Identification of immunogenic proteins and generation of antibodies against *Salmonella Typhimurium* using phage display

Torsten Meyer¹, Thomas Schirrmann¹, André Frenzel¹, Sebastian Miethe¹, Janin Stratmann-Selke²,³, Gerald F Gerlach⁵, Katrin Strutzberg-Minder⁵, Stefan Dübel¹ and Michael Hust¹*

**Abstract**

**Background:** Solely in Europe, *Salmonella Typhimurium* causes more than 100,000 infections per year. Improved detection of livestock colonised with *S. Typhimurium* is necessary to prevent foodborne diseases. Currently, commercially available ELISA assays are based on a mixture of O-antigens (LPS) or total cell lysate of *Salmonella* and are hampered by cross-reaction. The identification of novel immunogenic proteins would be useful to develop ELISA based diagnostic assays with a higher specificity.

**Results:** A phage display library of the entire *Salmonella Typhimurium* genome was constructed and 47 immunogenic oligopeptides were identified using a pool of convalescent sera from pigs infected with *Salmonella Typhimurium*. The corresponding complete genes of seven of the identified oligopeptides were cloned. Five of them were produced in *E. coli*. The immunogenic character of these antigens was validated with sera from pigs infected with *S. Typhimurium* and control sera from non-infected animals. Finally, human antibody fragments (scFv) against these five antigens were selected using antibody phage display and characterised.

**Conclusion:** In this work, we identified novel immunogenic proteins of *Salmonella Typhimurium* and generated antibody fragments against these antigens completely based on phage display. Five immunogenic proteins were validated using a panel of positive and negative sera for prospective applications in diagnostics of *Salmonella Typhimurium*.

**Background**

*Salmonella* spec. is a genus of the *Enterobacteriaceae*. Two species are in the genus Salmonella: *S. bongori* and *S. enterica* [1]. *Salmonella enterica* is classified in serogroups and serovars on the basis of their O- and H-antigens (somatic and flagellar antigens) [2,3]. So far, 2800 *Salmonella enterica* gene families and more than 2500 serovars are known. More than 1500 serovars belong to the subspecies *Salmonella enterica* subspecies *enterica* [4]. These pathogens cause foodborne gastrointestinal infections, usually through raw poultry and pork, but it can also be found in non-alcoholic beer or seafood. The subspecies *enterica* is the cause of 99% of human *Salmonella* infections. The prevalent serovars are Typhimurium and Enteritidis [4-7]. The most reported phage types for *Salmonella Typhimurium* are DT193, U302 and DT104. Infections with the latter two phage types increased in 2009 [5]. Human infections with phage type DT104 are particularly critical, because these strains are resistant to most of the commonly used antibiotics [6]. In Europe, *Salmonella* caused more than 130,000 reported infections in 2008 and 108,614 cases in 2009. In the US more than a million cases are estimated to occur [5,8].

Improved detection of livestock colonised with *S. Typhimurium* would be very helpful to prevent foodborne diseases. In particular, infections in swine are difficult to diagnose, because the animals develop either no or only slight symptoms [9]. Only through continuous monitoring of the herds infections of humans can be prevented. Established methods for *S. Typhimurium* diagnostics are classically time-consuming, using microbiological cultures on
different liquid and solid media [10,11], specific fluorescence labeled DNA probes [12], PCR [13] or recently, a quantum dot-based bead assay [14]. Currently, high throughput diagnostic of S. Typhimurium is performed by indirect ELISA [9,15,16]. The commercially available ELISA kits e.g. SALMOTYPE® or Enterisol®-ELISA use a mixture of O-antigens of Salmonella enterica subspecies enterica serovars. They are based on the system established by Nielson et al. [15]. Because of this mixture, cross-reactions occur with other bacteria [15]. In addition, the sensitivity varies between the different ELISA assays [17]. For a sensitive and specific ELISA, immunogenic and species specific proteins are required [18]. The improvement of detection methods, as well as the development of new vaccines would be facilitated by the identification, characterisation and validation of previously unknown immunogenic proteins.

