Distribution of the *clpX* gene in *Brachyspira* species and reactivity of recombinant *Brachyspira pilosicoli* ClpX with sera from mice and humans

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Previously, a *clpX* gene encoding a predicted 67 kDa membrane-associated ATPase subunit of the Clp protease (ClpX) was identified in a porcine strain (95/1000) of the intestinal spirochaete *Brachyspira pilosicoli*. In the current study, the distribution of this large *clpX* gene was investigated in a collection of strains representing all seven *Brachyspira* spp. Using PCR with internal primers, an 878 bp portion of the gene was detected in 29 of 35 strains (83%) of *B. pilosicoli*, 6 of 24 strains (25%) of *Brachyspira hyodysenteriae*, 14 of 16 strains (88%) of *Brachyspira intermedia*, 6 of 17 strains (35%) of *Brachyspira innocens*, 1 of 6 strains (17%) of *Brachyspira murdochii*, 1 of 2 strains (50%) of *Brachyspira aalborgi* and not in the single strain of *Brachyspira alvinipulli*. The whole gene was sequenced from 20 *Brachyspira* spp. strains and compared with the *clpX* gene from *B. pilosicoli* 95/1000 (GenBank accession no. AY466377). The genes had 99.3–99.7% nucleotide sequence similarity and the predicted products had 99.7–100% amino acid sequence similarity. The *clpX* gene from WesB, a human strain of *B. pilosicoli*, was cloned and expressed as a histidine-tagged fusion protein in *Escherichia coli* BL21. The purified protein was used to vaccinate mice and their sera were found to recognize the expected ~67 kDa protein in whole-cell preparations of WesB. Sera from mice vaccinated with formalin-treated whole-cell proteins of WesB reacted with the recombinant protein. These results indicate that ClpX is both conserved and immunogenic and hence might be useful as a subunit vaccine component for *Brachyspira* spp. infections. Sera from humans with no known exposure to *B. pilosicoli* reacted with the recombinant ClpX protein, indicating that it is unlikely to be useful as a reagent for serological detection of *Brachyspira* spp. infections.

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INTRODUCTION

The genus *Brachyspira* includes seven species of anaerobic intestinal spirochaetes, several of which are pathogenic in humans and/or in various species of animals and birds (Hampson & Stanton, 1997). The most common and important pathogenic species are *Brachyspira hyodysenteriae*, the agent of swine dysentery (Hampson *et al.*, 2006a), and *Brachyspira pilosicoli*, the agent of intestinal spirochaetosis – a disease of many species including humans, poultry, pigs, dogs and horses (Hampson & Duhamel, 2006).

Intestinal spirochaetosis is characterized by the end-on attachment of large numbers of spirochaetal cells to the luminal surface of the large intestine epithelium, to form a 'false brush border' (Harland & Lee, 1967; Mikosza & Hampson, 2001). The basis of the observed attachment is unknown, but it has been suggested that spirochaetal outer-membrane proteins may have an important role in this interaction (Trott *et al.*, 2001). To date, relatively few outer-membrane proteins of *Brachyspira pilosicoli* or other

Brachyspira species have been characterized. Recently, Trott *et al.* (2003) identified a membrane-bound 61 kDa clip protease (ClpX) homologue in porcine *Brachyspira pilosicoli* strain 95/1000 by screening a *Brachyspira pilosicoli* genome library using a monoclonal antibody generated against a *Brachyspira pilosicoli* membrane vesicle preparation. They described the ClpX protein as an inner-membrane protein of *Brachyspira pilosicoli* but with a low-density presentation in the outer membrane that accounted for the reactivity of the protein with the monoclonal antibody. ClpX ATPase is a member of a group of molecular chaperones involved in the conformational integrity of proteins under conditions of normal growth as well as under stress (Gottesman *et al.*, 1993; Skinner & Trempy, 2001).

In the current study, the distribution of the *clpX* gene amongst a large collection of *Brachyspira* spp. strains was determined by PCR analysis and the whole gene was sequenced in 20 strains of *Brachyspira* species. The *clpX* gene was expressed in *Escherichia coli* and the purified recombinant protein was used to immunize mice and to

evaluate its reactivity with sera from mice and humans. The long-term aim of the study was to investigate recombinant ClpX as a potential subunit vaccine component for *Brachyspira* infections and/or as a reagent for *Brachyspira*-specific serological assays.

METHODS

Ethics. This work was conducted with the approval of the Murdoch University Animal and Human Ethics Committees.

