 bp), Lane 2: No template control, and Lanes 3-6: Positive and negative samples, (**B**) AGE of PCR product for serogrouping of *D. nodosus* (Serogroup B). Lane M: 100 bp DNA ladder. Lane 1: Negative control, Lanes 2-7: Positive samples, and Lane 8: Positive control, (**C**) AGE of PCR products for serogrouping of *D. nodosus* (Serogroup E). Lane M: 100 bp DNA ladder. Lane 1: Positive control (363 bp), Lanes 2-7: Positive samples, and Lane 8: Negative control, and (**D**) AGE showing mixed serogroup infection with PCR product for serogroup B&E. Lane M: 100 bp DNA ladder. Lane 1: Positive control, Lanes 2, 5, 6: Positive samples for serogroup B, Lane 3: Negative control, Lane 4: Positive samples for serogroup B&E, and Lane 7: Positive samples for serogroup E

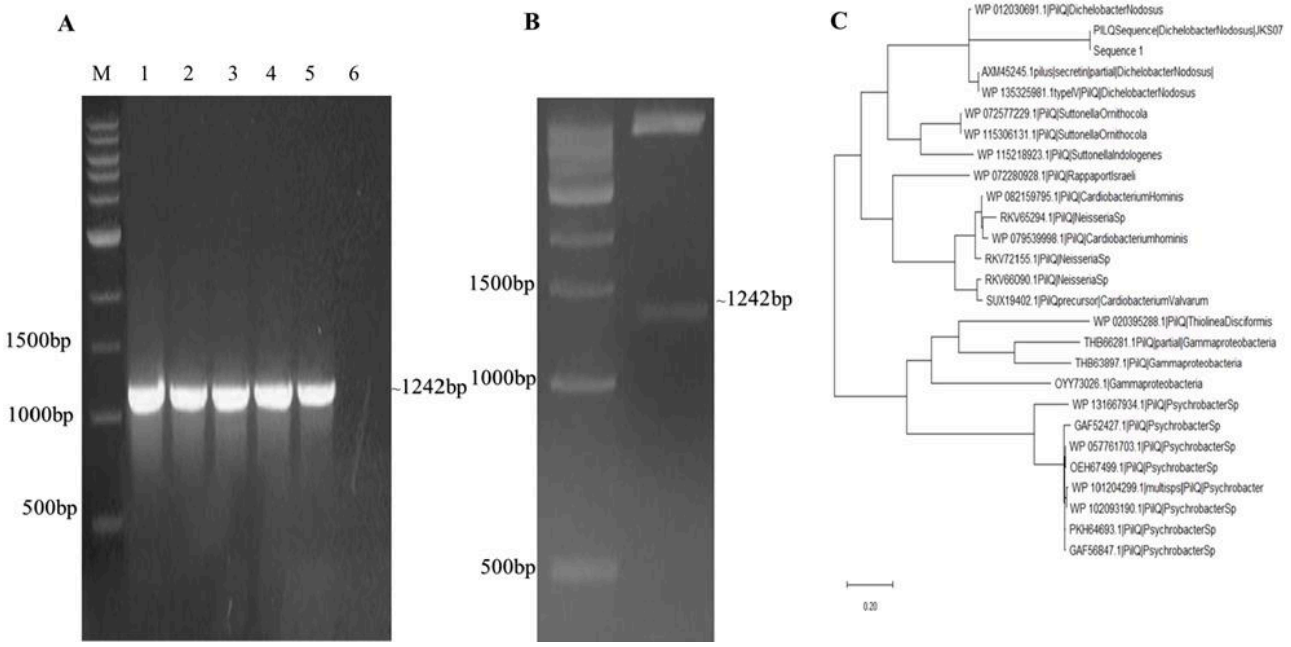

Fig. 2: (**A**) Amplification of *pilQ gene* fragment by polymerase chain reaction (M: 1 Kb plus DNA ladder). Lanes 1-5: Positive samples, and Lane 6: Negative template control, (**B**) AGE of recombinant plasmid releasing *pilQ* gene fragment after restriction digestion (M: 1 Kb plus DNA ladder). Lane 1: Digested recombinant plasmid showing bands for vector and released insert, and (**C**) Molecular phylogenetic analysis of *pilQ* sequence by the Neighbour-Joining method using the MEGAX program. *pilQ* belonging to the same genus were clubbed under the same clade, and the same was observed with our sequence of interest. The JKS07 and other *D. nodosus* sequences forming an Operational Taxonomical Unit (OTU) showed close similarity to the pilQ OTU of *Sutonella ornithicola*

(3.36%) samples. The majority of the flocks carried serogroup B, while few flocks were infected with serogroup E.

