Original Article

Mimotopes of heat shock proteins of *Salmonella enterica* serovar Typhi identified from phage-displayed peptide library

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Abstract
Background: Heat shock proteins (HSPs) are known to be involved in the pathogenesis of *Salmonella enterica* serovar Typhi (*S.* Typhi), the causative agent of typhoid fever. The objective of this study was to apply a phage display library to identify mimotopes of two HSPs, HSP90 and DnaK in *S.* Typhi.
Methodology: A 12-mer random peptide library expressed on the surface of the filamentous phage, M13, was used to select the mimotopes of two *S.* Typhi heat shock proteins by biopanning with monoclonal antibodies (mAbs), DnaK and HSP90. The immunogenicity of the selected peptides was determined through binding affinity with polyclonal antibodies from pooled typhoid-confirmed patients' sera and purified HSPs mAb using Western blotting and ELISA.
Results: Five rounds of biopanning resulted in enrichment of phage clones expressing the binding motifs TDxSTRP and FPSHYWLYPPPT, respectively. The selected peptides showed strong immunoreactivity with patients' sera. Thus, monoclonal antibodies against HSP and patient sera can select common mimotopes from the random peptide library.
Conclusion: These findings may provide fundamental information for further studies on diagnostic application or vaccine design against this aetiologic agent of typhoid fever.

Key Words: Phage Display, Heat Shock Proteins, *S.* Typhi

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Introduction

Typhoid fever, a disease caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) remains a major global health problem [1]. Although typhoid fever is an old disease, the roles of various components of human immune response to *S.* Typhi are not completely understood. Heat shock proteins (HSPs), which represent the most highly conserved proteins found in both prokaryotic and eukaryotic cells, are involved in the regulatory response for cells' physiological growth. Microbial HSPs are usually intracellular proteins constitutively expressed under various physiological conditions. However, they are rapidly synthesised in response to a variety of environmental stresses including temperature changes, nutrient deprivation, anoxia, inflammation, viral infection, irradiation, oxidising agents, heavy metal and malignant transformation [2-5]. These stress conditions result in the unfolding or misfolding of polypeptides when the normal physiological functions of microbial cells are affected. High levels of heat shock proteins are required to maintain protein homeostasis in the stressed cells [6, 7]. Many stimuli other than heat can induce the synthesis of HSPs, such as amino acid analogues [8], glucose analogues [9], heavy metals [10], protein kinase C stimulators [11], nitric oxide [12] and others. Hence, HSPs are also known as stressed proteins. Generally, HSPs can be divided into two groups, molecular chaperones and energy-dependent proteases [13, 14].

Studies on the role of HSPs in pathogenesis in *Salmonella* have mostly been in *S.* Typhimurium infection in mice with very little information about *S.* Typhi infection. For example, HSPs such as DnaK together with DnaJ are required for invasion of epithelial cells and survival within macrophages and are responsible for causing systemic infection in the mouse model [14]. *S.* Typhimurium has been shown to respond to starvation, anaerobiosis and heat shock by inducing synthesis of more than

100 proteins [15]. Buchmerier and Heffron [16] reported that over 30 proteins of *S.* Typhimurium, including GroEl and DnaK, were induced and synthesized during infection of macrophages. Comparative analyses have shown that both *S.* Typhimurium and *S.* Typhi share homologous regions of genes which are responsible for infection and survival within their hosts [17]. Mills and Finlay [18] also suggested that *S.* Typhi and *S.* Typhimurium use similar mechanisms for invasion and intracellular trafficking in cultured human epithelial cells. One conserved region, the *htr*A gene, which encodes a heat shock protein in both *S.* Typhimurium and *S.* Typhi, is essential for survival and replication in host tissues [19]. Tang *et al*. [20)] have shown that HSPs of *S.* Typhi are targets for the humoral response in patients with typhoid fever. On the other hand, the epitope of the GroEL analogue in *S.* Typhi was shown to react with anti-sera of typhoid fever [21]. Based on these observations, it is suggested that HSPs of *S.* Typhi might be involved in the pathogenesis of typhoid fever.

Phage display technology has commonly been applied to rapidly identify antigenic epitopes of pathogenic microorganisms [22]. With this approach, peptide or protein is expressed as a fusion entity with a coat protein of bacteriophages, resulting in display of the fusion polypeptide on the surface of the virion, while the DNA encoding the fusion polypeptide resides within the virion. Phages displaying peptides are then allowed to interact with antibodies immobilised on a solid support and the binding phages are subsequently eluted and can be specifically enriched by several cycles of affinity selection. The identity of the fusion peptides can then be determined by sequencing the inserts present in the genome of the recombinant phage [23]. This report describes the isolation of two HSP mimotopes, namely HSP90 and a DnaK analogue of *Salmonella* Typhi, using a random phage-displayed peptide library, and determination of their immunogenicity by Phage ELISA and Western blot.

Materials and Methods

Monoclonal antibodies and Sera

Two mouse monoclonal antibodies (mAbs) against HSP90 and DnaK, i.e. anti-HSP90 (SPA 830; *Achyla ambisexualis* HSP90) and anti-DnaK (SPA 880; *E. coli* DnaK), respectively, were used according to the manufacturer's instructions (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada). The polyclonal antibodies used in this study were pooled typhoid patient sera. Medical diagnosis of these patients was confirmed by high antibody titers (1:640) for one or both O and H antigens, as determined by the Widal test. Pooled normal sera were obtained from 10 normal healthy, asymptomatic individuals who were blood donors from the University Hospital Blood Bank.

Selection of binding peptides by biopanning

A 12-mer random peptide library (New England BioLabs Beverly, MA) was used in this study. *Escherichia coli* ER2537 was used as the host strain for amplification of the phage library. Two independent affinity selections were conducted with mAbs HSP90 and DnaK, respectively. Microtiter plate wells were separately coated with these antibodies (100 μg/ml mAbs in 0.1M $NaHCO_3$ [pH8.6] overnight at 4°C. Nonspecific binding was blocked by incubating with 200 μl of blocking buffer (5 mg/ml BSA, 0.1M $NaHCO_3$, 0.02% NaN_3) in each well for 1 hr and then the wells were washed six times with TBS (50 mM Tris-HCl pH7.5, 150 mM NaCl).

