APPENDIX 1
ABBREVIATIONS USED FOR AMINO ACIDS

Amino Acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX 2

PATIENT SERA PROFILE

(All sera were obtained from The Blood Bank and The Departmant of Medical Microbiology , University Hospital, Kuala Lumpur)

PATIENT LABEL	SEX	AGE	WIDAL TEST 'O' TITRE	'H' TITRE
16	M	23	>2560	>2560
19	F	18	1280	>2560
20	F	21	>2560	>2560
22	M	12	>2560	1280
24	F	20	1280	>2560
25	F	47	>2560	1280
031	M	38	>2560	>2560
034	M	25	>2560	>2560
041	F	14	>2560	1280
043	F	18	>2560	>2560
049	M	28	>2560	>2560
69159	M	8	>2560	>2560
84650	M	16	>2560	>2560
84993	F	6	1280	>2560
M265	M	44	>2560	>2560
M353	F	27	>2560	>2560
M148	F	43	>2560	>2560
26552	M	34	>2560	>2560
NormLKY -18-2	F	25	<40	<40
norm-SD	M	23	<40	<40
LKY19-2	F	19	<40	<40
norm-18/2	F	26	<40	<40

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 99

Amino terminus is printed on the left

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 1 A 1(1,2)PLAQ      <
 2 A 2(1,2)GLAQ      <
 3 A 3(1,2)KDKFGNDRK<
 4 A 4(1,2)KFGNDRKRG<
 5 A 5(1,2)GNDRKRGND<
 6 A 6(1,2)DRKRGNDKT<
 7 A 7(1,2)KRGNDKTGK<
 8 A 8(1,2)GNDKTGKGR<
 9 A 9(1,2)DKTGKGRND<
10 A10(1,2)TGKGRNDKS<
11 A11(1,2)KGRNDKSGT<
12 A12(1,2)RNDKSGTTK<
13 A 1(3,4)DKSGTTKDG<
14 A 2(3,4)SGTTKDGSR<
15 A 3(3,4)TTKDGSREE<
16 A 4(3,4)TDGSREEED<
17 A 5(3,4)GSREEEDKE<
18 A 6(3,4)REEEDKENG<
19 A 7(3,4)EEDKENGDK<
20 A 8(3,4)DKENGDKES<
21 A 9(3,4)ENGDKESKN<
22 A10(3,4)GDKESKNDG<
23 A11(3,4)KESKNDGDG<
24 A12(3,4)SKNDGDGTT<
25 A 1(5,6)NDGDGTTTT<
26 A 2(5,6)DGTTTTQSK<
27 A 3(5,6)GTTTTQSTE<
28 A 4(5,6)TTTQSTEGK<
29 A 5(5,6)TQSTEGKGN<
30 A 6(5,6)STEGKGNDK<
31 A 7(5,6)EGKGNDKRG<
32 A 8(5,6)KGNDKRGDE<
33 A 9(5,6)NDKRGDKEE<
34 A10(5,6)KRGDKEEKS<
35 A11(5,6)GDKEEKSCS<
36 A12(5,6)KEEKSCSDS<
37 A 1(7,8)EKSCSDSKQ<
38 A 2(7,8)SCSDSKQGT<
39 A 3(7,8)SDSKQGTSN<
40 A 4(7,8)SKQGTSNSD<
41 A 5(7,8)QGTSNSDET<
42 A 6(7,8)TSNSDETGK<
43 A 7(7,8)NSDETGKED<
44 A 8(7,8)DETGKEDKG<
45 A 9(7,8)TGKEDKGKE<
46 A10(7,8)KEDKGKEGT<
47 A11(7,8)DKGKEGTED<
48 A12(7,8)GKEGTEDGT<
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49 B 1(1,2)PLAQ      <
50 B 2(1,2)GLAQ      <
51 B 3(1,2)EGTEDGTGQ<
52 B 4(1,2)TEDGTGQDE<
53 B 5(1,2)DGTGQDEDE<
54 B 6(1,2)TGQDEDEGQ<
55 B 7(1,2)QDEDEGQGR<
56 B 8(1,2)EDEGQDRGY<
57 B 9(1,2)EGQDRGYSY<
58 B10(1,2)QDRGYSYNK<
59 B11(1,2)RGYSYNKET<
60 B12(1,2)SYNKETGE<
61 B 1(3,4)YNKETGEES<
62 B 2(3,4)KETGEESDK<
63 B 3(3,4)TGEESDKKS<
64 B 4(3,4)EESDKKSNR<
65 B 5(3,4)SDKKSNREE<
66 B 6(3,4)KKSNREEKG<