Spirochaetal reference strains and culture conditions. A total of 101 *Brachyspira* species strains were used, comprising 35 strains of *Brachyspira pilosicoli* (from humans, pigs, dogs and chickens), 24 strains of *Brachyspira hyodysenteriae*, 17 strains of *Brachyspira innocens*, 16 strains of *Brachyspira intermedia*, six strains of *Brachyspira murdochii*, two strains of *Brachyspira aalborgi* and one strain of *Brachyspira alvinipulli*. The strains were obtained as frozen stocks from the culture collection held at the Reference Centre for Intestinal Spirochaetes, Murdoch University, Australia. The cells were thawed and plated onto trypticase soy agar (BBL) containing 5% (v/v) defibrinated ovine blood. The plates were incubated for 7–10 days at 37 °C in anaerobic conditions generated using anaerobic Gaspak Plus sachets (BBL). The purity of each culture was examined by phase-contrast microscopy and pure cells were propagated in 10 ml and then 100 ml Kunkle's pre-reduced anaerobic broth, containing 2% (v/v) fetal bovine serum and 1% (v/v) ethanolic cholesterol solution (Kunkle *et al.*, 1986).

Preparation of genomic DNA from *Brachyspira* spp. strains. Approximately 2×10^9 cells from mid-exponential phase spirochaetal growth were used to prepare chromosomal DNA using a DNeasy tissue kit according to the manufacturer's instructions (Qiagen). RNA was removed by adding 20 µl RNase A (100 mg ml^{-1}) to each sample, followed by incubation at room temperature for 2 min. DNA concentrations were calculated by measuring the A_{260} of the solution in a Lambda 25 UV/Vis spectrometer using UV WINLAB software (PerkinElmer).

Primer design and specification. Five sets of primers (one set for gene distribution analysis, three sets for sequencing and one set for cloning) were designed to be complementary to the internal and external regions of the *clpX* gene (Table 1). The oligonucleotides were designed using PRIMER DESIGNER software (Education and Scientific Software), SEQED version 1.0.3 (Applied Biosystems) and the AMPLIFY program version 1.2 (University of Wisconsin). Primers were purchased from GeneWorks.

PCR. The PCR amplification mixtures consisted of $1 \times$ PCR buffer, 1.5 mM MgCl_2 , 0.6 U *Taq* DNA polymerase, 0.06 U *Pfu* DNA polymerase, 0.25 mM each dNTP (Promega), 0.5 µM of the primer set and 50–100 ng chromosomal DNA template (genomic DNA) in a total volume of 25 or 50 µl. Reactions were loaded into 0.2 ml thin-walled thermocycling tubes (Multiply-Pro) and Ultrapure water (Fisher Biotech) was used to make up the reaction volume.

Thermocycling conditions consisted of an initial template denaturation for 5 min at 94 °C, followed by 30–35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and primer extension at 72 °C for 1–1.5 min. The final cycle had the extension time increased to 7 min to complete synthesis of all strands. Amplified products were separated by electrophoresis in 1.2% (w/v) agarose in $1 \times$ TAE buffer [40 mM Tris/acetate (pH 8.0), 1 mM EDTA], stained by immersion in a 1 µg ethidium bromide ml^{-1} solution and viewed over UV light.

Distribution analysis. PCR was used to amplify the 878 bp internal portion of the *clpX* gene between nt 369 and 1246 from all 101 *Brachyspira* species strains using primer pair CLPX.369.DF1 and CLPX.1246.DR1 (Table 1).

Sequencing reactions. The full *clpX* gene was sequenced from 12 strains of *Brachyspira pilosicoli*, three strains of *Brachyspira innocens*, two strains each of *Brachyspira hyodysenteriae* and of *Brachyspira intermedia* and one strain of *Brachyspira aalborgi*. Initially, an external primer set, CLPX.–140.SF1 and CLPX.+63.SR3 (Table 1), amplifying 1863 bp that included the coding region of *clpX*, was applied to chromosomal DNA from 20 *Brachyspira* spp. strains in 50 µl PCRs. The PCR products were purified using an UltraClean PCR Clean-up kit (Mo Bio Laboratories). The purified PCR products then were sequenced using the three sequencing primer sets (Table 1) using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems) and an ABI 373A DNA sequencer (PE Applied Biosystems), according to the manufacturer's specifications.