An aliquot of 10^{11} pfu/ml phage solution (10 μl) was added to each coated well. The mixture was incubated for 1 hour at 37°C and the wells were washed extensively for 10 times (5-min interval) with TBST (TBS with 0.1% Tween-20). Bound phages were eluted with 100 μl of elution buffer (0.1M glycine, [pH2.2] in 0.1% of BSA) for 10 minutes at room temperature and neutralised immediately with 1M Tris-HCl (15μl [pH9.1]) to pH7. The level of specific phage enrichment was determined by calculating the ratio of eluted phage titres, which was based on the plaque numbers on LB/IPTG/Xgal plates of *E. coli* ER2537. The biopanning procedure was repeated for another four rounds, with a gradual increase of Tween-20 in the wash buffer up to 0.5% (vol/vol). After 5 rounds of biopanning, individual plaques were picked and used to infect *E. coli* ER2738 cells for amplification (as previously described in Tang *et al.*, 2003 [8]). Streptavidin and BSA were used as positive and negative controls, respectively.

Preparation, amplification, and titration of the selected phages

The selected phage clones were amplified to a high titer (approximately 10^{12} pfu/ml) and purified twice by precipitation with 20% (w/vol) polyethylene glycol 8000 (PEG 8000)-2.5M NaCl [9].

Plaque amplification

Individual blue plaques from the fourth and fifth rounds of biopanning were randomly picked from Luria–Bertani agar plates (used in output titration) and used to infect *E .coli* ER 2738 cells. The culture was grown in LB broth (supplemented with tetracycline [20 μg/ml]) at 37°C for 4.5 hours before being centrifuged at 10,000 rpm for 15 minutes. The upper 80% of phage-containing supernatant was collected and stored in 4°C until use.

DNA sequencing

Phage single-stranded DNA (ssDNA) was extracted according to the method previously described by Tang *et al*. [24]. The ssDNA (10μl) was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and an automated sequencer (ABI PRISM 377; Perkin Elmer). The sequencing primer was 5'-CCCTCATAGTTAGCGTAACG-3' (provided in the phage peptide library kit).

Reverse Phage-ELISA

A reverse phage enzyme-linked immunosorbent assay (ELISA) was carried out to verify the binding specificity and affinity of the selected phage to mAbs HSP 90 and DnaK. Briefly, 100 μg/ml of mouse HSP antibody (anti-HSP90/anti-DnaK) was added to each well and then incubated overnight at 4°C. The wells were then blocked with 0.1M $NaHCO_3$, pH 8.6, 5 mg/ml BSA and 0.02% NaN_3 at 4°C for 2 hours, followed by thorough washing with 0.5% TBST washing buffer (TBS with 0.5% (v/v) Tween-20). The selected phage clones with a titer of 10^{12} pfu/μl in 100μl of TBS (pH 7.0) were incubated with the immobilised antibodies for 1 hour at room temperature. The plate was washed six times with washing buffer, and then diluted horseradish peroxidase (HRP) conjugated anti-bacteriophage M13 antibody (Pharmacia) (in blocking buffer to 1:2500) was added to the wells. After two hours of incubation at room temperature with agitation, the wells were washed six times with washing buffer. The binding was detected by adding substrate ABTS [2, 2'-azino-bis (3-cthylbenz-thiazoline-6-sulfonic-acid); Sigma] to each well. The optical density at 414 nm (OD_{414}) was measured with an EL-320 microplate reader (Titertek Multiskan II; Flow Labs, Mt. Waverley, Victoria, Australia).

Direct Phage-ELISA

Direct phage-ELISA was used to verify that there was no cross-reactivity between the secondary antibody and the binding phages. The microtiter plate was coated overnight with selected phage clones (10^{12} pfu/μl) in 150 μl coating buffer [0.1M $NaOCH_3$ (pH 8.6). The wells were blocked by blocking buffer, followed by five washes with 0.5% TBST and diluted relevant antibodies (anti-HSP90/ anti-DnaK, 100μg/ml) were added to the wells. After incubation, the wells were washed five times and secondary antibody (HRP-linked anti-mouse IgG-1: 2500) was added. The binding was detected by adding the substrate ABTS and the OD_{414} was measured.

Screening of immunoreactivity of the selected phage clones with pooled human typhoid sera

The same phage clones used for determination of binding affinity with mAb were also screened for their immunoreactivity with typhoid patients' sera (TPS). Ten TPS were pooled together and diluted in 0.1M $NaHCO_3$ (pH8.6) to 1:10 before coating the wells of microtiter plates. The wells were blocked with blocking buffer for two hours. After washing five times with 0.5% TBST, phage clones with titer of 10^{12} pfu/μl in 100μl of TBS (pH 7.0) were added to the wells and incubated for 1 hour at room temperature. The subsequent washing and detection steps were performed as described earlier.

Screening of heterogeneous immunoreactivity of the selected phage-displayed peptide with individual typhoid patient sera

Representative phage clones HA5(2), HB5(2) and DKB5(3), DKC5(1), with the highest signal in phage-ELISA both from both anti-HSP90 and anti-DnaK categories, respectively, were chosen for further evaluation for their immunoreactivity with individual typhoid patient sera.

The 10 TPS were coated individually on separate wells with the dilution mentioned above.

All the subsequent steps were as described above for the phage-ELISA.

SDS-PAGE and Western blotting assay

Immunopositive phage clones tested with ELISA on monoclonal antibodies, HSP90 or DnaK and polyclonal antibodies (sera from typhoid fever patients) were subjected to Western blot analysis to confirm their binding specificity and affinity. Selected phage clones with a titer of 10^{11} pfu/ml were denatured at 95°C for 5 minutes in the presence of SDS-PAGE sample buffer (1.0M Tris-HCl [pH6.8], 10% Glycerol, 0.0025% Bromophenol blue, 2% SDS). The denatured samples were subjected to SDS-PAGE on 12% Tris-Glycine gel. After separation, the samples were transferred to a polyvinylidene difluoride transfer membrane (PVDF, Amersham Biosciences). The membrane was then blocked with 3% Blotto (3% skim milk powder) for 1 hour at room temperature, washed with 0.1% TBST and probed with anti-HSP mAb overnight at 4°C. Alkaline phosphatase-linked anti-mouse immunoglobulin G (IgG) was used as the secondary antibody to probe the membrane for 2 hours at room temperature. The binding was detected with one-step NBT-BCIP (Pierce, USA).

