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In vitro effect of single-domain antibody, selected from the phage display library of Indian desert camel, on *Pasteurella multocida* LPS-induced activation of bovine peripheral leukocytes

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Abstract
The objective of the present study was to assess the ability of single-domain antibody clone 26 (dAb Cl 26) selected from phage display library to reduce *Pasteurella multocida* (vaccine strain p52) LPS - induced effects on buffalo leukocytes *in vitro*. The dAb Cl26 was expressed to an optimum level and later purified under denaturing condition using Ni-NTA chromatography. The protein concentration of purified dAb Cl-26 was estimated by BCA method and found to be 280 µg/ml. LPS of vaccine strain (p52) of *P. multocida* was extracted and concentration was found to be 38 µg/ml. The purity of extracted LPS was assessed for nucleic acid and protein contamination and was found to be negative. The purified dAb Cl-26 showed reactivity with *Pasteurella multocida* (p52) LPS by Indirect ELISA as well as Dot-Immunoblotting.
For *in vitro* testing of purified dAb Cl-26 on *P. multocida* (p52) LPS-induced activation of bovine peripheral leukocytes, Buffalo peripheral blood plastic adherent mononuclear cells were exposed to LPS alone and in combination with different concentration of dAb Cl-26 and the expression level of various genes namely TNFα, IL-1β and IL-6 were studied by qRT-PCR. The dAb Cl-26, in a dose dependent manner, reduced the LPS-induced TNFα, IL-1β and IL-6 transcripts in buffalo peripheral leukocytes *in vitro*.

Keywords: LPS, single-domain antibody, *pasteurella multocida*, phage display library

1. Introduction
Lipopolysaccharide (LPS) or endotoxin is a major component of the cell-wall of Gram negative bacteria and contributes to toxicity when it is released into the blood circulation after a bacterial infection. This fatal syndrome is known as sepsis. Despite introduction of new antibiotics from time to time, Gram- negative (G –ve) sepsis remains associated with high morbidity and mortality in man and animals throughout the world [1]. LPS from Gram-negative bacteria is the major etiological agent of septic shock, which is a serious and often fatal dysregulation of the innate immune response. Endotoxins are amphipathic macromolecules located in the outer layer of the outer membrane of G –ve bacteria, which on release from bacteria *in vivo* or administered in an isolated form, exert both physiological and pathophysiological effects in higher organisms, and thus represent important virulence factors of G –ve bacteria [2]. Therapies for this life-threatening syndrome (G–ve sepsis) remain unsatisfactory. *Pasteurella multocida*, a Gram-negative facultative bacterium is an important animal pathogen causing diseases in various domestic animals. Haemorrhagic septicaemia (HS), a type of pasteurellosis, is an infectious and acute disease of domestic animals. The disease occurs mainly in Asian and African countries [3]. To-date only a few potential antigens of *P. multocida* have been identified. Proteins, polysaccharides, LPS and fimbrie have been identified as antigens of this organism, LPS is a major virulence factor and played an essential role in causing diseases as HS in buffaloes [4].
Phage display system is robust and has potential to provide biotechnologically and therapeutically useful Ab fragments [5, 6]. Presently, phage displayed Ab fragments, particularly dAbs, are offering possible solutions to these problems. The dAb fragments can be readily produced in the soluble form in *E. coli* host. Camelid and shark dAbs against many clinically and biotechnologically useful antigens (Ags) have been produced [7]. The dAbs, also called nanobodies (Nbs) selected from the immune libraries fulfill all the properties of a practical

binders such as economic production, humanized sequence, stable and soluble behavior in aqueous solution, high affinity, specificity, etc. [8]. These beneficial properties stimulated several research groups in universities and pharmaceutical and biotech companies to employ Nbs as a research tool and/or to develop future diagnostic and therapeutic applications.

Phage display library of LPS-immunized Indian desert camel has already been produced previously and several LPS-binding clones have already been selected from the library [9, 10]. Recently, further characterization of some LPS-binding dAb clones has revealed that dAb clone 26 binds LPS with good affinity [11]. In the present study, *in vitro* effects of the dAb Cl 26 on *P. multocida* (vaccine strain p52) LPS-induced effects on buffalo peripheral leukocytes were studied.