67 B 7(3,4)SNREEKGKE<
68 B 8(3,4)REEKGKEDE<
69 B 9(3,4)EKGKEDEGE<
70 B10(3,4)GKEDEGETW<
71 B11(3,4)EDEGETWTR<
72 B12(3,4)EGETWTRGK<
73 B 1(5,6)ETWTRGKKG<
74 B 2(5,6)WTRGKKGGD<
75 B 3(5,6)RGKKGGDRR<
76 B 4(5,6)KKGGDRRKQ<
77 B 5(5,6)GGDRRKQDT<
78 B 6(5,6)DRRKQDTTG<
79 B 7(5,6)RKQDTTGGT<
80 B 8(5,6)QDTTGGTSE<
81 B 9(5,6)TTGGTSEEG<
82 B10(5,6)GGTSEEGEE<
83 B11(5,6)TSEEGEEKT<
84 B12(5,6)EEGEEKTED<
85 B 1(7,8)GEEKTEDGQ<
86 B 2(7,8)EKTEDGQKR<
87 B 3(7,8)TEDGQKRNK<
88 B 4(7,8)DGQKRNKDT<
89 B 5(7,8)QKRNKDTTT<
90 B 6(7,8)RNKDTTTDG<
91 B 7(7,8)KDTTTDGGE<
92 B 8(7,8)TTTDGGEEQ<
93 B 9(7,8)TDGGEEQGR<
94 B10(7,8)GGEEQGRGR<
95 B11(7,8)EEQGRGRQQ<
96 B12(7,8)QGRGRQQEE<
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GENERAL NET SYNTHESIS SCHEDULE NUMBER : 99

Amino terminus is printed on the le

```
 97 C 1(1,2)PLAQ      <
 98 C 2(1,2)GLAQ      <
 99 C 3(1,2)RQRQQEETS<
100 C 4(1,2)RQQEETSDY<
101 C 5(1,2)QEETSDYDR<
102 C 6(1,2)ETSDYDREK<
103 C 7(1,2)SDYDREKQE<
104 C 8(1,2)YDREKQERK<
105 C 9(1,2)REKQERKGG<
106 C10(1,2)KQERKGGKG<
107 C11(1,2)ERKGGKGTE<
108 C12(1,2)KGGKGTEEK<
109 C 1(3,4)GKGTEEKEK<
110 C 2(3,4)GTEEKEKKR<
111 C 3(3,4)EEKEKKRED<
112 C 4(3,4)KEKKREDHT<
113 C 5(3,4)KKREDHTRE<
114 C 6(3,4)REDHTREEG<
115 C 7(3,4)DHTREEGGG<
116 C 8(3,4)TREEGGGGR<
117 C 9(3,4)EEGGGGRSK<
118 C10(3,4)GGGGRSKDK<
119 C11(3,4)GGRSKDKGD<
120 C12(3,4)RSKDKGDNE<
121 C 1(5,6)KDKGDNEDD<
122 C 2(5,6)KGDNEDDNG<
123 C 3(5,6)GNEDDNGKR<
124 C 4(5,6)EDDNGKRER<
125 C 5(5,6)DNGKRERDW<
126 C 6(5,6)GKRERDWCG<
127 C 7(5,6)RERDWCGEE<
128 C 8(5,6)RDWCGEESN<
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APPENDIX 4 PEPTIDE SYNTHESIS SCHEDULE

(the duration of the schedule is for 9 days, the example shown below is for Day 1, the schedule for the subsequent 8 days follow the same format)

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 99 Page 4

Bulk solutions for activator and/or additives (256 wells)

Chemistry Group 1 data for synthesis coupling 1

Activator : DCC requires 526.1 mg in 8.5 ml of DMF

Additive 1: HOBt requires 454.6 mg in 33.0 ml of DMF

WEIGHTS FOR INDIVIDUAL AMINO ACID SOLUTIONS

AA	#	Amino acid description	Batch	Weight (mg) Target	Actual	DMF (ml)	DCC (ml)	HOBt (ml)
D	18	Fmoc-L-Asp(OtBu)-OH	.....	76.2	.....	0.00	0.62	2.47
E	42	Fmoc-L-Glu(OtBu)-OH.H2O	.....	182.7	.....	0.00	1.37	5.49
G	36	Fmoc-Gly-OH	.....	105.6	.....	0.00	1.18	4.74
K	36	Fmoc-L-Lys(Boc)-OH	.....	166.4	.....	0.00	1.18	4.74
N	12	Fmoc-L-Asn(Trt)-OH	.....	76.6	.....	0.00	0.43	1.71