Results

Sequence analysis of phage clones selected from biopanning with mAb HSP90

An increase of phage titers from the first to the fifth round of biopanning indicates that the phages expressing specific ligands were enriched (data not shown). Fifteen and nineteen individual phage clones were randomly selected from the fourth and fifth rounds of biopanning respectively, for further analysis. The encoded peptide sequences were deduced from the DNA sequencing and are summarised in Table 1. A population of diverse peptide sequences was isolated.

Phages recovered from the fourth (4th) and fifth (5th) rounds of biopanning against mAb HSP90 were named Hy4 (n) and Hy5 (n) respectively (y=group of different consensus motif, n=members within a group). Most of the sequences contained a common motif with at least 4 of the 6 amino acids with the sequence TDxSTRP (x=any amino acid) (Table 1). In the 4th round of biopanning, 40% of the phage clones (HA4) carried the peptide sequence TDxSTRP with x=any amino acid, while the remaining phage clones were equally distributed into 3 groups with different binding peptide sequences [HB4 - DxSTRP=20%; HC4 – TDxT=20%; HD4 – TDxSTR=20%) (Table 1).

Table 1. Peptide ligands of mAb HSP90 obtained in the fourth and fifth rounds of biopanning.

Phage clone	Peptide Sequences																F	%
HA4(1)			W	P	M	N		**D**	D	**S**	**T**	**R**	**P**	R				
HA4(2)			D	S	M	N	**T**	**D**	T	**S**	**T**	**R**	**P**	R				
HA4(3)					F	Q	**T**	**D**	M	**S**	**T**	**R**	**P**	R	G	A	1x	40
HA4(4)				Y	T	Y	**T**	**D**	Y	**S**	**T**	**R**	**P**	H	Y		1x	
HA4(5)					D	P	**T**	**D**	Y	**S**	**T**	**R**	**P**	H	M	W	1x	
HA4(6)					P	Q	**T**	**D**	G	**S**	**T**	**R**	**P**	R	G	L	1x	
HB4(1)					D	F	R		Y	**S**	**T**	**R**	**P**	F	A	L	1x	
HB4(2)					Q	R	Y		Y	**S**	**T**	**R**	**P**	R	A	L	1x	20
HB4(3)					M	I	S		Y	**S**	**T**	**R**	**P**	D	R	Q	1x	
HC4(1)					V	Q	**T**	**D**	G	**S**	**T**	K	S	H	R	H	1x	20
HC4(2)					I	R	**T**	**D**	E	**S**	**T**	K	R	L	P	P	1x	
HC4(3)				T	L	S	**T**	**D**	Q	**S**	**T**	T	R	N	W		1x	
HD4(1)	D	I	D	Y	S			D	Y	**S**	**T**	**R**					5x	20
HD4(2)					D			D	H	**S**	**T**	**R**	C	W	V	S	3x	52.6
HD4(3)	Y	P	L	H	L	N	**T**	**D**	Y	**S**	**T**	**R**					1x	
HA5(1-5)			W	P	M	N		**D**	D	**S**	**T**	**R**		R			1x	
HA5(6-8)				Y	T	Y	**T**	**D**	Y	**S**	**T**	**R**	**P**	H	Y		1x	
HA5(9)					F	S	**T**	**D**	W	**S**	**T**	**R**	**P**	R	L	V	1x	
HA5(10)			D	S	M	N	**T**	**D**	D	**S**	**T**	**R**	**P**	R			1x	
HB5(10)					D	F	R	**D**	Y	**S**	**T**	**R**	**P**	F	A	L	1x	
HB5(2)					M	I	S	**D**	Y	**S**	**T**	**R**	**P**	D	R	R	1x	
HB5(3)					W	T	R	**D**	N	**S**	**T**	**R**	**P**	I	V	R	1x	31.6
HB5(4)					T	D	S	**D**	Y	**S**	**T**	**R**	**P**	S	R	G	1x	
HB5(5)					T	Y	Q	**D**	F	**S**	**T**	**R**	**P**	R	S	F	1x	
HB5(6)				A	E	S	D	**D**	S	**S**	**T**	**R**		R	H		1x	
HD5(1)					Q	F	**T**		G	**S**	**T**	**R**	Q	H	A	G	1x	
HD5(2)					W	N	**T**	**D**	F	**S**	**T**	**R**	A	A	H	T	1x	15.8
HD5(3)	A	Y	K	L	P	Q	**T**	**D**	D	**S**	**T**						1x	

The percentage of yield for every group of phage clones carrying a particular binding motif was calculated by the formula: [(Frequency of each group of phage clones / Total number of phage clones isolated) x 100]. Conserved motifs are in bold. F, frequency; %, percentage.

Following the 5th round of biopanning, three classes, with 50% of them bearing the common motifs TDxSTRP, were obtained (Table 1). The peptide DxSTR was consistently found in all the phage clones.

Sequence analysis of phage clones selected from biopanning with mAb DnaK

Under the mAb DnaK category, 21 and 19 phage clones were randomly selected from the 4th and 5th rounds of biopanning, respectively.

Sequence analysis of the phage clones revealed several distinguishable motifs. In the 4th round of biopanning, four groups were isolated: FPSHYWLYPPPT [DKC4, 33.3%], NYKSPLAVPMT [DKB4, 28.6%], DLNTNRTQMVLH [DKA4, 19%] and KIVMFWHEPVYA [DKD4, 19%] (Table 2). Fifty percent of the phage clones under the DKC5-group carrying the peptides FPSHYWLPPPT were further enriched in the 5th round of biopanning (Table 2).

