Research article

Epidemiological survey of serum titers from adults against various Gram-negative bacterial V-antigens

Short title: Gram-negative bacterial V-antigen titers

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Abstract

The V-antigen, a virulence-associated protein, was first identified in *Yersinia pestis* more than half a century ago. Since then, other V-antigen homologs and orthologs have been discovered and are now considered vital molecules for the toxic effects mediated by the type III secretion system in infections caused by various pathogenic Gram-negative bacteria. After purifying recombinant V-antigen proteins including PcrV from *Pseudomonas aeruginosa*, LcrV from *Yersinia*, LssV from *Photobacterium luminescens*, AcrV from *Aeromonas salmonicida*, and VcrV from *Vibrio parahaemolyticus*, we developed an enzyme-linked immunoabsorbent assay to measure titers against each V-antigen in the sera collected from 186 adult volunteers. Different titer-specific correlation levels were determined for the five V-antigens. The anti-LcrV and anti-AcrV titers shared the highest correlation with each other, with a correlation coefficient of 0.84. The next highest correlation coefficient was between anti-AcrV and anti-VcrV titers at 0.79, while the lowest correlation was found between anti-LcrV and anti-VcrV titers, which were still higher than 0.7. Sera from mice immunized with one of the five recombinant V-antigens displayed cross-antigenicity with some of the other four V-antigens, supporting the results from the human sera. Thus, the serum anti-V-antigen titer measurement system

34 could potentially be used for epidemiological investigations on various pathogenic Gram-

35 negative bacteria.

36 Introduction

37 The type III secretion system (TTSS) plays an important role in the virulence of many
 38 Gram-negative bacteria [1-3]. Through the TTSS, Gram-negative bacteria inject their
 39 effector molecules to target eukaryotic cells and induce a favorable environment for their
 40 infections. Translocation is a mechanism through which effector molecules of the TTSS
 41 pass through the targeted eukaryotic cell membrane. During translocation, three bacterial
 42 proteins form a translocational pore structure called the ‘translocon’ [1-3]. A cap protein
 43 in the secretion apparatus of the type III secretion needle is one type of translocon protein,
 44 which for historical reasons is called the V-antigen in *Yersinia* spp. [4-8]. Briefly, the V-
 45 antigen, a virulence-associated protein, was identified as an antigenic component
 46 recognized by the immune system in *Yersinia pestis* plague-infected hosts more than half
 47 a century ago [4-8]. In the 1980s, the V-antigen of *Y. pestis* was identified as a low-
 48 calcium response (*lcr*)-associated protein (named LcrV) encoded in the plasmid
 49 associated with its virulence [9]. A homologous gene called PcrV was identified in the
 50 *Pseudomonas aeruginosa* genome [10], and it has been reported that the virulence
 51 associated with the TTSS can be inhibited by a specific antibody against LcrV in *Yersinia*
 52 and PcrV in *P. aeruginosa* [11, 12]. Because vaccinating mice with LcrV or PcrV had
 53 protective effects against lethal infections with *Yersinia* or *P. aeruginosa*, respectively,

later on, anti-PcrV immunotherapy was developed to target human infections with *P. aeruginosa* using immunoglobulins [13-24] and vaccines [25-27], from which and several projects have progressed to human clinical trials [28-31].

We recently published an epidemiological study on serum titers against PcrV in human volunteers [32], and another showing how the prophylactic administration of human serum-derived immunoglobulin with a high anti-PcrV titer significantly improved the survival rate, pulmonary edema, and inflammatory cytokine production in a *P. aeruginosa* pneumonia model [18]. The results of both studies imply that immunity against the V-antigen and its homologs might be necessary for preventing infections caused by pathogenic bacterial species utilizing the TTSS-virulence mechanism. The possession of V-antigen homologs has recently been reported in several Gram-negative bacteria, including *Aeromonas* spp., *Vibrio* spp., and *Photobacterium luminescens* (hereafter referred to as *Ph. luminescens*) [33]. Although specific immunity against the V-antigen or its homologs seems to be important for host immunity against such bacterial infections, insufficient information is available on human immunity against V-antigens. Therefore, here, we conducted epidemiological studies on serum titers against the V antigen and its homologs in *Y. pestis*, *Aeromonas salmonicida*, *Vibrio parahaemolyticus*, and *Ph. luminescens*. Potential associations for age, titer levels, and cross-reactivity

among the recombinant V-antigen homologs were evaluated, and for some species the titer levels against these antigens were highly correlated and some V-antigen homologs showed cross-reactivity. Speculation about specific immunity against multiple pathogens will also be discussed.