Phylogenetic analysis. Sequence data were edited using SEQED version 1.0.3 (PE Applied Biosystems) and compared with the sequence of the *clpX* gene of porcine *Brachyspira pilosicoli* strain 95/1000 strain (GenBank accession no. AY466377). The nucleotide and translated protein sequences were aligned using CLUSTALX and CLUSTALW multiple alignment software to obtain a similarity matrix of the 20 different strains of *Brachyspira* spp. A neighbour-joining phylogenetic tree based on a distance matrix was drawn using the BIOEDIT Sequence Alignment Editor (North Carolina State University) and MEGA 3.1 (Kumar *et al.*, 2004).

In silico analysis. The *clpX* gene was analysed using BLASTP (Altschul *et al.*, 1997), reverse-position-specific BLAST (RPS-BLAST; Marchler-Bauer

Table 1. PCR primers used to amplify all or part of the *clpX* gene in *Brachyspira* spp. strains

Primer set	Primer name	Sequence (5'→3')	Product size (bp)
Distribution analysis primers	CLPX.369.DF1	ATGAGCAAAAAAATAATAAGG	878
	CLPX.1246.DR1	TCAAGCCATATAAGTTTATC	
Sequencing primers (set 1)	CLPX.–140.SF1	AATATTGGCGATATAGTAAAGG	1863
	CLPX.697SR1	GACCAATACTTGAATAAGGCTC	
Sequencing primers (set 2)	CLPX.579SF2	GTCTTCCATTTGCACGTGCAG	–
	CLPX.1286SR2	GCTCCCATATTCATAGTAGAAG	
Sequencing primers (set 3)	CLPX.1162SF3	CCAGAGTTTATAGGAAGAATACC	–
	CLPX.+63.SR3	CATTATTTGCCAACTCCATTACTG	
Cloning primers	CLPX.4.CF1	AGGGGATCCAGCAAAAAAATAATAAGGAA	1782
	CLPX.1785.CR1	TAAGAATTCTCAAGCCATATAAGTTTATC	

et al., 2002) and CDART (Marchler-Bauer *et al.*, 2005) to find protein domain similarities and functional architecture. SIGNALP (EMBOSS) (Bendtsen *et al.*, 2004), PSORTB (Gardy *et al.*, 2005), TMPRED (Hofmann & Stoffel, 1993), PENCE (<http://www.cs.ualberta.ca/~bioinfo/PA/Subcellular>) and CELLO (<http://cello.life.nctu.edu.tw>) were used to predict subcellular localization. The EMBOSS programs IEP and GEECEE also were used to identify the isoelectric point and the molecular mass of the ClpX protein and to calculate the GC content of the *clpX* gene.

Preparation of recombinant histidine-tagged ClpX. The entire *clpX* gene encoding 595 aa was amplified from the genomic DNA of *Brachyspira pilosicoli* strain WesB using primers CLPX.4.CF1 and CLPX.1785.CR1 (Table 1) using the optimized conditions described above. The amplified product was restricted with *EcoRI* and *BamHI* (New England Biolabs) in a double digest according to the manufacturer's instructions. The restricted insert DNA was purified using an UltraClean PCR Clean-up kit and cloned into an Xpress Protein Expression System (Invitrogen) according to the manufacturer's instructions. Recombinant histidine (His₆)-tagged ClpX protein was expressed in *E. coli* BL21 Star (DE3)pLys (Invitrogen) in 2 × YT medium supplemented with 100 µg ampicillin ml⁻¹, 1 mM IPTG and 1 % (w/v) glucose. Affinity chromatography using a Ni-NTA column (Qiagen) was applied to purify the recombinant His₆-ClpX protein under denaturing conditions according to the manufacturer's instructions. The purified protein was dialysed against distilled water to remove denaturing agent and salts. The dialysed protein was then lyophilized and resuspended in PBS. Quantification of recombinant His₆-ClpX protein was carried out by electrophoresis of serial dilutions of the protein and BSA and lysozyme standards (100, 250, 500 and 1000 ng) on an SDS-polyacrylamide gel and capturing the gel image using a densitometer (proxPRESS Proteomic Imaging System; PerkinElmer Life Sciences). The image was analysed using the PROTEOME ID ANALYSER version 1.10 (PerkinElmer Life Sciences) to calculate protein concentration.