Table 2. Peptide ligands of mAb DnaK obtained in the fourth and fifth rounds of biopanning.

Phage clone	Peptide Sequences	F	%
DKA4(1-4)	D L N T N R T Q M V L H	4x	19.0
DKB4(1-6)	N Y K S P L F A V P M T	6x	28.6
DKC4(1-7)	**F P S H Y W L Y P P P T**	7x	33.3
DKD4(1-4)	K I V M F W H E P V Y A	4x	19.0
DKA5(1-3)	D L N T N R T Q M V L H	3x	15.8
DKB5(1-5)	N Y K S P L F A V P M T	5x	26.3
DKC5(1-11)	**F P S H Y W L Y P P P T**	11x	57.9

F, frequency; %, percentage.

Phagotopes isolated by biopanning against pooled typhoid patient sera (TPS)

The binding peptides derived from biopanning against TPS showed a diverse mixture of sequences. No common motif was observed in this selection. Peptide sequences TISNRDYIRPMD were enriched from 21 to 46%. Peptide sequences bearing HA- and DKC-homolog motifs (TDxSTRP and FPSHYHYWLYPPPT respectively) were also isolated (Table 3).

Table 3 Peptide ligands of mAb DnaK obtained in the fourth and fifth round of biopanning.

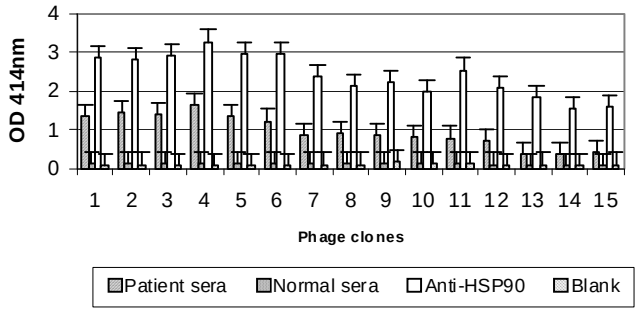
Phage clone	Peptide Sequences	F	%
TPS4 (1-3)	**T I S N R D Y I R P M D**	3x	21.4
TPS4 (4)	F P S H Y W L Y P P P T	2x	14.3
TPS4 (5)	**S Q M P E Y L L** K A D N	1x	7.1
TPS4 (6)	**W P M N T D D S T R P** R	1x	7.1
TPS4 (7)	Y T Y **T D Y S T R** P H Y	1x	7.1
TPS4 (8)	P Q **T D G S T R** P R G L	1x	7.1
TPS4 (9)	T L S Q D Q S E T L N Y	1x	7.1
TPS4 (10)	P H P S T M F D R Q E D	1x	7.1
TPS4 (11)	T S P H K T T L D L N A	1x	7.1
TPS4 (12)	D N H S P V N I A H K L	1x	7.1
TPS4 (13)	S R L P L S Q P S P N S	1x	7.1
TPS5 (1-6)	**T I S N R D Y I R P M D**	6x	46.1
TPS5 (7)	**S Q M P E Y L L** K A D N	3x	23.1
TPS5 (8)	**W P M N T D D S T R P** R	1x	
TPS5 (9)	Y T Y **T D** Y **S T R P** H Y	1x	38.5
TPS5 (10)	P Q **T D** G **S T R P** R G L	1x	
TPS5 (11)	D P **T D** Y **S T R P** H M W	1x	

F, frequency; %, percentage.

Analysis of binding affinity and specificity of selected phage clones with the selecting mAb using reverse Phage-ELISA and direct phage-ELISA

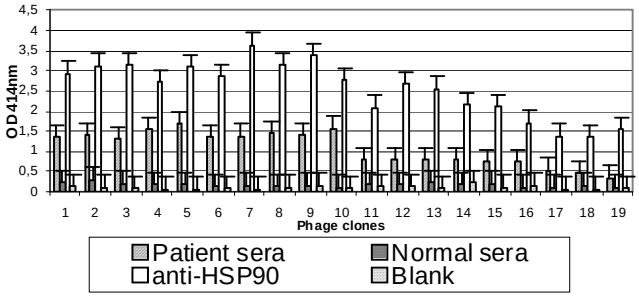
ELISA was carried out on all the randomly selected phage clones for their binding specificity and affinity to their respective mAbs. In the reverse Phage-ELISA, all the 15 individual phage clones from the 4th round of biopanning against anti-HSP90 reacted strongly with the antibody, with the ratio between the interaction of phage-antibody and phage-blank being more than 5 (Figure 1). The same result was observed by direct Phage-ELISA (data not shown). However, the reactivity among the immunopositive clones varied with each other according to the groups of peptides being distributed. Among the 4 groups of anti-HSP90-corresponding peptides, the motif TDxSTRP [HA4] appeared to have the strongest binding affinity with the mAb HSP90, with the majority of the peptides (6 out of 9) showing an OD_{414}nm reading above 2.5 (Figure 1). To verify that the reactive signals observed in these 4 groups of peptides were due to the relative binding affinity but not due to the titer of phage particles which may give false positive results, the phage clones (HA4, HB4, HC4 and HD4 and control phage (wild-type M13) were incubated at 10-fold serial dilutions with the antibody. The results showed that the relative binding affinity of the three classes of phage clones was independent of the phage titer (data not shown).

Figure 1. ELISA reactivity of selected anti-HSP90 derived phage clones from 4th biopanning with typhoid patients' sera, normal sera, HSP90 mAb and BSA (blank). Lanes 1-6 Phage clones HA4(1-6); lanes 7-9 clones HB4 (1-3), lanes 10-12 clones HD4 (1-3) and lanes 13-15 clones HC4 (1-3).



Screening of the binding affinity with phage clones derived from the 5th round of biopanning also revealed that the relative binding affinity was peptide sequence-based. Ten of seventeen clones from the HA5-group had OD_{414}nm readings above 2.5. Although phage clones from HB5 and HD5-groups also showed binding with the mAb HSP90, their overall reactivity was relatively lower than the HA5-group (Figure 2).