Cloning and expression of *pilQ* gene

The primers designed in the present study successfully amplified the 1242 bp N-terminal end of the *pilQ* gene of *D. nodosus* (Fig. 2A). The specific PCR product was subsequently cloned into the pET28a vector after restriction digestion of both with *Bam*HI and *Xho*I enzymes. *E. coli* DH5α cells were transformed with the recombinant plasmid using the heat-shock method. Successful cloning and transformation of the recombinant plasmid were confirmed by colony PCR and restriction digestion of the plasmid extracted from the DH5α cells (Fig. 2B). Molecular phylogenetic analysis using the Neighbor-Joining method was performed with MEGAX software (Fig. 2C). From the resulting tree, it was inferred that *pilQ* sequences belonging to the same genera clustered within the same clade, a pattern also observed for our sequence of interest. The JKS07 and other *D. nodosus* sequences forming an operational taxonomical unit (OTU) showed close similarity to the *pil*Q OTU of *Suttonella ornithocola*. The sequence was submitted to GenBank under accession no. MN067549.

After confirming the cloned *pilQ* gene in the pET28a vector, the recombinant plasmid was transformed into *E. coli* BL21 cells. The IPTG-induced *E. coli* BL21 cells harboring the recombinant plasmid demonstrated optimal expression of PilQ protein after 4 h of induction with 1 mM IPTG at 37°C. The size of the recombinant protein on the polyacrylamide gel was approximately 42.8 kDa (Fig. 3A). Initially, some leaky expression of the recombinant protein was observed, which was addressed by adding glucose to the medium at a final concentration of 10 mM.

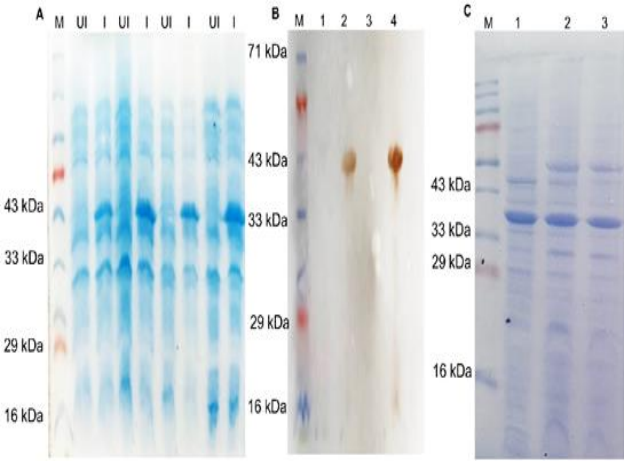

Fig. 3: (**A**) SDS-PAGE depicting 42.8 kDa protein in induced and uninduced cultures at 2, 4, 6, and 8 h intervals. M: Prestained protein marker; UI: Uninduced culture; I: Induced culture, (**B**) Western Blot using Anti His conjugated antibody for confirmation of recombinant PilQ protein. M: Marker, Lanes 1 and 3 ~ 42.8 kDa – r-PilQ was detected using anti-His antibody and DAB as conjugate, and (**C**) SDS-PAGE depicting ~ 42.8 kDa affinity purified r-PilQ protein. M: Marker, Lanes 1-3: purified r-PilQ protein

Table 3: Immunoinformatic analysis of antigenic determinants/epitopes identified in "PilQ" protein

Sno	Start position	Sequence	End position
1	16	DAEPVSF	22
2	27	PTAIILD	33
3	37	TTSALPSREVPI	48
4	52	GIFSVDVIP	60
5	65	TRAVVNL	71
6	75	MSYNVSRVGKDIYLSLPLSDAAPALSQ	101
7	126	QRKAYAYKLVPT	137
8	145	GGLLSFK	151
9	165	GGTLVATISD	174
10	184	RLDVADYATPVMR	196
11	256	YTGEPLSLNFQDIEVRAVLNI	276
12	282	QQNIVVSD	289
13	295	ITLRLDN	301
14	303	PWDQALDI	310
15	328	GRIIYIAP	335
16	364	IRIKYAKASDLQRI	377
17	392	GILAAQDAVLSARGSVT	408