Q	18	Fmoc-L-Gln(trt)-OH	.....	113.0	.....	0.00	0.62	2.47
R	28	Fmoc-L-Arg(PMC)-OH.3IPE	.....	194.3	.....	0.00	0.93	3.73
S	18	Fmoc-L-Ser(tBu)-OH'	.....	71.0	.....	0.00	0.62	2.47
T	30	Fmoc-L-Thr(tBu)-OH	.....	118.7	.....	0.00	0.99	3.98
Y	6	Fmoc-L-Tyr(tBu)-OH	.....	33.0	.....	0.00	0.24	0.96
						0.00	8.19	32.74

Amino acids weighed by :..... Date:.....

Solutions prepared by :..... Date:.....

Solutions dispensed by :..... Date:.....

Comments:-

PIN POSITIONS for Synthesis coupling 1

NEW PIN POSITIONS
A 3(1,2) TO A12(7,8) B 3(1,2) TO B12(7,8) C 3(1,2) TO C 8(5,6)

Well positions for amino acid dispensing

D A 5(1,2) A 9(1,2) A 4(3,4) A 4(7,8) A 7(7,8) A11(7,8) B 2(5,6)
B12(5,6) C 3(3,4)

E A 3(3,4) A 5(3,4) A 3(5,6) A 9(5,6) A 9(7,8) B 4(1,2) TO B 5(1,2)
B12(1,2) B 5(3,4) B 7(3,4) TO B 9(3,4) B 8(5,6) B10(5,6) B 7(7,8)
B12(7,8) C 7(1,2) C11(1,2) C 5(3,4) C12(3,4) C 7(5,6)

C A 4(1,2) A 1(3,4) A 6(3,4) A10(3,4) TO A11(3,4) A 7(5,6) A 8(7,8)
B 6(3,4) B 1(5,6) B 6(5,6) B 9(5,6) B 6(7,8) C 9(1,2) TO C10(1,2)
C 6(3,4) TO C 7(3,4) C 2(5,6) C 6(5,6)

K A 3(1,2) A 7(1,2) A12(1,2) A 7(3,4) A 4(5,6) A 6(5,6) A 8(5,6)
A 6(7,8) B10(1,2) B 2(3,4) B12(3,4) B 3(7,8) C 6(1,2) C 8(1,2)
C12(1,2) TO C 1(3,4) C 9(3,4) TO C10(3,4)

N A 9(3,4) A 5(5,6) A 3(7,8) B10(3,4) C 5(5,6) C 8(5,6)

Q A 1(7,8) B 3(1,2) B 6(1,2) B 4(5,6) B 1(7,8) B 8(7,8) B11(7,8)
C11(3,4) C 1(5,6)

R A 8(1,2) A 2(3,4) B 7(1,2) B 4(3,4) B11(3,4) B 3(5,6) B 2(7,8)
B 9(7,8) TO B10(7,8) C 5(1,2) C 2(3,4) C 8(3,4) C 3(5,6) TO C 4(5,6)

S A10(1,2) A 8(3,4) A 2(5,6) A10(5,6) TO A12(5,6) B 1(3,4) B 3(3,4)
C 3(1,2)

T A 6(1,2) A11(1,2) A12(3,4) TO A 1(5,6) A 2(7,8) A 5(7,8) A10(7,8)
A12(7,8) B11(1,2) B 5(5,6) B 7(5,6) B11(5,6) B 4(7,8) TO B 5(7,8)
C 4(3,4)

Y B 8(1,2) TO B 9(1,2) C 4(1,2)

ELSEVIER

Immunology Letters 62 (1998) 105–109

Immunology Letters

Rapid note

Immunogenic epitopes of *Salmonella typhi* GroEL heat shock protein reactive with both monoclonal antibody and patients sera

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Abstract

A series of 122, 9-mer overlapping peptides based on the sequence of the *Salmonella typhi* GroEL gene was synthesized on the surfaces of polyethylene pins and screened with monoclonal antibody to GroEL and with human sera from patients with typhoid fever and normal healthy blood donors. Three immunogenic epitopes corresponding to peptides EGQDRG*YSY*, *YSY*NKETGE and GKGTEEKEK