Figure 2. ELISA reactivity of selected anti-HSP90 derived phage clones from 5th biopanning with typhoid patients' sera, normal sera, HSP90 mAb and BSA (blank). Lanes 1-10: HA5 clones, lanes 11-16: HB5 clones, lanes 17-19: HD5 clones.



The binding specificity and affinity of anti-DnaK-derived phage clones by reverse Phage-ELISA also revealed strong binding in most of the selected phage clones with the mAb DnaK, but to different degrees among the groups. Among the 21 selected phage clones from the 4th round of biopanning, only 17 reacted positively with the mAb DnaK, while the other 4 demonstrated weak binding. The mAb DnaK was able to bind strongly with 3 groups of phage clones DKA4, DKB4 and DKC, which expressed the motifs DLNTNRTQMVLH, NYKSPLFAVPMT and FPSHYWLYPPPT, respectively (with OD_{414}nm above 2.0) (Figure 3).

Again, similar results were obtained from direct-Phage ELISA. Also, ELISA screening of phage clones isolated from the 5th round of biopanning showed the strongest binding among DKC5-phage clones (11 of 19) (Figure 4). Both Phage-ELISA and reverse Phage-ELISA showed that DKC5-phage clones demonstrated higher reactivity than DKA and DKB-clones.

Figure 3. ELISA reactivity of selected anti-DnaK derived phage clones from 4th biopanning with typhoid patients' sera, normal control sera DnaK MAb. Lanes 1-4: DKA4 clones, lanes 5-10: DKB4 clones, lanes 11-17: DKC4 clones, and lanes 18-21: DKD4 clones.

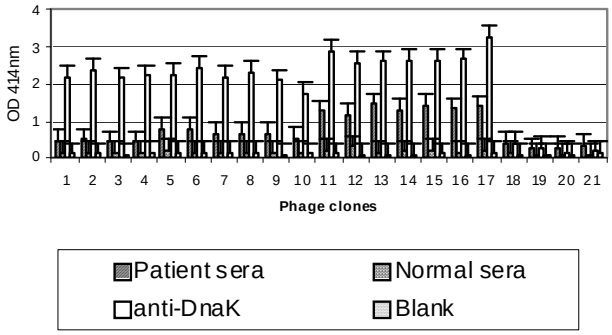
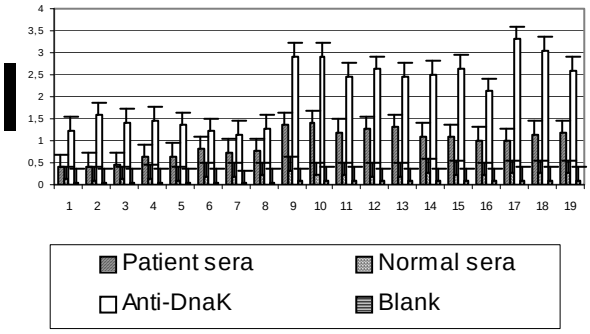


Figure 4. ELISA reactivity of selected anti-DnaK derived phage clones from 5th biopanning with typhoid patients' sera, normal control sera, DnaK MAb and BSA (blank). Lanes 1-3: DKA4 clones, lanes 4-8: DKB5 clones, and lanes 9-19: DKC5 clones.



Immunoreactivity of the selected phage clones with human typhoid sera

Representative phage clones HA5(2), HB5(2) and DKB5(3), DKC5(1) that gave the greatest signals with mAbs HSP90 and DnaK respectively were further tested for their immunoreactivity with human typhoid sera using ELISA. Healthy

individual sera were used as negative controls in the assay. Using a cutoff recognition of 3.0 (ratio of OD414nm reading in patient sera: OD414nm reading in normal sera > 3.0), immunopositive results were shown in all the selected phage clones above. Among the anti-HSP90-derived phage clones, phages under the HA-group gave the highest signal as indicated by the OD414nm reading which ranged from 0.8 to 1.4 (Figure 5). In the anti-DnaK category, phages in DKC-group displayed the strongest immunoreactivity with the patient sera (Figure 6). Levels of binding to negative controls were all significantly lower.

Figure 5. Heterogeneous reactivity of 10 samples of typhoid patients' sera with selected anti-HSP90 derived phage-displayed peptides. Phage clone HA5(1) carrying the internal binding motif TDxSTRP showed higher immunoreactivity with the 10 individual patients' sera compared to phage clones HB5(1) carrying the internal binding motif DxSTRP.

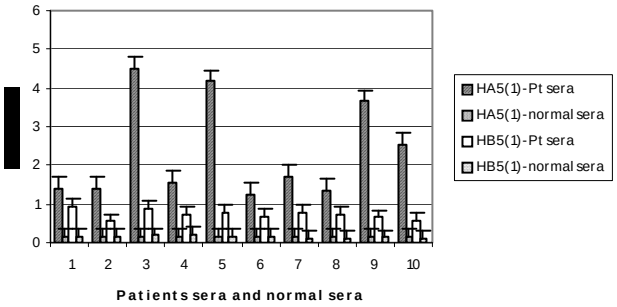
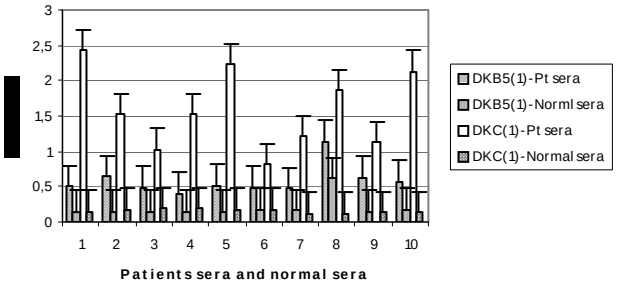


Figure 6. Heterogeneous reactivity of 10 samples of typhoid patients' sera with selected anti-DnaK derived phage-displayed peptides. Phage clone DKC(1) displaying the peptide FPSHYWLYPPPT showed higher immunoreactivity with the 10 individual patients' sera compare to phage clones DKB5(1) carrying the peptide NYKSPLFAVPMT.