2. Materials and Methods

2.1. LPS-binder dAb Cl 26

The LPS-binder dAb Cl 26 was originally selected from the phage display library of LPS-immunized Indian desert camel (*Camelus dromedaries* L.) [12-13]. The expression and purification of the dAb Cl 26 has been described earlier [14]. Briefly, the dAb Cl 26 was expressed in E. coli BL21 (DE3) cells under IPTG (1mM) induction. The recombinant dAb fused with 6xHis tag (dAb.6xHis) could be purified using Ni-NTA chromatography under denaturing condition as assessed by SDS-PAGE and dialyzed against PBS (pH 7.8) and stored at -80°C for further use. The concentration of the purified dAb Cl26 was estimated by BCA method using the commercial BCA reagents kit in a microtitre plate version [15].

2.2. Extraction and purification of *Pasteurella multocida* (p52) LPS

Pasteurella multocida (p52) LPS was extracted by the method of Westphal and Jann (1964) with minor modifications [16]. The LPS preparation so obtained was dialyzed against double glass distilled water (DGDW) by 6 changes of 2 liters of DGDW over a period of 3 days. The dialyzed LPS was collected in a sterile screw-capped plastic vial and stored at -20˚C until use. The LPS concentration was determined by phenol-sulphuric acid method described by Dubois *et al.* (1956) [17]. Glucose in concentrations ranging from 10 µg/ml to 100 µg/ml was used for construction of a standard curve of A485nm value vs. glucose concentration (µg/ml). LPS concentrations were interpolated from the standard curve. The purity of the extracted LPS extracted was accessed by resolving the LPS in 1.5% low endo-osmosis (LE) agarose in 1x tris- acetate-EDTA (TAE) buffer to detect the nucleic acid in the extracted LPS. The purity of LPS was also assessed by its resolution in SDS-PAGE to detect any protein contamination. The reactivity of purified dAb Cl 26 with extracted LPS was assessed using Indirect ELISA as well as Dot-Immunoblotting [18].

2.3. *In vitro* testing of dAb Cl 26 on *P. multocida* LPS-induced activation of bovine peripheral leukocytes

Peripheral blood plastic adherent mononuclear cells from apparently healthy Buffalos were separated and cultured [19]. Buffalo peripheral blood plastic adherent mononuclear cells were exposed to LPS alone and in combination with different concentration of dAb Cl-26 and the expression level of various genes namely TNFα, IL-1β and IL-6 were studied by qRT-PCR. Three different doses of 10 µg, 1 µg and 100 ng of dAbCl26 was mixed with 1 µg of purified *P. multocida* (p52) LPS and incubated for 1 hr at room temperature. Endotoxin-free water control group and LPS control group (1 µg of LPS/5 x 10^6 cells) were also included in the study. After incubation, the medium was aspirated from the cell culture plate and each formulation (Table 1) was added in duplicate wells. The plate was incubated in CO_2 incubator for 75 min.

After 75 min of incubation, RNA was extracted from the cells by using RNeasy plus Mini Kit (Qiagen, Germany, 74134) in an RNAse-free environment. The purified RNA of different samples was used as a template for the synthesis of first-stand cDNA for use in qRT-PCR. The first stand cDNA was synthesized using Superscript® III First-Stand Synthesis supermix for RT-qPCR (Invitrogen, USA) as per manufacturer instructions. For quantifying levels of TNFα, IL-1β and IL-6 transcripts in the above synthesized cDNA samples of various groups, the RT-qPCR was performed using the Stratagene Brilliant III Ultra- Fast SYBR® Green QPCR Master Mix (Agilent Technologies, USA, 600882) in the Stratagene Mx3005P QPCR Systems from Agilent. The primers used in the study are given in Table 2.

Table 1: Formulations for the *in vitro* functional effects of dAb clones on LPS-induced activation of buffalo peripheral blood plastic adherent mononuclear cells

Groups	Formulation	Amount/well	Cell count/ml/well
1	Endotoxin-free water control	300 µl	5 x 10^6
2	*P. multocida* (p52) LPS (1 µg) control	300 µl	5 x 10^6
3	Cl-26 (100 ng) + LPS (1 µg)	300 µl	5 x 10^6
4	Cl-26 (1 µg) + LPS (1 µg)	300 µl	5 x 10^6
5	Cl-26 (10 µg) + LPS (1 µg)	300 µl	5 x 10^6