were identified upon screening with the human sera. In addition, screening of the peptides with a monoclonal antibody to GroEL detected binding to a third peptide, K*GGKGTEEK*, which contains a common overlapping sequence to peptide *GKGTEEK*EK. Identification and definition of these epitopes will be important in delineating the biological and immunological functions of this protein and in designing better diagnostic tests and vaccines. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Epitopes; *S. typhi*; GroEL

In addition to their roles as molecular chaperones in normal physiological processes, heat shock proteins (HSPs) or stress proteins of microbial pathogens have been shown to be important in pathogenesis and host immune responses [1,2]. Many bacterial HSPs have been shown to be immunogenic proteins [3–6] and it was recently demonstrated that immunization with mycobacterial HSPs induced protective immunity against tuberculosis in mice [7]. We have recently shown that the major HSPs GroEL and DnaK were induced in *Salmonella typhi* following a shift in growth temperature from 37 to 45 and 55°C [6]. It was also noted that specific antibodies to these two HSPs were present in the sera of patients with typhoid fever [6]. The availability of molecular sequence information for many immunogenic proteins, together with methods to conveniently synthesize libraries of synthetic peptides on polyethylene pins [8], has enabled studies to define immunogenic or immunodominant epitopes within these proteins [9,10]. In this study we report the identification of three immunogenic 9-mer peptides within *S. typhi* GroEL following the screening of a synthetic peptide library with human sera (from patients and controls) and a monoclonal antibody against GroEL.

A total of 122 overlapping, 9-mer peptides (1 amino acid removed at a time) were synthesized in duplicate on the surfaces of multiple polyethylene pins using the Epitope Scanning Kit (Chiron Technologies, Clayton, Vic., Australia) according to the manufacturer's instructions. Peptides were selected from the hydrophilic regions of the published sequence of *S. typhi* GroEL

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PII S0165-2478(98)00028-5

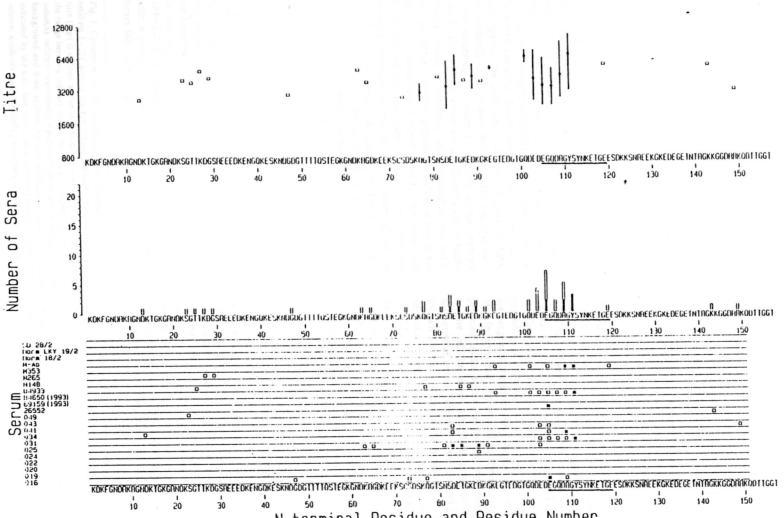
Titre
12800
6400
3200
1600
800
Number of Sera
20
15
10
5
0
Serum
N-terminal Residue and Residue Number
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

Fig. 1.