The most common method for the identification of immunogenic proteins is 2D-PAGE of cultured bacterial pathogens and immunoblot using sera from infected patients or animals followed by mass spectrometry or microsequencing [19-24]. However, this method is limited. Differentially expressed proteins, e.g. dependent on pathogen-host interaction, can not be detected. Furthermore, weakly expressed antigens may also not be identified. In these cases, antigen phage display may circumvent these limitations. Our approach for the identification of immunogenic proteins is phage display. Phage display technology was invented by George P. Smith [25]. This methods can be used both for the selection of antibodies [26-29] and for the identification of immunogenic proteins from genomic or cDNA libraries [30-34]. Here, the cloning of randomly fragmented genomic DNA or cDNA into phage display vectors should allow, in theory, the display of all polypeptides encoded by the genome of the donor or all polypeptides encoded by the transcripome of the donor, respectively [35,36]. In this study, we combined the identification of immunogenic proteins by M13 phage display using genomic libraries from S. Typhimurium with the selection of open reading frames without any subcloning steps (Figure 1 left part) in order to improve the library quality [37,38]. Afterwards, the genes corresponding to the identified immunogenic oligopeptides were cloned and produced in E. coli (Figure 1 middle part). Using our phage display based pipeline for the generation of human antibodies [39], we were able to generate human, recombinant antibodies against these antigens (Figure 1 right part).

**Results**

**Generation of the Salmonella Typhimurium genomic phage display library**

Sonication of Salmonella DNA did not lead to clonable DNA fragments, whereas the sonication of E. coli DNA as a control could be cloned without problems (data not shown). Therefore, genomic DNA was digested with a mixture of the 4 base pair cutters DpnI and AluI and the 6 base pair cutter AfeI to construct the Salmonella Typhimurium genome library (Figure 2A). The digested DNA was cloned into pHORF3 [38] resulting in a library with $1.6 \times 10^6$ independent clones. The insert rate and size was analysed by colony PCR (Figure 2B), which indicated that more than 90% of the clones contain an insert. The shortest inserts had a size of about 40 bp while the longest inserts had a length of 2500–3000 bp with an average length of about 500–800 bp. For the selection of open reading frames, the library was packaged with Hyperphage [37,40,41]. The packaged library was also analysed by colony PCR (Figure 2C) resulting in shorter DNA fragments compared to the initial library.

**Selection of immunogenic Salmonella Typhimurium oligopeptides**

Pooled convalescent serum from pigs infected with Salmonella Typhimurium was used for the panning procedure. In total, two panning rounds were performed and 184 oligopeptide phage clones were analysed by ELISA for binding to serum IgGs. 58 oligopeptide phage clones with different gene fragments were found to bind to the porcine convalescent serum IgGs but not to the control serum IgGs. For 19 gene fragments, no homologous NCBI database hit was found. 31 of the identified proteins showed a higher similarity to other Salmonella enterica serovars (Table 1). One of the selected gene fragments showed the best NCBI database hit to Salmonella Typhimurium, but the gene fragment was not in frame (Table 2). Seven oligopeptide phage clones with a good signal to noise ratio (three- to four-fold over background) in the ELISA and the highest homology to genes from Salmonella enterica spp. enterica serovar Typhimurium were selected for further characterisation (Table 3). Binding of the oligopeptide phage to convalescent serum IgGs, including the non-Salmonella Typhimurium hits and the out of frame Salmonella Typhimurium hit, was verified in an additional ELISA with piglet serum as negative control (data not shown).

**Cloning of the complete ORFs of the identified oligopeptides and antigen production**

The corresponding complete protein coding sequence of the identified gene fragments was amplified from genomic DNA (Figure 3A) and cloned into pET21A+. When the ORF contained a leader peptide, it was replaced by the pelB leader peptide by cloning into pET21A + pelB. It was not possible to amplify the putative carbohydrate kinase encoding gene from the genomic DNA. The antigens were produced in 500 mL scale shake flasks, purified by IMAC and verified by SDS-PAGE (Figure 3B). Five of six antigens
cloned into pET21A + were produced and purified successfully. It was not possible to produce the hypothetical protein STM14.

Analysis of the identified immunogenic proteins with different pig sera
The five full size antigens were tested with sera from ten Salmonella Typhimurium positive classified pigs, using the commercial SALMOTYPE Pig Screen ELISA, and sera from four negative classified pigs. In addition, they were tested with the pooled immune positive sera used for the selection procedure and with a piglet serum as negative control (Figure 4).

9 out of 10 positive sera bound better to the antigen putative dihydroxyacid dehydratase compared to the negative sera. This means, that 9 of the 10 positive sera had a higher ELISA O.D. value compared to the negative serum with the highest ELISA O.D. value.