Materials and methods

Construction of five recombinant Gram-negative bacterial V-antigens (PcrV, LcrV, AcrV, VcrV, and LssV)

Five recombinant V-antigens were constructed. Details on the PCR primers and cloning sites are listed in **Table 1**. The coding regions in the V-antigens were polymerase chain reaction (PCR)-amplified with specific primers containing the restriction enzyme sites for insertion into a protein expression vector. PCR-amplified genes were cloned into the pCR2.1 cloning vector and *E. coli* TOP10F cells via TOPO cloning (Thermo Fisher Scientific, Waltham, MA, USA). After digesting the purified plasmids containing each individual cloned gene with restriction enzymes, the inserted coding regions of each gene were transferred to the multi-cloning site of expression vector pQE30 (Qiagen, Hilden, Germany) for the expression of a hexahistidine-tagged protein in *E. coli* M15. The various

89 endotoxin-free Gram-negative bacteria V-antigens were prepared as reported previously

90 (Fig. 1) [17, 20].

91

92 **Table 1.** Gene sources, primer sets for V-antigen gene cloning, and characteristics of

93 the recombinant V-antigens used in this study.

Gene	Gene source	Restriction enzyme site for expression vector	Cloning PCR primers*	Coding region size (bp)	V-antigen proteins	Amino-acids	MW (kDa)	Ref
<i>pcrV</i>	<i>Pseudomonas aeruginosa</i> PA103	<i>Bam</i> HI <i>Hind</i> III	5'- <u>CGGGATCC</u> ATGGAAGTCA GAAACTTTAA-3' 5'- <u>AAGCTT</u> CTAGATCGCGCTG AGAATGT-3'	927	PcrV	306	33.8	[34-36]
<i>lcrV</i>	<i>Yersinia pestis</i> pCD1 plasmid	<i>Bam</i> HI <i>Hind</i> III	5'- <u>CGGGATCC</u> ATGATTAGAGC CTACGAACAAAACCCACA A-3' 5'- <u>AAGCTT</u> TCATTTACCAGAC GTGTCATCTAGCAGACG-3'	1,023	LcrV	338	38.6	[9]
<i>acrV</i>	Plasmid JF2267 of <i>Aeromonas salmonicida</i> subsp. <i>Salmonicida</i> ,	<i>Sph</i> I <i>Sal</i> I	5'- <u>GCATGC</u> ATGAGCACAATCC CTGACTAC-3' 5'- <u>GTCGACTC</u> AAATTGCGCCA AGAATGTCG-3'	1,134	AcrV	375	41.6	14
<i>vcrV</i>	<i>Vibrio parahaemolyticus</i> , ATCC 17802D-5	<i>Bam</i> HI <i>Pst</i> I	5'- <u>CGGGATCC</u> ATGACGGATAT GACAACAAC-3' 5'- <u>CTGCAGT</u> TAAATGGCTCGT AGGATTTCTTG-3'	1,866	VcrV	619	68.3	-
<i>lssV</i>	<i>Photobacterium luminescens</i> subsp. <i>luminescens</i> , ATCC 29999	<i>Bam</i> HI <i>Hind</i> III	5'- <u>GGATCC</u> ATGGAAATAGGC CATATCAA-3' 5'- <u>AAGCTT</u> TTAAATGCGCCG AGAATAT-3'	1,020	LssV	337	38.3	-

94 *: letters with underlining designate the restriction enzyme site

95

96 **Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**
 97 **separation of extracted recombinant hexahistidine-tagged V-antigen proteins.**

98 Recombinant PcrV from *P. aeruginosa*, LcrV from *Y. pestis*, AcrV from *Aeromonas*
 99 *salmonicida*, VcrV from *V. parahaemolyticus*, and LssV from *Ph. luminescens* were
 100 SDS-PAGE separated using a 10% Bis-Tris-gel.

101

102 **Survey participants and study background**

103 This study was approved by the Kyoto Prefectural University of the Medicine Ethics
 104 Committee. Surgical adult patients (n=186) who were undergoing anesthesia in the
 105 central operating division of the Kyoto Prefectural University of Medicine, from April
 106 2012 to March 2013, participated in this study as volunteers, as previously reported [32].
 107 Briefly, serum was prepared from the remaining small amount of each blood sample
 108 collected for arterial blood gas analysis on the induction of anesthesia, and then stored at
 109 -80°C .