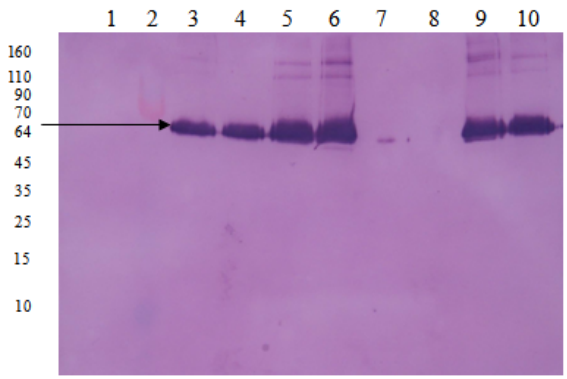
Heterogeneous immunoreactivity of the selected phage-displayed peptide with individual typhoid patient sera (TPS)

All 10 TPS recognized the selected anti-HSP90 and anti-DnaK-derived binding peptides with variable reactivity. Selected phage clones under the HA-group (with displayed peptides TDxSTRP) generally showed higher reactivity to typhoid patients' sera compared to phage clones under the HB group (with displayed peptides DxSTRP). Within each group of peptides, there was heterogeneous reactivity among the sera tested (Figure 5). A similar observation was obtained for individual TPS tested against anti-DnaK-derived phage clones. Phage clones from DKC-group bearing peptides FPSHYWLYPPPT gave higher immunoreactivity as compared to phage clones in the DKB group (NYKSPLFAVPMT) (Figure 6). Levels of binding to normal sera were all significantly lower.

Western blot analysis of binding specificity of selected peptide to MAb

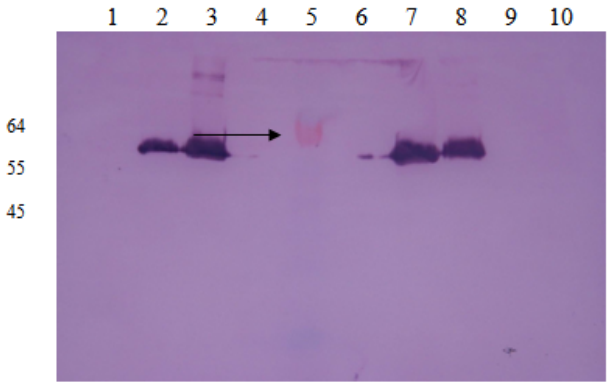
Western blot analysis with mAb HSP90 showed that the peptide fused to protein pIII of M13 phage was consistently detected in all three selected classes of binding motifs isolated. A major band was observed for each clone at approximately 66 kDa, representing the displayed peptides fused to the phage pIII coat protein of approximately 64 kDa (Figure 7). No binding was observed with phage clone from the HC-group (Figure 7, lane 8). Also, the HSP90 did not recognise pIII on wild-type M13 phage (Figure 7, lane 1), which rules out the possibility of cross-reactivity between the phage protein component and the mAb.

Figure 7. Western blot analysis of phage clones selected after the 5th round of biopanning against anti-HSP90 mAb. Lanes 1-10: Wild-type M13, protein ladder, HA4(1), HA4(2), HB4(1), HB4(2), HC4(1), HC4(2), HD4(1), HD4(2).



Similarly, when anti-DnaK derived peptides were subjected to Western blot analysis, it was found that phage clones from DKB (Figure 8, lanes 3 and 7) and DKC (Figure 8, lanes 2 and 8)-groups reacted with mAb DnaK. No band was detected for phage clones from DKA (Figure 8, lanes 4 and 6) or DKD (lane 1 and 9)-groups with this mAb. Also, no binding was observed with peptides derived from wild-type M13 phage (lane 10).

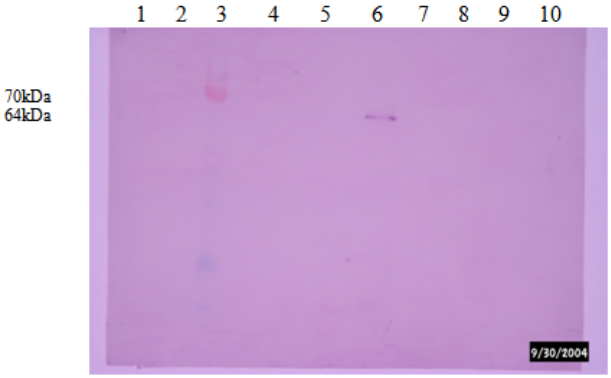
Figure 8. Western blot analysis of phage clones selected from 5th biopanning against anti-DnaK mAb. Lanes 1-10: DKD4(1), DKC4(1), DKB4(1), DKA4(1), Protein ladder, DKA4(2), DKB4(2), DKC4(2), DKD4(2), Wild-type M13. (Note: The faint marking in lane 6 was due to spillover from lane 7. Repeated runs of analysis show absence of binding to DKA).



Reactivity of selected peptides against typhoid patients' sera

In order to confirm the immunoreactivity of anti-HSP90 and anti-DnaK selected peptides by Phage-ELISA, the binding affinity of these peptides was evaluated with pooled typhoid patients' sera (TPS) via Western blotting. Only a faint band was observed with the phage clone in the HA-group, while others did not display binding (Figure 9, lane 6).

Figure 9. Western blot analysis on selected peptide derived from biopanning against anti-HSP90 and anti-DnaK mAb. Lanes 1-10: Wild-type M13, BSA, protein ladder, DKB5(1), DKB5(1), HA5(1), HB5(1), BSA, BSA, BSA.



The two motifs (HA and DKC) were then used for screening of recognition of these peptides by a series of individual TPS. Peptides displayed on the phage clone HA were recognized by all the 10 individual sera while those from phage clone DKC had binding to only 3 sera (data not shown). None of the 10 normal control sera from healthy individuals recognized any of the selected motifs from HA and DKC.