[11] utilizing the ExPASy programme (Hopp and Wood hydrophobicity plot) accessed through the University of Geneva www server. The method of synthesis used was Fmoc chemistry and *N*-α-Fmoc-protected amino acids were purchased from Cambridge Research Biochemicals, Cambridge, UK. The success of the syntheses was monitored by the simultaneous synthesis of a positive (PLAQ) and a negative (GLAQ) control peptide and subsequently testing their binding to the supplied monoclonal antibody. The synthesized peptides, on pins configured to a 96 well microtitre plate format, were then tested for binding to human sera and anti-GroEL monoclonal antibody by a modified ELISA method as described previously [9]. Human sera were tested at a dilution of 1 in 10000 and the monoclonal antibody at 1 in 5000. Serum samples were obtained from 17 patients with a culture-confirmed diagnosis of typhoid fever from the University Hospital, Kuala Lumpur, Malaysia and from four healthy blood donors from the University Hospital Blood Bank. A monoclonal antibody against the *Esherichia coli* GroEL protein (SPA 870, StressGen, Vancouver, BC, Canada) was also used in the screening. In order to identify which peptides gave ELISA signals which are significantly above background values, a consensus plot algorithm was used. This algorithm identifies peptides which give significant binding by calculating a mean value for the lower half of all the values and then adding three times the standard deviation to this mean. All values above this cut-off value are treated as significant. The algorithm is useful when assessing the results of multiple sera assays and when human sera is used, which frequently yield high background binding.

On testing of synthetic peptides with sera from 17 patients with typhoid fever and comparing the reactivities with normal sera from 4 healthy blood donors, three immunodominant regions were identified corresponding to peptides EGQDRGYSY, YSYNKETGE (Fig. 1) and GKGTEEKEK (Fig. 2). Peptides EGQ-DRGYSY and YSYNKETGE were overlapping peptides in the region between N-terminal residues 105 and 119 among the synthesized peptides (Fig. 1) and they are found within the same hydrophilic region of GroEL in the region of amino acid residues 191–203 and 199–214 [11] respectively. Screening of the peptides with a monoclonal antibody to *E. coli* GroEL (which bears 90% homology to *S. typhi* GroEL, [11]) detected binding to peptide KG*GKGTEEK*, which contains a common overlapping sequence to peptide *GKG-TEEK*EK, the third peptide identified during screening with human sera (Fig. 2). This peptide was in the region between N-terminal residues 205 and 213 among the synthesized peptides (Fig. 2) and corresponding to the region of amino acid residues 375–392 of the protein [11].

The immunological relevance of the *S. typhi* GroEL heat shock protein was demonstrated in our previous study which showed that antibodies specific to this protein were abundantly present in sera from patients with typhoid fever [6]. This observation with *S. typhi* is in agreement with other studies showing that pathogen stress proteins are immunodominant antigens and are targets for the host immune response. Many bacterial HSPs have been shown to be immunogenic proteins, e.g. GroEL of *S. typhimurium* [3], GroEL and GroES of *Campylobacter jejuni* [4], and GroEL and GroES homologs of *Helicobacter pylori* [5]. The fact that HSPs are targets of the immune response in a broad spectrum of infections may be related to the abundance of these proteins under stress conditions. For example, GroEL is one of the most abundant proteins expressed by salmonellae within infected macrophages [3], which are known to be critical antigen-presenting cells in the immune system. Given the potential importance of GroEL, it was of interest to identify and characterize immunogenic epitopes within this protein, as has been immunogenic epitopes within this protein, as has been done with other pathogens e.g. the hsp60 of *Chlamydia trachomatis* [10]. In this study we identified three immunodominant epitopes following screening with sera from patients with typhoid fever. One of these epitopes was also identified by a monoclonal antibody to *E. coli* GroEL, a protein which possesses 90% homology with the *S. typhi* GroEL [11].