110

111 **Anti-V antigen titer measurements**

We developed an enzyme-linked immunoabsorbent assay (ELISA) for measuring the serum anti-V antigen titers. Micro-well plates (Nunc C96 Maxisorp; Thermo Fisher Scientific) were coated for 2 h at 4°C with recombinant V-antigen proteins (recombinant PcrV from *P. aeruginosa*, LcrV from *Yersinia*, AcrV from *Aeromonas*, VcrV from *Vibrio*, and LssV from *Ph. luminescens*) suspended in coating buffer (1.0 µg/mL in coating solution; 0.0M NaHCO₃, pH 9.6) [32] (**Fig.1**). The plates were washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (P9416; Sigma-Aldrich, St. Louis, MO, USA), and then blocked with 200 mL of 1% bovine serum albumin/PBS overnight at 4°C. Samples (serial dilution: 1280×) were applied to the plates (100 mL/well) and then incubated overnight at 4°C. Peroxidase-labeled anti-human IgG (A8667, Sigma- Aldrich) was applied at 1:60,000 for 1 h at 37°C. After six washes, the plates were incubated with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (A3219; Sigma-Aldrich) at room temperature for 30 min. After adding 0.5 M H₂SO₄ at 100 mL/well to the plates, the optical density (O.D) at 450 nm was measured with a microplate reader (MTP-880Lab; Corona Electric, Hitachinaka, Japan). To ensure no cross-reactivity with *Escherichia coli* proteins, competitive ELISA with a soluble fraction of *E. coli* M15 lysate was performed, and no significant effect on titer measurement was observed.

130

131 **Immunizing mice with the V-antigens**

132 Certified pathogen-free, male ICR mice (4 weeks old) were purchased from Shimizu
 133 Laboratory Supplies, Co, Ltd., Japan. Mice were housed in cages with filter tops under
 134 pathogen-free conditions. The protocols for all animal experiments were approved by the
 135 Animal Research Committee of Kyoto Prefectural University of Medicine, Kyoto, Japan,
 136 before undertaking the experiments (authorization number: M29-592). Three mice per
 137 group were intradermally immunized with one of the five recombinant V-antigen proteins
 138 (10µg/dose) adjuvanted with complete Freund's adjuvant in the first injection, and four
 139 weeks later with incomplete Freund's adjuvant as a second injection. Eight weeks after
 140 the first injection, the immunized mice were euthanized, and peripheral blood samples
 141 were collected from them. Serum titers against the five V-antigens were individually
 142 measured by ELISA, as described above.

143

144 **Phylogenetic and cluster analyses**

145 The five V-antigens were phylogenetic analyzed using ClustalW (Genome Net,
 146 <https://www.genome.jp/tools-bin/clustalw>), or RStudio (version 1.2, Boston, MA, USA.
 147 <https://www.rstudio.com>) with R version 3.4.3 (The R Foundation, <https://www.r->

[project.org](#)). Unrooted trees were prepared using the neighbor-joining method and rooted trees were prepared using the unweighted pair group method with arithmetic mean, and heat maps where the individual values contained in a matrix are represented as colors with dendrograms were made using the heat map function of R language. The predicted three-dimensional structures were generated with the Cn3D macromolecular structure viewer at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>).

Results

Anti-V antigen titers and volunteer age distribution

The 186 volunteers consisted of 111 (59.7%) males and 75 (40.3%) females, and the following age distribution: 20–29 years (13, 7.0%), 30–39 years (20, 10.8%), 40–49 years (26, 14.0%), 50–59 years (23, 12.4%), 60–69 years (40, 21.5%), 70–79 years (42, 22.5%), and 80 years plus (22, 11.8%). No study participant had an active infection. The titers against the V-antigen across the age distribution of the 186 participants are shown in **Fig. 2**. There was no statistically significant correlation in the linear regression between age and each anti-V antigen titer, although high anti-PcrV titers were more common at over 50 years of age in the population, as reported previously [32]. As an overall trend, two

separate titer peaks in the 40–50 and 70–80 age groups were observed in the age distribution of all the anti-V-antigen titers.

Fig. 2. Age distribution of human V-antigen titers

The diluted serum (1,280×) was used for ELISAs, and the O.D. 450 nm values were measured. O.D. values of 0.5 or more are indicated by red dots, while yellow dots represent values between 0 and 0.3.