Discussion

Microbial heat shock proteins have been shown by a number of studies as major antigens responsible for a number of infections [25, 26]. This is not surprising because as infections develop, the sudden change of temperature from 25°C to 37°C and the host immune response raised against the pathogen serve as stresses that stimulate the synthesis of HSPs. As a result of accumulation of these proteins, microbial HSPs become prominent antigens that elicit a major portion of the immune repertoire. In this study, we have presented a comparative study on the mimotope identification using mAbs against the heat shock proteins, HSP90 and DnaK, and pooled patient sera to assess the diversity of mimotopes that could be detected. Previous studies have shown that HSPs of *S.* Typhi are targets for the humoral response in typhoid fever patients and sera from these patients are able to recognise the HSP90 and DnaK-analogue of *S.*

Typhi (20). Panchanathan *et al*. [21] identified the GroEL analogue epitope in *S.* Typhi that showed significant interaction with typhoid sera.

In this study, a 12-mer random peptide library displayed on filamentous phage M13 was used to map the mimotopes of HSP90 and DnaK. Phage-displayed peptide libraries have routinely been used to define ligand binding sites and study antigen-antibody interactions [23]. Although the identified epitopes may not be structurally similar to the original identity of HSP90 and DnaK analogue in *S.* Typhi, the data obtained suggest that these are most probably HSPs mimotopes. First, both the typhoid patient sera (TPS) and purified HSP monoclonal antibodies (anti-HSP90 and anti-DnaK) selected similar binding motifs from the affinity selection process. Following 5 successive biopannings, mAbs HSP90 and DnaK were able to isolate phage clones displaying the consensus peptide sequences TD_STRP and FPSHYWLYPPPT respectively. The same groups of peptide sequences were also selected with pooled typhoid patient sera (TPS). This indicates that both the HSP mAbs and certain subsets of antibodies within the patients' sera shared similar binding characteristic to the mimotopes isolated. Hence, it is postulated that the peptides isolated from the random peptide library were the mimotopes of *S.* Typhi HSP90 and DnaK-analog.

Secondly, immunoreactivity screening with Phage-ELISA using TPS indicated strong binding between the selected phage-displayed peptides (both from anti-HSP90 and anti-DnaK category) and the TPS. In particular, HA phage clones, bearing the displayed peptides TDxSTRP, had the highest reactivity with TPS as compared to clones from the HB, DKC and DKB- groups. Western blot analysis confirmed that the binding was directed to the fusion peptide on the pIII minor coat protein as there was no binding to the wild type M13 phages. These observations suggest that phage clones expressing the motifs were recognised by sera from typhoid patients as a result of specific binding of the peptides to the anti-sera.

Thirdly, all 10 individual typhoid positive sera recognised the HA-, HB-, DKB- and DKC-phage clones, as demonstrated by Phage-ELISA assay. However, signals given by different individual TPS against a particular motif were variable, indicating a heterogeneous humoral response generated against particular epitopes within *S.* Typhi HSPs. Among the different motifs isolated, phage clones bearing the peptide TDxTSRP (HA-group) were consistently recognised by all 10 TPS in both Phage-ELISA and Western blot assays, suggesting that this peptide is a putative mimotope of the immunodominant HSP90 of *S.* Typhi.

The affinity selection with pooled typhoid patients' sera generated a diverse population of 12-mer sequence motifs compared to those derived from monoclonal antibodies. This may be due to the following reasons: First, pooled typhoid fever patient sera may consist of a broad spectrum of antibodies against various antigens of *S.* Typhi, such as Vi polysaccharide, flagellum, porin or OMP as well as to HSPs. The titer of HSP antibodies may be overwhelmed by the higher titer of antibodies against other more dominant antigens. Secondly, the titer of the HSP antibodies would have been diluted in pooled patient sera, particularly with those anti-sera that contain very low or no antibodies against HSPs. When individual TPS were used in the Phage-ELISA assay, there were considerable diverse OD readings as a result of different levels of recognition by different TPSs. Also, Western blot assay using individual TPS generated more intense bands against the fused peptides compared to faint bands developed by pooled TPS. Based on these observations, it is believed that the antibodies against *S.* Typhi HSPs in some of the TPS were diluted when they were pooled together, thus giving a low yield of phage clones expressing HSP-specific peptides, when the antibodies were used for affinity selection.

In summary, screening of the HSP mimotopes from the random peptide library using anti-HSP mAb and TPS as ligates has yielded several peptides which represent *S.* Typhi HSP90 and DnaK mimotope characteristics. In the HSP90 category, the TDxSTRT (HA), DxSTRP (HB) and TDxST (HD) HSP90 mimotopes were reactive with HSP90 mAb determined by Phage-ELISA and Western blot assays, suggesting they bound specifically with the mAb. All the three groups of binding motifs resembled the property of linear but discontinuous epitopes that consist of several amino acid residues that are discrete in the primary sequence. Two regions within the motif were separated by an amino acid of any type (x). The region identified in HA contained the same

motif (DxST) shared by HB (DxSTRP) and HD (TDxST), suggesting that Aspartic acid (D), Serine (S) and Threonine (T) are critical residues involved in the binding if HSP90 mAb and TPS. However, stronger interaction was thought to be attributable to the addition of amino acid residues Arginine (R) and Proline (P) in the N-terminal part of the internal interval, -x-. The crucial contribution of these 2 residues for TPS and mAb bindings was further confirmed by Western blot assay. Different results were produced when the three fusion peptides were tested against mAb and TPS. All fusion peptides from HA, HB and HD-groups were reactive with HSP90 mAb. However, anti-sera from typhoid fever patients recognised only the HA-peptide. Similar observations were obtained when the assay was tested with individual TPS. The addition of amino acid residues, R and P, seemed to increase the binding affinity of the mimotope DxST of *S.* Typhi HSP90 to the TPS.