Preparation of mouse polyclonal antiserum against *Brachyspira pilosicoli* strain WesB. A whole-cell bacterin was prepared from *Brachyspira pilosicoli* strain WesB as described previously (Hampson *et al.*, 2000). A total of 10⁸ formalin-treated WesB cells were emulsified in an equal volume of Freund's incomplete adjuvant (Sigma) in a total volume of 100 µl and administered subcutaneously into five 5-week-old male C3H/HeJ mice (Western Australian State Animal Resource Centre) three times at 2-week intervals. Three weeks after the last inoculation, the mice were euthanized by methoxy-flurane inhalation, followed by cervical dislocation. Blood was collected by cardiac puncture and the serum was separated and stored at -20 °C until used in Western blot analysis.

Immunization of mice using recombinant His₆-ClpX protein. Ten C3H/HeJ male mice of 5 weeks of age were obtained from the Western Australian State Animal Resource Centre and housed in two groups each comprising five mice. The five mice in the vaccinated group were injected subcutaneously twice with a 2-week interval with 100 µg His₆-ClpX protein emulsified in an equal volume of Freund's incomplete adjuvant in a total volume of 100 µl. The second group of five mice was left unvaccinated. Three weeks after the last inoculation, all of the mice were euthanized and the blood was collected as described above. Aliquots of 5 µl of the sera from the vaccinated mice were adsorbed by mixing with 5 µl recombinant His₆-ClpX protein at 300 ng µl⁻¹ in PBS and incubating at 37 °C on a rotary mixer for 1 h.

Western blot analysis using sera from immunized mice. The mouse sera were analysed by Western blot analysis against both whole-cell protein preparations of *Brachyspira pilosicoli* and the recombinant His₆-ClpX protein. For preparation of whole-cell

proteins, cells of *Brachyspira pilosicoli* strain WesB were suspended in PBS at a concentration of 10⁸ cells ml⁻¹ and sonicated on ice for three cycles of 30 s with a 2 min rest between each cycle. The sonicate was centrifuged at 10 000 g for 10 min and the supernatant was separated for a blot assay (Hampson *et al.*, 2000). The total protein concentration was quantified using a Bio-Rad protein assay kit, according to the manufacturer's instructions. Ten micrograms of cell protein was separated by 12.5 % (w/v) denaturing SDS-PAGE in a Tris/glycine buffer system. The loaded protein was then transferred electrophoretically to a 0.2 µm nitrocellulose membrane. The membrane was blocked with TBS-T (Tris-buffered saline supplemented with 0.05 %, v/v, Tween 20) containing 5 % (w/v) skimmed milk powder for 1 h, followed by three washings with TBS-T. The membrane was assembled into a multi-screen apparatus (Bio-Rad) for multiple serum analysis. Mouse sera were diluted 100-fold in 100 µl TBS-T or 50-fold for the adsorbed sera, added to the membrane through the wells of the multi-screen probe and incubated for 1 h at room temperature with gentle mixing. The membrane was reacted with a 1 : 2500 dilution of goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) for 1 h at room temperature, followed by three washes with TBS. Immunodetection of proteins was carried out using 10 ml developing buffer [100 mM Tris/HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl₂] containing 0.33 mg nitro blue tetrazolium chloride (Sigma) ml⁻¹ and 0.165 mg 5-bromo-4-chloro-3-indolyl phosphate (Sigma) ml⁻¹. For Western blotting using recombinant His₆-ClpX protein, 10 µg of the recombinant protein was used. All other procedures were the same as described above.

Western blot immunoreactivity of His₆-ClpX protein with human sera. Human sera were also reacted in Western blots with recombinant His₆-ClpX protein as described for mouse sera, except that anti-human IgG-alkaline phosphatase conjugate was used.

The 33 human sera tested were obtained as part of a previous study (Brook *et al.*, 2001); they comprised 12 samples from migrant individuals from developing countries who, at the time of sampling, were colonized by *Brachyspira pilosicoli* as determined by faecal culture and PCR, five samples from migrant individuals who were negative for *Brachyspira pilosicoli* and 16 samples from healthy, culture-negative individuals from Perth, Western Australia.

RESULTS AND DISCUSSION

Distribution of the *clpX* gene in *Brachyspira* spp.