Correlations among the anti-V antigen titers

We next analyzed whether any correlations existed among the five anti-V antigen titers. The anti-LcrV and the anti-AcrV titers showed the highest correlation with a correlation coefficient 0.84, followed by the anti-AcrV and anti-VcrV titers at 0.79, and the anti-LcrV titers and anti-VcrV titers at 0.74 (**Fig. 3**) No statistically significant correlations were detected between anti-PcrV and any of the other anti-V-antigens.

Fig. 3. Serum titer correlations between two V-antigens

The diluted serum (1,280×) was used for ELISAs, and the O.D. values at 450 nm were measured. The titer correlation of the two V-antigens was mapped in an X–Y plot.

A cluster analysis was prepared from the correlation coefficient values, and phylogenetic trees and a heat map were made (**Fig 4A**). The unrooted and rooted phylogenetic trees and the heat map show that anti-PcrV had the most unique profile among the five anti-V-antigen titers. The anti-LssV titer is located between anti-PcrV and three other anti-V-antigen titers. Higher homology in antigenicity was observed among anti-AcrV, anti-LcrV, and anti-VcrV titers.

Fig. 4. Phylogenetic trees and heat maps showing the correlations among the five anti-V-antigen titers

A. Human serum titers. The correlation coefficients of the serum titer correlations shown in **Fig. 3** were matrixed. Phylogenetic trees (an unrooted tree, neighbor-joining method; and a rooted tree, unweighted pair group method with arithmetic mean) and a heat map were constructed. **B.** Anti-V-antigen serum titers from mice immunized with one of the five V-antigens. Phylogenetic trees (unrooted tree, neighbor-joining method; and a rooted tree, unweighted pair group method with arithmetic mean) and a heat map were constructed.

Cross-reactive antibodies against V-antigens and anti-V antigen titers in the serum from immunized mice

To evaluate potential cross-antigenicity among the five V-antigens, the serum titers from a mouse immunized with one of five recombinant V-antigen proteins were measured by ELISA (**Fig 5**). Cluster analysis was applied to the analysis by using the O.D. values from the ELISA, from which phylogenetic trees and a heat map were made (**Fig 4B**). LssV and AcrV showed higher cross-antigenicity with each other, unlike VcrV, which was less cross-reactive against the other V-antigens (**Fig. 4B and 5**). While the sera from the AcrV-immunized mice reacted with PcrV, the sera from PcrV-immunized mice did not react with AcrV.

Fig. 5. ELISA plate displaying the anti-V-antigen serum titers from mice immunized with one of the five V-antigens.

Three mice per group were intradermally immunized with one of five recombinant V-antigen proteins (10µg/dose) adjuvanted with complete Freund's adjuvant as the first immunization and, four weeks later, with incomplete Freund's adjuvant as the second immunization. Eight weeks after the first immunization, mice were euthanized and peripheral blood samples were collected. Serum titers against the five V-antigens were

measured by ELISA. The plates were incubated with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) at room temperature for 30 min, and this photo was taken.

Next, to determine whether correlations among the V-antigens have some association with the identity of the primary protein sequences, we investigated the identity of these sequences for the five V-antigens using the BLOSUM substitution score matrix in ClustalW (**Fig. 6**). In this map, the overall similarities of two out of five of the V-antigens for the whole molecules ranged between 21 and 49%. The similarity of the amino-terminal domain (14–51%) and central domains (14–45%) can be seen to be low in comparison with the carboxyl-terminal domain that showed 48–84% similarity. VcrV is unique with a long extra sequence (over 160 amino acids, aa) at the amino-terminal domain, and an extra sequence (of 80 aa) in the central domain of the sequence alignment.

Fig. 6. Clustal W sequence alignment of the five primary V-antigen sequences

The overall sequence similarities of two of the five V-antigens across the whole molecules are between 21 to 49%. The similarity of the amino-terminal domain (14–51%) and central domains (14–45%) is low in comparison with the carboxyl-terminal domain,

which has a 48–84% similarity range. VcrV is unique with an extra-long amino-terminal domain sequence (over 160 aa), and an extra sequence (80 aa-) in the central domain of the sequence alignment.

We also made phylogenetic trees of V-antigens based on the primary amino acid sequence similarity scores of whole molecules, amino-terminal domains, central domains, and carboxyl-terminal domains (**Fig. 7**). In the cluster analysis of the carboxyl domains with the highest similarity scores (48–84%) for their amino-acid sequences, AcrV, LssV, and PcrV are closer than VcrV and LcrV. These phylogenetic analyses using the primary amino-acid sequences did not match well with the phylogenetic trees constructed from the correlations among the serum IgG titers of the five V-antigens (**Fig. 4A**). In particular, VcrV, which has an extremely long central domain containing regions that are missing in the other V-antigens, occupies a separate position in the phylogenetic trees made from the primary amino-acid sequences. Therefore, these findings suggest the titer cross-antigenicity in human sera may not be correlated with similarity in the amino-acid primary sequences.