Table 2: Bovine Primers used in Rt-qPCR

Primer name	Sequence (5′-3′)	Tm (°C)
Bo β-Actin forward	CGCACCACCGGCATCGTGAT	75.8
Bo β-Actin reverse	TCCAGGGCCACGTAGCAGAG	69.9
Bo TNFα forward	TCTTCTCAAGCCTCAAGTAACAAGT	63.3
Bo TNFα reverse	CCATGAGGGCATTGGCATAC	67.3
Bo IL-1β forward	AAATGAACCGAGAAGTGGTGTT	63.5
Bo IL-1β reverse	TTCCATATTCCTCTTGGGCTAGA	64.6
Bo IL-6 forward	TCAGCTTATTTTCTGCCAGTCTCT	64.2
Bo IL-6 reverse	TCATTAAGCACATCGTCGACAAA	66.3

3. Results

3.1 Expression and purification of dAb Cl26

The expression of dAb Cl 26 in BL21 (DE3) cells under IPTG induction was detected as band of approximately 17 kDa in polyacrylamide gels (Fig. 1). The recombinant dAb.6xHis clones were purified using Ni-NTA chromatography under denaturing condition and the purified dAb Cl 26 was detected as a single band in Coomassie's Brilliant Blue R-250 stained gel (Fig. 2). The concentration of the purified dAb Cl 26 as estimated by BCA was found to be 280 µg/ml. The purified dAb Cl 26 was stored at -20°C for further use.

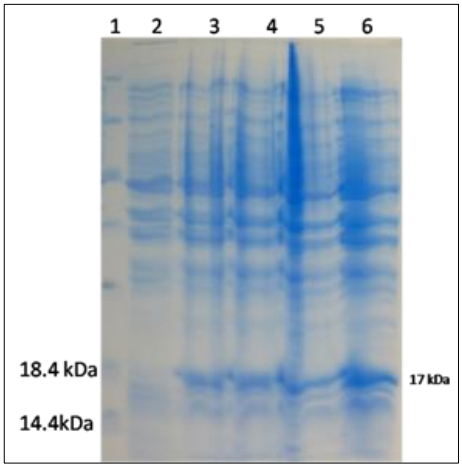

Fig 1: SDS-PAGE followed by Coomassie's brilliant blue R-250staining of 15% resolving gel to show protein profiles of uninduced and IPTG-induced bacterial lysates of dAb Cl 26 [Lane 1: Protein molecular marker; Lane 2: uninduced bacterial lysate; Lane 3-6: IPTG-induced bacterial lysate after 10 hours of growth]

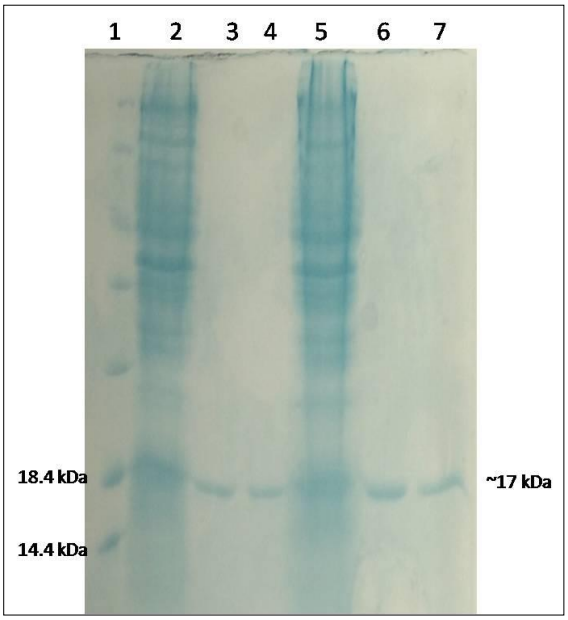

Fig 2: Coomassie's brilliant blue R-250 staining of 15% resolving gel showing purity of eluted dAb Cl 26 [Lane 1: Protein molecular marker; Lane 2,5: IPTG induced dAb Cl 26; Lane 3,4,6,7: Purified dAb Cl 26 showing band of about ~17 kDa]

3.2 Extraction of *Pasteurella multocida* (p52) LPS

The LPS concentration was determined by phenol-sulphuric acid method described by Dubois *et al*. (1956) and was found to be 38 µg/ml (Fig. 3). The purity of the extracted LPS was accessed by electrophoresis and SDS-PAGE. The extracted LPS was found to be free of Nucleic acid and protein contamination (Fig. 4 and Fig. 5).