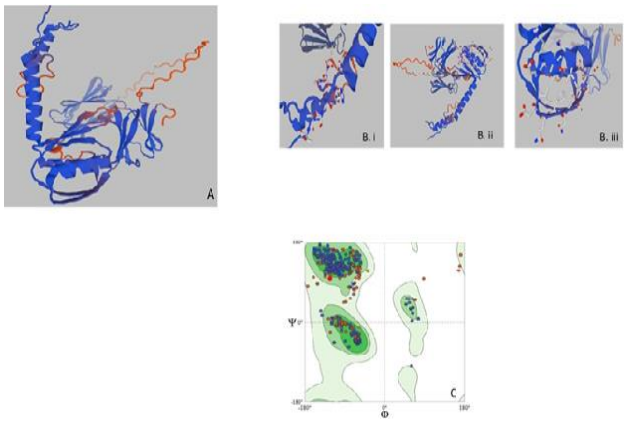

Fig. 4: (**A**) Immunoinformatic analysis of the Predicted 3D Model of PilQ Protein with Highlighted Antigenic Regions, (**B.i**, **B.ii**, and **B.iii**) The predicted 3D structure of PilQ protein of *Dichelobacter nodosus* antigenic determinants on 3D structure of PilQ protein of *Dichelobacter nodosus*, and (**C**) Ramachandran Plots showing Mol Probity Score=0.99, Clash Score=0.00 and Ramachandran favoured=91.95%

The expressed r-PilQ protein of *D. nodosus* was further confirmed by western blotting using an anti-His tag antibody. The analysis revealed an immunogenic band at approximately 42.8 kDa, confirming the 6X histidine-tagged r-PilQ protein (Fig. 3B). The expressed protein was purified under denaturing conditions by Ni-NTA affinity chromatography from a bulk culture of the r-PilQ clone in LB broth, as the expressed protein formed inclusion bodies. SDS-PAGE analysis of the purified protein indicated purification to near homogeneity (Fig. 3C).

Immunoinformatic analysis of the amino acid sequence of the PilQ protein identified seventeen antigenic determinants/epitopes capable of eliciting a protective immune response. The list of these determinants is depicted in Table 3. The predicted three-dimensional (3D) model of the PilQ protein, along with antigenic regions, was generated using the automated protein structure homology-modelling platform SWISS-MODEL (http://swissmodel.expasy.org), as depicted in Figs. 4A-C. The Ramachandran plot demonstrates that the PilQ protein predominantly contains antiparallel, parallel, and right-twisted β-sheets, followed by right-handed α-helices. The predicted 3D model generated via SWISS-MODEL further supports the hypothesis that the PilQ protein can effectively present antigenic regions to the immune system. The presence of diverse secondary structures, such as antiparallel and parallel β-sheets, along with right-handed α-helices, may enhance the protein's ability to interact with immune cells and elicit a robust humoral and cellular immune response. Given the structural and antigenic properties of PilQ, it holds promise as a vaccine candidate. The ability of PilQ to maintain its immunogenicity *in vivo*, as indicated by its structural stability and antigenic profile, makes it a compelling subject for further research in vaccine development. However, experimental validation in animal models and clinical trials would be essential to confirm its efficacy and safety.

Discussion

Footrot stands as one of the most economically significant and contagious diseases affecting sheep and goats. It has been enzootic in the Kashmir Valley for the past two decades. Concerted efforts have been made to understand the epizootiology of footrot in Kashmir, particularly serogroups B, C, E, and I of *D. nodosus*. However, serogroup B has been predominantly reported from footrot lesions (Wani *et al*., 2019). Transformation-mediated serogroup conversion and serogroup-specific vaccination could account for the prevalence and emergence of different serogroups in a defined geographical area (Kennan *et al*., 2005). Therefore, continuous surveillance, especially for different serogroups of *D. nodosus*, is of paramount importance (Smith *et al*., 2022).

In this study, sheep from organized farms and those reared by farmers in four districts of Kashmir Valley (Ganderbal, Budgam, Srinagar, and Pulwama), as well as migratory sheep flocks of the Bakerwal nomadic tribe, were inspected between December 2018 and April 2019. Footrot was recorded at 6.7% in sheep flocks across the four districts, while its prevalence was found to be 0.67% and 3.36% in organized sheep farms and Bakerwal sheep flocks, respectively. This prevalence was lower compared to previous reports, which ranged from 11-19% in Kashmir Valley (Farooq *et al*., 2010; Rather *et al*., 2011; Kabili *et al*., 2014). The reduced prevalence might be attributed to unfavorable climatic conditions during the study period and good care and management of individual sheep, especially in organized government farms. Conversely, Bakerwal sheep had been migrating from drier areas where the incidence of footrot is lower. In the current study, *D. nodosus* was detected in 59.45%, 56.3%, and 60% of samples from organized sheep farms, farmer-reared sheep flocks in the four districts, and migratory Bakerwal sheep, respectively. The lower detection of *D. nodosus* might be due to crude DNA extraction methods, the presence of PCR-inhibitory

substances like humic acid in the DNA samples, or a very low number of bacterial cells, as swabs may not reach the deeper regions of the hoof where bacterial load is comparatively higher (Cagatay and Hickford, 2005).