Fig. 7. Phylogenetic trees and heat maps based on the primary amino-acid sequences

(whole molecule, amino-terminal, center, and carboxyl-terminal) of the V-antigens.

Phylogenetic analyses of the primary amino-acid sequences of whole molecules, amino-terminal, center, and carboxyl-terminal domains. A. Complete primary sequence. B. Amino-terminal domain, C. Center domain, D. Carboxyl-terminal domain. The amino acid positions in the amino-terminal, center, and carboxyl-terminal domains are as follows: LcrV: #1-#164, #165-#278, #279-#326 respectively; PcrV: #1-#142, #143-#256, #257-#294, respectively; AcrV: #1-#162, #163-#316, #317-#361, respectively; VcrV: #1-#361, #362-#558, #559-#607, respectively; LssV: #1-#170, #171-#280, #281-#325, respectively.

As we have previously reported, a blocking monoclonal antibody (called Mab166) against PcrV recognizes a conformational structure but not a primary amino-acid sequence [15]. As shown in **Fig. 8**, the predicted three-dimensional structures of the V-antigens have similar dumbbell-like structures with two globular domains on either end of a grip formed by two coiled-coil motifs [37]. The grip that connects the two globular domains contains an antiparallel coiled-coil structure comprising a central coiled-coil region and a carboxyl-terminal coiled-coil region. The carboxyl-terminal is folded into a

single long α -helix. Therefore, there is a possibility that the correlations we have observed may be dependent on the sequence similarity of the three-dimensional structures associated with the conformational epitopes.

Fig. 8. The predicted three-dimensional V-antigen structures.

The predicted three-dimensional structures were generated by the Cn3D macromolecular structure viewer at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>).

Discussion

Yersinia LcrV has been recognized as a V-antigen with immunoprotective characteristics in *Yersinia* infections since the 1950s [4, 5, 7, 8], but it took almost 50 years until LcrV, *P. aeruginosa* PcrV, and *Aeromonas* AcrV were anatomically visualized as distinct structures on the tip of the needle of the injectisome of the type III secretion apparatus [38-41]. Because specific antibodies binding to a particular portion of this structure can inhibit the translocation of type III secretory toxins in *Yersinia* and *P. aeruginosa* [11, 42], gaining better understanding of the interactions between V-antigens and the host's

humoral immunity against the virulence of bacterial type III secretion is important if potential non-antibiotic treatments for infections in various hosts are to be developed.

Cross-antigenicity among *Yersinia* spp. was reported nearly 40 years ago. In 1980, cross-immunity to *Y. pestis* was noted in mice that had been orally infected with *Y. enterocolitica* serotype O3 [43]. In 1983, it was also reported that partial protection in mice against *Y. pestis* infection by the *Y. enterocolitica* V-antigen was linked to the partial cross-reactivity of V-antigens [44]. Later, DNA sequencing of the most common serotypes of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* revealed that two evolutionary distinct types of V-antigen exist in *Yersinia* spp. [45]. One type is represented by the *Y. enterocolitica* serotype O8 strains WA, WA-314, and NCTC 10938 (LcrV-YenO8), while the other type comprises *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serotypes O3, O9, and O5 (LcrV-Yps). By raising monospecific antisera against both types of V-antigen (*Y. enterocolitica* serotypes O3 and O8), anti-V-antigen serum was protective only if the immunizing V-antigen was the same type as the V-antigen produced by the infecting strain. The difference in protective immunity between the two types was caused by the presence of a hypervariable region between amino acids 225 and 232. The protectivity of the V-antigen was later confirmed and refined using the recombinant V-antigen of *Y. pseudotuberculosis* and monospecific anti-V-antigen serum

[46]. In that study, antiserum against the *Y. pseudotuberculosis* V-antigen provided mice with passive immunity to a challenge infection with *Y. pestis* or *Y. pseudotuberculosis* but not *Y. enterocolitica* O8 (strain WA). These past studies on cross-antigenicity to *Yersinia* V-antigens in mice imply that the structure of the key domain, but not the overall primary amino acid sequence similarity *per se*, is important for protective immunity.