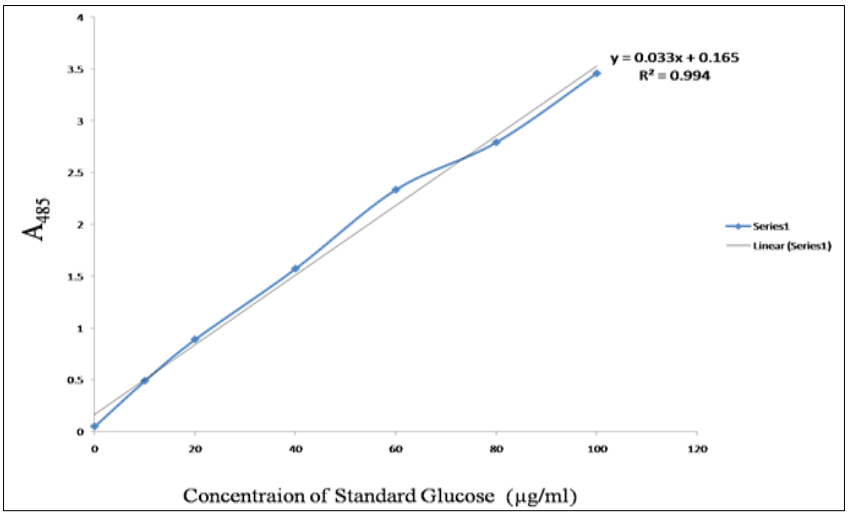

Fig 3: Standard curve of Glucose concentration

Fig 4: AGE of extracted LPS [Lane 1: 100 bp DNA ladder; Lane 2: LPS]

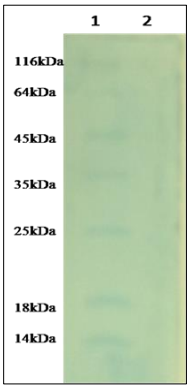

Fig 5: Coomassie's brilliant blue R-250 staining of LPS [Lane 1: Protein marker; Lane 2: LPS]

3.3. *In vitro* effect of dAb Cl 26 on *P. multocida* LPS-induced activation of bovine peripheral leukocytes

In the present study, buffalo peripheral blood plastic adherent mononuclear cells were exposed to LPS alone and in combination with different concentration of dAb Cl 26 and the expression level of various genes namely TNFα, IL-1βand IL-6 were studied by qRT-PCR. Quantity of total RNA extracted from different samples was in the range of 35 ng/µl – 60 ng/µl from $5x10^6$ cells/ml/well. RT-qPCR was performed using 1 ng cDNA. Relative transcript levels of bovine TNFα, IL-1β and IL-6 genes as determined by real-time RT-qPCR are shown in figure 6-8 respectively. The dAb Cl 26 showed dose dependent decrease in the LPS induced gene expression of TNFα, IL-1β and IL-6.