In the organized sheep farms, serogroup B of *D. nodosus* was found to be predominant (95.45%) followed by mixed infection of serogroups B and E (4.5%). Similarly, serogroup B was also predominant (83.17%), in sheep flocks of the four districts, followed by serogroup E (13.8%) and mixed infection of serogroups B and E (3.7%). The migratory Bakerwal sheep also exhibited similar results. These findings align with reports from earlier studies in Kashmir (Hussain *et al*., 2009; Farooq *et al*., 2010; Rather *et al*., 2011; Kabili *et al*., 2014). In the present study, serogroup B was exclusively detected in samples from Budgam, Pulwama, and Srinagar districts, whereas both serogroups B and E were detected in Ganderbal. Rather *et al*. (2011), also reported the detection of serogroup E alongside with B in Srinagar and Budgam; similarly, Farooq *et al*. (2010) detected both serogroups in Pulwama district. Interestingly, we observed serogroup E exclusively in sheep flocks of Wayul (Ganderbal). These findings might be due to the limited sample size from different districts or a changing epizootiological scenario of footrot in Kashmir, possibly resulting from treatment and culling of footrot-affected sheep or serogroup conversion of *D. nodosus*. Consequently, a bivalent vaccine targeting serogroups B and E of *D. nodosus* is currently needed for effective footrot control (Smith *et al*., 2022).

Another objective of this study was to produce a recombinant immunogenic protein, PilQ, from *D. nodosus*, which could serve as a vaccine candidate offering protection against all ten serogroups given, its conservation across serogroups of *D. nodosus*. PilQ is a homododecameric outer membrane complex crucial for the translocation of the fimbriae across the outer membrane and is involved in type IV and type II fimbrial biogenesis, fimbrial extrusion, natural transformation, and protease secretion (Collins *et al*., 2004; Han *et al*., 2007). Bacterial fimbriae act as major immunogenic proteins that confer protective immunity. However, this immunity is serogroup specific, with no cross-protection observed for other serogroups (Egerton *et al*., 2002; Dhungyel *et al*., 2013). To overcome this limitation, multivalent vaccines have been developed, but these also provide limited protection due to antigenic competition (Hunt *et al*., 1994). Therefore, a universal vaccine against all the serogroups of *D. nodosus* is necessary to prevent antigenic competition. In pursuit of this, we aimed to produce *pilQ* recombinant footrot antigen in *E. coli*. An outer membrane domain of the *pilQ* gene was successfully cloned and expressed using the pET28a plasmid in *E. coli* BL21. The pET system was chosen for its robust capabilities in cloning, expression, and purification of recombinant proteins in *E. coli*. Initially, some leaky expression was observed, which was resolved by adding 10 mM glucose to the LB medium. Optimal expression was recorded after 4 h of induction at 37°C. The overexpressed protein formed inclusion bodies, necessitating its purification under denaturing conditions by treating the cells with lysis buffer containing 8 M urea. Expression was found to be lower at low temperatures. The His-tagged protein was successfully purified using Ni-NTA agarose after optimal binding overnight at 4°C. The eluted recombinant PilQ (r-PilQ) protein was found to be nearly homogenous, suggesting its potential as a vaccine candidate. Further research is required to assess the protective immune response to the r-PilQ protein in laboratory animals and ultimately formulate a serogroup-independent recombinant vaccine to combat severe ovine footrot.

Bivalent vaccines have demonstrated remarkable efficacy in controlling diseases such as virulent ovine footrot. This success can largely be attributed to their focused approach. Unlike polyvalent vaccines, which target multiple serogroups simultaneously, bivalent vaccines concentrate on just two specific serogroups. This precision maximizes the immune response by minimizing antigenic interference. The targeted immunization strategy of bivalent vaccines has shown significant promise, especially in flocks with limited serogroup diversity. In a pivotal study by Dhungyel *et al*. (2013), vaccine trials were conducted across 12 flocks, and it was observed that virulent footrot was eradicated in five flocks that initially had fewer than three serogroups present. This outcome highlighted the potential of bivalent vaccines to effectively eradicate disease in specific outbreak contexts, thereby offering a potent solution for managing virulent ovine footrot. Schiller *et al*. (2013) concluded that bivalent vaccines, with their straightforward and simple formulation, tend to cause fewer side effects and are easier to manufacture than their polyvalent counterparts.

The prevalence of ovine footrot was found to be low during the study period. It was lower in the organized farms and migrating Bakerwal sheep flocks than in the sheep flocks in the four districts of Kashmir Valley. Serogroup B of *D. nodosus* was identified as predominant. Serogroup E was detected exclusively in some isolated flocks reared by farmers. The *pil*Q gene was successfully expressed in *E. coli* and purified to near homogeneity, indicating its potential for further exploitation as a serogroup-independent vaccine candidate against footrot.

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Conflict of interest

The authors declare no conflict of interest.

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