Other than *Yersinia* LcrV, only two studies about *P. aeruginosa* PcrV have reported on antibody titers against *P. aeruginosa* PcrV in human serum [32, 47]. No other studies investigating antibody titers against other V-antigens have been reported as yet. *V. parahaemolyticus*, a Gram-negative marine bacterium, the V-antigen titers against it of which were examined in the present study, causes food-borne gastroenteritis [48, 49].

Among *Vibrio* spp., *V. harveyi* (a Gram-negative bioluminescent marine bacterium) is ubiquitous in the marine environment. It is considered one of the important bacterial species that form the normal flora of healthy shrimp and it carries a V-antigen homolog gene in its genome [50]. This bacterium is sometimes recognized as causing high mortality in shrimps in the worldwide shrimp fishing industry [51, 52]. As a halophilic *Vibrio* species, *V. alginolyticus*, which causes wound infections, was first recognized as pathogenic to humans in 1973 [53]. Recent studies have proposed that *V. alginolyticus* possesses the same TTSS gene organization as *V. parahaemolyticus* and *V. harveyi* [54,

55]. *Aeromonas* spp., such as *A. salmonicida* and *A. hydrophila*, are not pathogenic to humans but cause infections in salmon and trout and carry the AcrV V-antigen homolog. However, *A. hydrophila* sometimes causes gastroenteritis in humans, who acquire infections with it by ingesting food or water containing sufficient numbers of this organism [56-58]. *Ph. Luminescens* (a gammaproteobacterium within the *Enterobacteriaceae* family) is also not pathogenic to humans, but is a lethal pathogen of insects and possesses a pathogenicity island encoding the TTSS including the LssV V-antigen homolog [59-62]. It lives in the gut of an entomopathogenic nematode within the *Heterorhabditidae* family. However, human infections with *Photorhabdus* spp. have recently been reported in the USA and Australia, suggesting that these bacteria are emerging human pathogens [63]. In comparison with the infections caused by *P. aeruginosa*, infections caused by *Y. pestis* or *V. parahaemolyticus* are less common. This makes it interesting that titers against V-antigens from non-human pathogens were also detected in this study. Anti-AcrV showed a high correlation with anti-LcrV, and some correlation was detected between anti-LssV and anti-VcrV. The titers against the antigens from non-human pathogens might result from cross-antigenicity among the V-antigen homologs. Our cluster analysis with a heat map of anti-V-antigen titers from the 182 adult

volunteers displayed a higher correlation between LcrV, AcrV, and VcrV than the correlation between PcrV and LssV and the other V-antigens (**Fig. 4A**).

Immunizing mice with one of the five recombinant V-antigens resulted in cross-antigenicity of the sera against the V-antigens (**Figs. 4B and 5**). This result shows that VcrV is unique, while AcrV, LssV, and LcrV share some degree of cross-antigenicity with each other, although the results differed slightly from the cross antigenicity observed with human sera. In our previous experiments on anti-PcrV titers in human sera, the administration of extracted IgG derived from high titer anti-PcrV sera protected against challenge with lethal pneumonia in a murine model [18]. Therefore, despite no similar immunological experiments being performed with other V-antigens, our study has shown that the cross-reactive antigenicity we observed among the various V-antigens with serum-specific anti-V antigen titers could potentially afford some degree of immunological protection against various Gram-negative bacteria. Further investigation of the conformational blocking epitopes in the needle cap structure of the type III secretory apparatus and the immunological aspects of the structural antigenicity of a critical portion of the V-antigens should provide better understanding on how to effectively block the TTSS-associated virulence associated with various Gram-negative pathogens.

362

363 **Conflict of interest**

364 T. Sawa has patents associated with PcrV immunization (World Patent No.
365 WO0033872; European Patent No. EP1049488; U.S. Patent No. 6309651; U.S. Patent
366 No. 6827935, and Japan Patent No. 2017-020501). Until 2011, T. Sawa received a patent
367 fee from the Regents of the University of California related to the development of a
368 therapeutic monoclonal antibody at KaloBios Pharmaceutical. There are no current
369 financial relationships existing with any organization associated with this study.

370

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377

378 **Author Contributions**

379 **Conceptualization:** Teiji Sawa.

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Fig 1

Fig 2

Fig 3

A. human serum titers

B. murine serum titers

Fig 4

ELISA coating protein

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Fig 5

Fig 6

A. whole molecule

B. amino-terminal

C. center

D. carboxyl-terminal

Fig 7

PcrV

LcrV

AcrV

VcrV

LssV

Fig 8