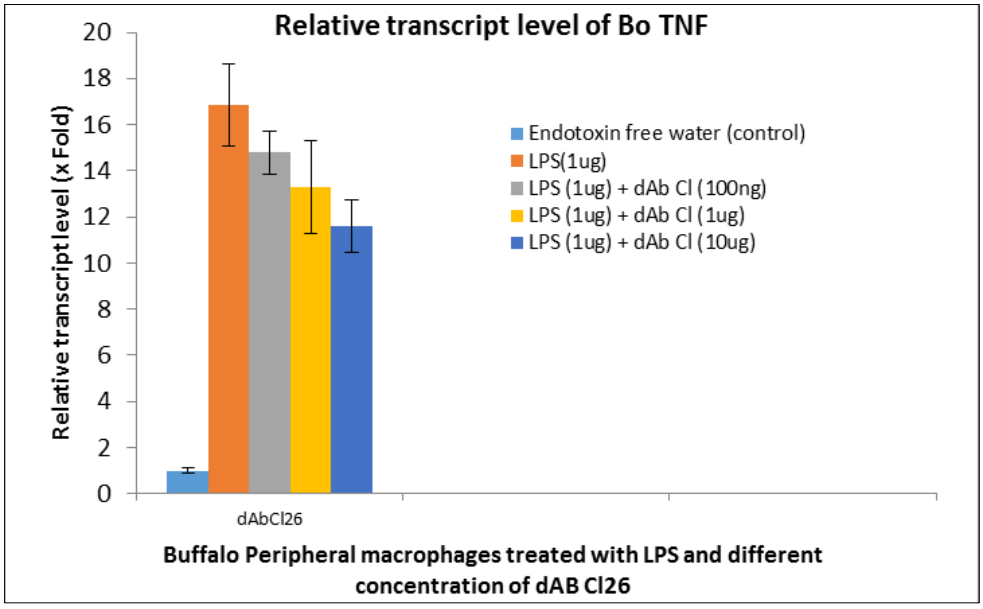

Fig 6: Relative transcript levels of Bo TNF determined by RT-qPCR

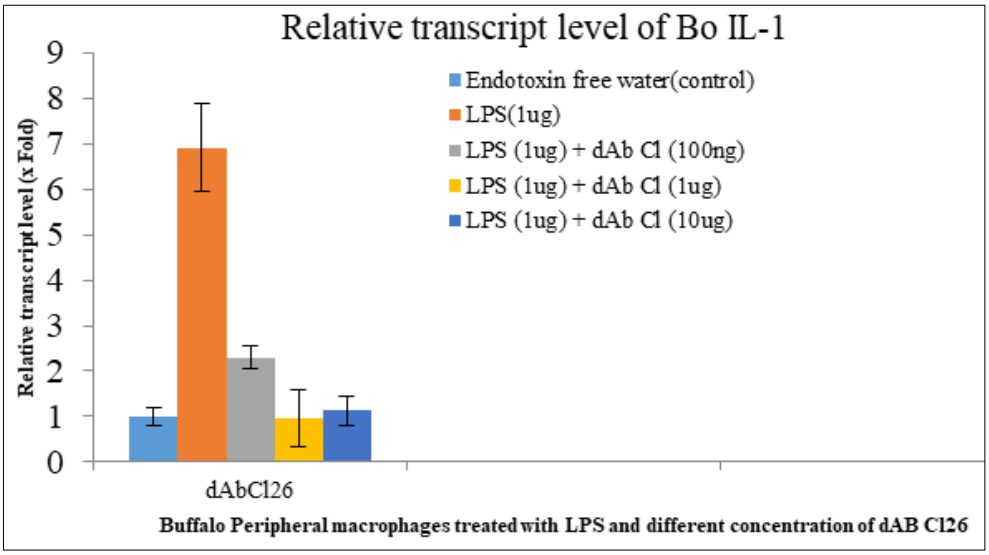

Fig 7: Relative transcript levels of Bo IL-1β determined by RT-qPCR

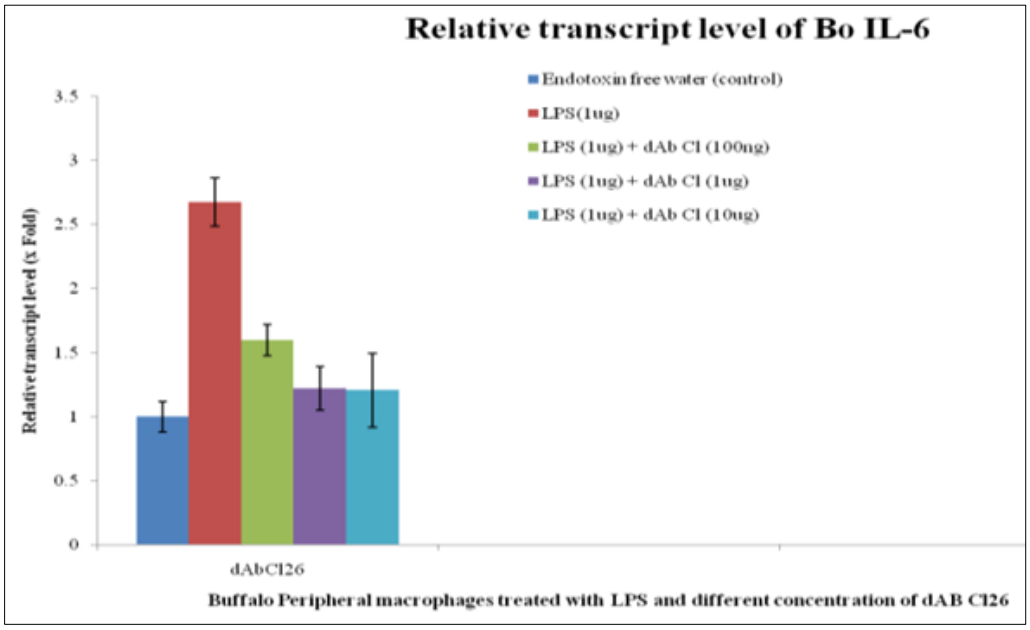

Fig 8: Relative transcript levels of Bo IL-6 determined by RT-qPCR

4. Conclusion

These observations led us to suggest that the dAb Cl 26 could be a promising candidate for reducing or alleviating the ill-effects of endotoxaemia in animals. After necessary modifications in the dAb Cl 26, it should be tested for its therapeutic potential in the intended host species.

5. Acknowledgement

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6. Competing interests

Authors have declared that no competing interests exist.

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