Yersinia outer-membrane protein B (YopB): a tool for identification of *Yersinia pestis* isolates

Yersinia species that are pathogenic to humans include *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. *Y. pestis* is the cause of bubonic plague, which is transmitted by fleas. Common to all of these species is the presence of a 70 kb virulence plasmid that harbours a type III secretion system, and several secreted and translocated proteins called *Yersinia* outer proteins or Yops (Cornelis, 2002). The plasmid-encoded type III secretion system enables *Yersinia* to survive and proliferate extracellularly in host lymphatic tissues (Trülsch *et al.*, 2004). Three of the Yops – YopB, YopD and LcrV – are required for translocation of the others across the target-cell membrane (Viboud *et al.*, 2003). The hydrophobic YopB and YopD thus seem to be central for translocation of the effectors and for the formation of a channel in lipid membranes. Presumably they play different roles in pore formation (Cornelis, 2000). Indeed, YopB alone can disturb artificial membranes, whereas YopD cannot (Francis & Wolf-Watz, 1998). Accurate laboratory identification guarantees the safe control and handling of specimens and is essential for surveillance of the spread of *Y. pestis* in natural plague foci (Anisimov *et al.*, 2004). The present study was aimed at developing a *Y. pestis*-specific PCR for the *yopB* gene and a specific immunoassay for

detection or typing of *Y. pestis* strains. The isolates used to characterize these assays were from human patients and rodent samples from 1994 outbreak regions (Batra *et al.*, 1996), and from surveillance studies of the Deccan plateau region (U. Tuteja, J. Shukla & H. V. Batra, unpublished data) (Table 1). Plasmid DNA was extracted from cultures by alkaline lysis (Maniatis *et al.*, 1982). The entire 70 kb/low-calcium response locus plasmid sequence (GenBank accession no. AF074612) was used to design *Y. pestis*-specific primers for the *yopB* gene using DNASIS software. The primer sequences were 5'-AAAAATGGCGGGGTGAGTT-3' (forward) and 5'-AAAACCTCGGCTCCTT-TAGC-3' (reverse). PCR amplification was carried out in a 50 µl reaction mixture containing the extracted bacterial plasmid (50 ng) isolated from the *Y. pestis* isolates and standard strains, 200 µM each dNTP, 1 µM each primer, 1 U *Taq* polymerase and 10 × buffer (Qiagen) using a Perkin Elmer model 480 thermal cycler. Amplification consisted of 30 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min. PCR products were analysed on a 1.5 % agarose gel (Fig. 1). The *yopB* gene 700 bp amplicon was detected from standard *Y. pestis* A1122, but other *Yersinia* (Fig. 1) and non-*Yersinia* (not shown) species were negative. All 18 isolates from human and rodent samples from outbreak regions were able to produce

the 700 bp amplification product, while two of the isolates from rodents from the surveillance region were negative for the *yopB* gene (Table 1). The 700 bp PCR product was cloned into the pQE-32 expression vector by ligation using *Sma*I and Klenow fragment blunt-ended DNA, and transformed into *Escherichia coli* SG13009 cells (Sambrook *et al.*, 1989). Recombinant clones were confirmed by restriction analysis and PCR (using *Sma*I and the same primers as above) (not shown). Expression of recombinant YopB protein was obtained by inoculating cultures, growing them overnight in LB broth and then inducing expression with 1 mM IPTG. Whole-cell lysates of the bacteria were prepared and expression of the recombinant protein was examined by 10 % SDS-PAGE (Laemmli, 1970). Recombinant protein was purified by Ni-NTA affinity chromatography (Qiagen). The truncated recombinant YopB (rYopB) protein obtained was estimated to be 28 kDa by SDS-PAGE and the concentration of the protein was 4 mg ml⁻¹ by the Lowry method. To characterize the recombinant protein and develop an antigen-based immunoassay, rabbits (for polyclonal antibodies) and BALB/c mice (for mAbs) were immunized with four doses of 500 and 50 µg recombinant protein, respectively. Total

Table 1. *Y. pestis* isolates, their origin, and the results of PCR, dot-ELISA and Western blotting