The 878 bp internal portion of the 1785 bp *clpX* gene was amplified by PCR from 29 of 35 strains (83 %) of *Brachyspira pilosicoli*, six of 24 strains (25 %) of *Brachyspira hyodysenteriae*, 14 of 16 strains (88 %) of *Brachyspira intermedia*, six of 17 strains (35 %) of *Brachyspira innocens*, one of six strains (17 %) of *Brachyspira murdochii*, one of two (50 %) strains of *Brachyspira aalborgi* and not from the single strain of *Brachyspira alvinipulli*. Given the known important functional roles of ClpX in other bacterial species, it is likely that a form of the *clpX* gene was present in all of the strains tested, but that heterogeneity at the primer sites used prevented PCR amplification. In any case, it was established that the gene was common in strains and species from throughout the genus and that this broad distribution represents an important advantage if ClpX were to be used as a component of a subunit vaccine.

Comparative analysis of the *clpX* gene and protein

The *clpX* gene in the 20 strains in which it was sequenced was highly conserved, sharing 99.3–99.7 % similarity at the nucleotide level and 99.7–100 % at the amino acid level. The nucleotide sequence for *clpX* in *Brachyspira pilosicoli* strain 95/1000 was identical to those in *Brachyspira intermedia* strains OR2 and OF11, whilst the peptide sequences of *clpX* in nine strains including *Brachyspira pilosicoli* strains 'S. jonesii', WesB and Q98.0026, *Brachyspira innocens* strains B256^T, Q91-12233 and Q91-1530-1, *Brachyspira intermedia* strains OR2 and OF11 and *Brachyspira hyodysenteriae* strain 155.11 were identical to those in *Brachyspira pilosicoli* strain 95/1000. The other strains had one or two amino acid differences (Table 2) and these occurred throughout the molecule. Five strains had a substitution of asparagine for serine at position 154. The general conservation of the protein across the different strains and species again is advantageous for subunit vaccine development.

The dendrogram constructed based on the *clpX* sequences is shown in Fig. 1. The sequences started at the ATG start codon and ended at the stop codon. The genes from six of the 12 strains (50 %) of *Brachyspira pilosicoli* were located in cluster IV, whilst the genes from the others, including 95/1000, were found in the other three clusters, together with those from other *Brachyspira* spp. The general imperfect match of the dendrogram with the accepted phylogeny of the *Brachyspira* spp. may have been associated with recent mutational changes in *clpX* or with horizontal gene transfer and genetic recombination between the species. There was no consistent clustering of *Brachyspira pilosicoli* strains according to the host species of origin, consistent with strains being able to cross species boundaries and potentially being zoonotic (Hampson *et al.*, 2006b).

In silico analysis of the *clpX* gene and protein

A BLASTP similarity search on ClpX found 38–44 % peptide identity between the *Brachyspira pilosicoli* strain 95/1000 ClpX and the ATP-dependent ClpX of a variety of bacteria including *Thermotoga maritima*, *Thermotoga naphthophila*, *Rickettsia conorii*, *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Streptococcus thermophilus*, *Borrelia burgdorferi*, *Borrelia garinii*, *Legionella pneumophila*, *Treponema denticola*, *Clostridium perfringens*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Leptospira interrogans*, *Salmonella enterica* and *Francisella tularensis*. As described by Trott *et al.* (2003), the *Brachyspira* ClpX had an extra ~200 amino acids at the N-terminal end of the protein compared with the ClpX protein in these other species and, consequently, the similarities were all in the C-terminal end of the molecule. The only similarity found at the N-terminal end was to the ATP-dependent zinc metalloprotease of *Bacillus halodurans*, which had about 8 % amino acid similarity with the N-terminal protein sequence from the ClpX of *Brachyspira pilosicoli* strain 95/1000.

RPS-BLAST analysis identified a 409 aa domain (residues 175–583) that was similar to the ATP-dependent Clp protease ATP-binding subunit clpX of *Bacillus halodurans* (GenBank accession no. Q9K8F4). The conserved domain architecture retrieval tool (CDART) identified a 105 aa region (residues 274–378) of the ClpX polypeptide that showed functional and architectural similarity to the Ruvb domain of the *Thermus thermophilus* Hb8 protein (GenBank accession no. 1HQCA).