In the case of the DnaK-analogue, two immunoreactive mimotopes were mapped with characteristics of continuous linear epitopes formed by 12 amino acid residues. They were FPSHYWLYPPPT (DKC) and NYKSPLFAVPMT (DKB). The former epitope demonstrated a stronger immunoreactivity against the mAb DnaK and TPS compared to the latter, suggesting the DKC-peptide possessed a higher binding affinity than the DKB-peptide. In contrast to the binding motifs selected by anti-HSP90, binding peptides selected by anti-DnaK involved the entire 12-mer peptide instead of few residues. Although peptides from the DKA-group were positive in Phage-ELISA, they failed to show any binding with anti-DnaK and TPS in Western blot. Peptides from phage clones DKD showed no specific binding in either the Phage-ELISA or in Western blot. This might be due to the relatively low affinity of TPS and DnaK mAb against these peptides. The deduced peptide sequences of the immunodominant phage clones match to hypothetical proteins, putative inner membrane proteins, and replication protein A of the *Salmonella enterica* genomes currently listed in GenBank. The roles of these proteins in *Salmonella* pathogenesis need further investigation.

In summary, a random peptide library was able to isolate mimotopes with characteristics of *S.* Typhi HSP90 and a DnaK analogue. These findings will provide fundamental information for further studies on diagnostic application or vaccine design against this aetiologic agent of typhoid fever. Further work is needed to understand the roles of these potential mimotopes in the pathogenesis of typhoid fever.

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References

1. Crump JA, Stephen LP, Mintz ED (2004) The global burden of typhoid fever. Bull WHO 82:346-353.
2. Kaufmann, SHE (1990) Heat shock proteins and the immune response. Immunol Today 11:129-136.
3. Parsell DA, Lindquist S (1993) The function of the heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Ann Rev Genetics 27:437-496.
4. Pirkkala L, Nykanen P, Sistonen L (2001) Role of the heat shock transcriptional factors in regulation of the heat shock response and beyond. FASEB J 15:1118-1131.
5. Donati YRA, Slosman DO, Polla BS (1990) Oxidative injury and the heat shock response. Biochem Pharmacol 40:2571-2577.
6. Barrett MJ, Alones V, Wang KX, Phan L, Swerdlow, R.H (2004) Mitochondria-derived oxidative stress induces a heat shock protein response. J Neurosci Res 78(3):420-429.
7. Hoffmann F, Rinas U (2004) Roles of heat-shock chaperones in the production of recombinant proteins in Escherichia coli. Adv Biochem Eng Biotechnol 89:143-161.
8. Lee Y, Nagao RT, Lin CY, Key JL (1996) Induction and Regulation of Heat-Shock Gene Expression by an Amino Acid Analog in Soybean Seedlings. Plant Physiol 110(1):241-248.
9. Lee J, Bruce-Keller AJ, Kruman Y, Chan SL, Mattson MP (1999) 2-Deoxy-D-glucose protects hippocampal neurons against excitotoxic and oxidative injury: evidence for the involvement of stress proteins. J Neurosci Res 57(1):48-61.
10. Noda M, Wataha JC, Lewis JB, Lockwood PE, Komatsu H, Sano H (2003) Hg2+ and Ni2+ alter induction of heat shock protein-72 in THP-1 human monocytes. J Biomed Mater Res A 67(1):240-245.
11. Ding XZ, Smallridge RC, Galloway RJ, Kiang JG (1996) Increase in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and [Ca2+]i. J Investig Med 44: 144-153.
12. Sun J, Liao JK (2004) Induction of angiogenesis by heat shock protein 90 mediated by protein kinase Akt and endothelial nitric oxide synthase. Arterioscler Thromb Vasc Biol 24(12):2238-2244.
13. Spiess C, Beil A, Ehrmann M (1999) A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell 97:339.
14. Takaya A, Tomoyasu T, Matsui H, Yamamoto T (2004) The DnaK/DnaJ chaperone machinery of *Salmonella*

enterica serovar Typhimurium is essential for invasion of epithelial cells and survival within macrophages, leading to systemic infection. Infect Immun 72(3):1364-1373.
15. Spector MP, Aliabadi Z, Gonzalez T, Foster JW (1986) Global control in *Salmonella* typhimurium: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. J Bacteriol 168(1):420-424.
16. Buchmeier NA, Heffron F (1990) Induction of *Salmonella* stress proteins upon infection of macrophages. Science 248(4956):730-732.
17. Porwolik S, Boyd EF, Choy C, Cheng P, Florea L. Proctor E, McClelland M (2004) Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J Bacteriol 186(17):5883-5898.
18. Mills SD and Finlay BB (1994) Comparison of *Salmonella* Typhi and Salmonella typhimurium invasion, intracellular growth and localization in cultured human epithelial cells. Microb Pathog 17(6):409-423.
19. Johnson K, Charles I, Dougan G, Pickard D, O'Gaora P, Costa G, Ali T, Miller I, Hormaeche C (1991) The role of a stress-response protein in *Salmonella* typhimurium virulence. Mol Microbiol 5:401–407.
20. Tang SW, Abubakar S, Devi S, Puthucheary S, Pang T (1997) Induction and characterization of heat shock proteins of Salmonella typhi and their reactivity with sera from patients with typhoid fever. Infect Immunol 65: 2983-2986.
21. Panchanathan V, Naidu BR, Shamala D, Pasquale AD, Mason D, Pang T (1998) Immunogenic Epitopes of *Salmonella* Typhi GroEL Heat Shock Protein Reactive with Both Monoclonal Antibody and Patient Sera. Immunol Lett 62:105-109.
22. Folgori A, Tafi R, Meola A, Felici F, Galfre G, Cortese F, Monaci P, Alfredo N (1994) A general strategy to identify mimotopes of pathological antigens using random peptide libraries in human sera. EMBO J 13:2236-2243.
23. Smith GP, Scott JK (1993) Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol 217:228-257.
24. Tang SS, Tan WS, Devi S, Wang LF, Pang T, Thong KL (2003) Mimotopes of the Vi antigen of Salmonella enterica serovar typhi identified from phage display peptide library. Clin Diagn Lab Immunol 10:1078-1084.
25. Kaufmann SHE (1991) Heat shock proteins and pathogenesis of bacterial infections. Springer Semin Immunopathol 13:25-36.
26. Friedland JS, Shattock R, Remick DG, Griffin GE (1993) Mycobacterial 65kD heat shock protein induces release of proinflammatory cytokines from human monocytic cells. Clin Exp Immunol 91:58-62.

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