Although the exact role of HSPs in pathogenesis and immunity to microbial infections appears to be complex and is not completely understood, their involvement is well established, particularly with enteric pathogens. With *S. typhi*, it has been shown that a deletion mutation which inactivates htrA, a gene encoding a stress protein, resulted in the attenuation of the *S. typhi* strain used and its subsequent use as a candidate vaccine strain in humans [12]. It has also been shown that a GroEL homolog of *H. pylori* was bound to urease, a recognized virulence factor of this pathogen [13] and

Fig. 1. Consensus plot of binding of synthetic peptides to human sera and to monoclonal antibody against GroEL. The plot covers N-terminal residues 1–151 among the synthesized peptides. All sera tested have been plotted and the values taken are average of the duplicate assays. The titre (top) is defined as the inverse dilution which would give an absorbance of > 1 above background. The bars shown in the titre output show the range between the sera values. Middle plot shows number of sera where empty columns indicate indefinite values above background and filled columns indicate statistically significant values above background. Similarly, in the lower plot empty squares indicate indefinite values above background and filled squares indicate statistically significant values above background. The region containing the immunodominant peptides is indicated by the solid line below the N-terminal residue number. SD28/2: Norm LKY 19/2 Norm 18/2, and 26552 = normal sera; M-Ab = monoclonal antibody against GroEL; all others = sera from typhoid fever patients.

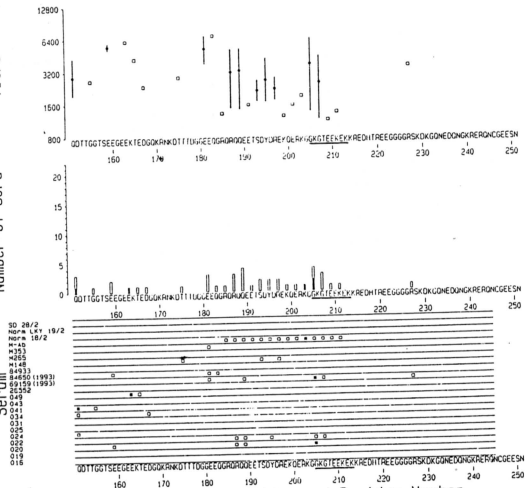

Fig. 2. Consensus plot of binding of synthetic peptides to human sera and to monoclonal antibody against GroEL. The plot covers amino acid residues 151–245 within the GroEL protein. All sera tested have been plotted and the values taken are average of the duplicate assays. The titre (top) is defined as the inverse dilution which would give an absorbance of > 1 above background. The bars shown in the titre output show the range between the sera values. Middle plot shows number of sera tested where empty columns indicate indefinite values above background and filled columns indicate statistically significant values above background. Similarly, in the lower plot empty squares indicate indefinite values above background and filled squares indicate statistically significant values above background. The region containing the immunodominant peptide is indicated by the solid line below the N-terminal residue number. SD28/2, Norm LKY 19/2, Norm 18/2, and 26552 = normal sera; M-Ab = monoclonal antibody against GroEL; all others = sera from typhoid fever patients.

that the GroEL of *S. typhimurium* was responsible for the binding of the bacterium to intestinal mucus [14]. In addition to their serving as target antigens during immune responses, and perhaps in contrast to their role as virulence factors for the pathogen, host HSPs are also believed to play an important role in antigen presentation and processing by facilitating the assembly of functional antigen-MHC Class I and -MHC Class II complexes [15] thereby playing an important role in the early inititiation of specific immunity in the host during the onset of infection. Importantly, using a DNA immunization approach, Lowrie et al. recently showed that immunization of mice with genes encoding mycobacterial hsp65 and hsp70 proteins induced strong protective immunity against tuberculosis [7].

In conclusion, epitope mapping performed on the *S. typhi* GroEL protein succeeded in defining three immunodominant epitopes. Given their well-established importance in pathogenesis and immunity, it is hoped that epitope mapping studies of these stress proteins will form the basis of future studies to evaluate their roles in bacterial infections. With regards to typhoid

fever, these findings may also be of value in the design of diagnostic tests and peptide-based vaccines.

Acknowledgements

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