Results from SIGNALP showed that the *Brachyspira* ClpX had no signal peptide in the 70 aa of its N-terminal sequence. PSORT predicted ClpX as a bacterial inner-membrane protein, whilst PSORT-B recognized it as a protein with unknown localization in the cell. PENCE and CELLO recognized ClpX as a cytoplasmic protein with high

Table 2. Amino acid substitutions in ClpX proteins of eight strains of *Brachyspira pilosicoli* and of *Brachyspira aalborgi* strain 513^T and *Brachyspira hyodysenteriae* strain 884 compared with *Brachyspira pilosicoli* reference strain 95/1000 (GenBank accession no. AY466377)

No. of strains	Strain ID	Position of different amino acid	Amino acid in sequenced strain	Amino acid in strain 95/1000
2	HRM2B	154	Asparagine	Serine
3	V1H91			
	513 ^T	225	Aspartic acid	Glutamic acid
	M1			
	P43/6/78 ^T			
1	Margaret	483	Asparagine	Aspartic acid
1	884	569	Aspartic acid	Glutamic acid
1	HRM7	154	Asparagine	Serine
		444	Methionine	Isoleucine
2	D17	8	Lysine	Glutamic acid
	16242.94	154	Asparagine	Serine

Fig. 1. Dendrogram constructed by the neighbour-joining method from the MEGA 3.1 software using the entire 1785 bp *clpX* gene. The tree comprised *clpX* genes from 20 strains of intestinal spirochaetes. The number adjacent to the nodes shows the bootstrap percentage. Bar, 0.0005 nucleotide substitutions per site.

prediction scores. The TMPRED software predicted a possible 24-residue transmembrane helix at aa 396–419. Searches with IEP and GEECEE predicted a molecular mass for ClpX of 67.42 kDa, a pI of 6.74 and a GC content of 28.06 mol%.

These *in silico* results generally agreed with those of Trott *et al.* (2003). As the *Brachyspira* ClpX protein had no signal peptide or obvious transmembrane region, it is likely to be cytoplasmic, but may be associated with the inner membrane. An inner-membrane and possible outer-membrane localization was suggested by Trott *et al.* (2003), based on

partitioning of the molecule in different cell fractions. The general conservation of amino acid sequence at the N-terminal end emphasizes the unique nature of the ClpX proteins in *Brachyspira* spp., but no clues were found as to the likely function of the unusual N-terminus.

Expression and immunogenicity of the His₆–ClpX protein in mice

The native ClpX protein had a predicted molecular mass of 67.42 kDa according to its amino acid sequence and an

Fig. 2. Detection of His₆–ClpX protein. (a) Coomassie brilliant blue-stained gel showing purified recombinant His₆–ClpX protein. (b) Immunoblot detection of purified recombinant His₆–ClpX protein using anti-His antibody and anti-mouse IgG–alkaline phosphatase conjugate. (c) Western blot reactivity of mouse serum raised against a bacterin preparation of *Brachyspira pilosicoli* strain WesB and reacted with purified recombinant His₆–ClpX protein. M, Molecular mass markers (kDa).

apparent molecular mass of 71.5 kDa as a His-tagged protein (Fig. 2). In Western blot analysis, the recombinant His₆-ClpX protein reacted strongly with mouse sera raised against whole-cell proteins of *Brachyspira pilosicoli* strain WesB (Fig. 2). Likewise, sera from the mice vaccinated with the recombinant His₆-ClpX protein reacted strongly with a ~67 kDa band in the whole-cell preparations of *Brachyspira pilosicoli* strain WesB (Fig. 3). Sera absorbed with recombinant His₆-ClpX protein no longer reacted with this band (data not shown). These results are encouraging for future development of *Brachyspira* ClpX as a vaccine subunit, as they demonstrate that native ClpX stimulates an immune reaction and that the recombinant His-tagged ClpX is immunogenic. Further work is required to evaluate whether vaccination with recombinant ClpX can provide protection against *Brachyspira* spp. infections.

Reactivity of human sera with recombinant His₆-ClpX protein

All of the human sera, whether or not the individuals were colonized with *Brachyspira pilosicoli*, reacted with the ClpX recombinant protein in Western blots (data not shown). This broad serological reactivity probably represented cross-reactivity, most likely resulting from previous exposure of these adults to other bacteria expressing forms of ClpX, although the possibility of prior exposure of all individuals to *Brachyspira pilosicoli* cannot be ruled out. These findings suggest that ClpX is unlikely to be a suitable serological reagent for use in detecting exposure to *Brachyspira* species, although it is possible that the unique N-terminal end of the molecule could be more serologically conserved. Further work is required to investigate this possibility.

Fig. 3. Western blot reactivity of mouse sera raised against the recombinant His₆-ClpX protein and reacted with whole-cell protein extracts of *Brachyspira pilosicoli*. Lanes 1–5, normal mouse sera (negative controls); lanes 6–10, serum samples from individual immunized mice. M, Molecular mass marker (kDa).

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