Region	Isolate	Recovered from:		<i>yopB</i> PCR (700 bp)	mAb dot-ELISA	mAb Western blot (41 kDa protein)
		Species	Place (India)			
Outbreak region	4, 8, 9, 101, 102, 103, 104, 105, 106, 107, 108	Human	Surat	+	+	+
	111, 112, 114, 115, 116	<i>Rattus rattus</i>	Mamla	+	+	+
	113	<i>Rattus rattus</i>	Surat	+	+	+
	117	<i>Tetera indica</i>	Mamla	+	+	+
Surveillance region	2H, 3H, 12H, 18H, 25H	<i>Tetera indica</i>	Hosur	+	+	+
	24H	<i>Tetera indica</i>	Hosur	–	–	–
	9R	<i>Tetera indica</i>	Palamner	–	–	–
	10R	<i>Tetera indica</i>	Palamner	+	+	+

Fig. 1. PCR amplification of the *yopB* gene in *Y. pestis* isolates. Lanes: 1, *Y. kristensenii*; 2, *Y. intermedia*; 3, *Y. fredericksonii*; 4, *Y. enterocolitica*; 5, *Y. pseudotuberculosis*; 6–11, *Y. pestis* isolates (112, 111, 10R, 9R, 24H and 8); 12, standard *Y. pestis* A1122; 13, molecular mass marker (200 bp step ladder).

immunoglobulin serum antibody titres were determined 7 days after the last booster by dot-ELISA (Khushiramani *et al.*, 2004). After the fourth dose, rabbit and mice antibody titres were determined as 1:64 000 by dot-ELISA. The hyperimmune sera were used in Western blotting (Towbin *et al.*, 1979) to check their reactivity against the native *Y. pestis* Yop proteins and the recombinant protein. Rabbit polyclonal antibodies raised against rYop detected bands of 28 kDa in cell lysates from IPTG-induced recombinant *E. coli* SG13009 and 41 kDa for native antigens of *Y. pestis* (Fig. 2). This finding suggested that the antigenicity of the recombinant protein was maintained. In another study, the same recombinant protein was also found to be biologically active. Both the truncated rYopB and rLcrV in the *in vitro* studies inhibited LPS-induced tumour necrosis factor- α , gamma interferon, keratinocyte-derived chemokine, IP-10, interleukin 12 and NO production in murine peritoneal macrophages (Sharma *et al.*, 2004; Sodhi *et al.*, 2004).

To check variation at the epitope level, mAbs were prepared following a standard PEG fusion protocol (Köhler & Milstein, 1975). mAbs produced by the hybridoma were screened by dot-ELISA. A total of ten stable mAbs was obtained against truncated rYopB protein. The class of immunoglobulin was determined by dot-ELISA using a mouse Typer isotyping kit (Bio-Rad). One of the clones was IgM type, whilst the rest were IgG type (subtypes IgG1, IgG2a, IgG2b, IgG3 and IgG4) having

lambda and kappa light chains. The mAbs generated were tested for specificity using *Y. pestis* A1122, *Y. pseudotuberculosis* 1A, *Y. enterocolitica* strain O:8, *Yersinia kristensenii*, *Yersinia fredericksonii*, *Yersinia intermedia*, *E. coli*, *Salmonella typhi*, *Staphylococcus aureus*, 'Salmonella abortus' and *Klebsiella pneumoniae* strains in dot-ELISA and Western blotting. In another study, it was observed that interaction of YopB with the host cell triggered a

pro-inflammatory signalling response that was counteracted by multiple effectors in the host cell (Viboud *et al.*, 2003). The YopB protein expressed in the present study could be used to enhance the immune response in eukaryotic cells. All clones were specific to *Y. pestis* when tested against other *Yersinia* and non-*Yersinia* species by dot-ELISA. Western blot reactions with mAbs to recombinant proteins were observed at the expected size of 41 kDa for the YopB protein of standard *Y. pestis* A1122 and isolates. The results obtained with mAb-based immunoassays correlated fully with the PCR results when tested with all of the Indian isolates (Table 1). mAbs generated in the present study could be utilized to study the exact mechanism of action of YopB proteins and their receptors. As these mAbs are specific to *Y. pestis* and as variation in the *yopB* gene of isolates was also observed, this PCR and immunoassay could be used as simple, rapid and cost-effective methods for the typing of *Y. pestis* strains.

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Fig. 2. Reactivity of polyclonal antibody. Lanes: 1, molecular mass marker (PMW-M, 14.3–97.4 kDa; Bangalore Genei); 2, standard *Y. pestis* A1122; 3, *Y. pestis* isolate recovered from a human patient from a plague outbreak; 4 and 5, *Y. pestis* isolates (9R and 24H) recovered from rodents from surveillance regions; 6, standard *Y. pseudotuberculosis* 1A; 7, *Y. enterocolitica*; 8, *Y. intermedia*; 9, *Y. fredericksonii*; 10, *Y. kristensenii*; 11, *E. coli*.

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