7 out of 10 positive sera bound better to the antigen putative electron transfer protein alpha compared to the negative sera. Here, the piglet serum revealed a high antigen binding capacity compared to the mixture of positive sera.

7 out of 10 positive sera bound better to the antigen 2,4-dieonyl-CoA-reductase compared to the negative sera.

8 out of 10 as positive classified sera bound better to the antigen phage tail-like protein compared to the negative classified sera.

8 out of 10 as positive classified sera bound better to the antigen putative dimethyl sulphoxide reductase compared to the negative classified sera.

Not all individual positive sera bound significantly better than all four individual negative sera. However, in general the positive sera showed better binding to all identified immunogenic proteins compared to the negative sera.

Generation of recombinant human antibodies against the identified immunogenic proteins
Antibody fragments against all five antigens were selected using the human naive antibody gene library.
Monoclonal binders were identified by antigen ELISA using soluble scFv fragments (data not shown). These binders were sequenced to identify unique binders and analysed using VBASE2 (www.vbase2.org) [42]. Human antibodies were successfully generated against all five antigens.

The best binders were recloned into the pOPE101-XP vector [43], produced in 1.6 L scale in the LEX system and IMAC purified (Table 4). The yields were between 0.5 mg/L and 12 mg/L. Afterwards, the purified scFv were analysed by titration ELISA (Figure 5). The EC50 values of the scFv (monovalent) are given in Table 4. For four scFv it was not possible to determine the EC50, since the maximal binding was not reached in the titrations ELISA.

**Analysis scFv binding to Salmonella proteins by immunoblot**

Binding to linear epitopes was analysed by SDS-PAGE of the antigens, followed by a Western Blot and an immunostain using the purified scFv. All binders to putative dihydroxyacid dehydratase, phage tail-like protein and...
putative dimethyl sulphoxide reductase bound linear epitopes. Three of the four binders to putative electron transfer protein alpha and the binder against 2,4-dienoyl-CoA-reductase did not bind in the immunoblot (Table 4).

**Discussion**

Antibody phage display for generation of recombinant antibody fragments [39,44-47] and the identification of immunogenic proteins by phage display [30-32,38,48,49] are established methods. But in this work, for the first time a complete phage display based pipeline from antigen identification to the generation of the corresponding antibody fragments was shown. Oligopeptide phage

### Table 1 Selected immunogenic *Salmonella* enterica proteins (without *Salmonella* serovar Typhimurium), including in frame and out of frame with gIII gene fragments

| Protein                                                                 | In frame with gill | pHORF 3 insert size [bp] | NCBI reference sequence | Assigned *Salmonella* serovar     |
|------------------------------------------------------------------------|--------------------|--------------------------|-------------------------|----------------------------------|
| ATP-dependent Clp protease ATP-binding subunit                         | yes                | 232                      | ZP_04657262             | Tennessee                        |
| outer membrane ferrichrome receptor protein precursor                  | yes                | 58                      | YP_002635837            | Paratyphi C                      |
| Rhs-family protein                                                     | yes                | 100                     | ZP_04657973             | Tennessee                        |
| hypothetical protein SentesTyphi_03066                                 | yes                | 294                     | ZP_03357516             | Typhi                            |
| crotonobetaine/carnitine-CoA ligase                                    | no                 | 135                     | YP_002635709            | Paratyphi C                      |
| transposase B                                                          | no                 | 144                     | ZP_03224077             | Kentucky                         |
| DNA polymerase I                                                       | no                 | 348                     | ZP_03381877             | Typhi                            |
| nitrate reductase 2, gamma subunit                                     | no                 | 89                      | ZP_03366843             | Typhi                            |
| putative phage terminase, large subunit                                | no                 | 648                     | YP_002216031            | Dublin                           |
| putative electron transfer flavoprotein alpha subunit                  | yes                | 58                      | YP_002636467            | Paratyphi C                      |
| hypothetical protein                                                   | no                 | 414                     | ZP_06535866             | Typhi                            |
| *Salmonella entericaenterica*_12643                                    |                    |                         |                         |                                  |
| exonuclease V subunit gamma                                            | no                 | 86                      | ZP_04653610             | Tennessee                        |
| bacteriophage Mu tail sheath protein                                   | no                 | 182                     | ZP_02663881             | Schwarzen-grund                  |
| pts system, glucose-specific iibc component                            | no                 | 109                     | ZP_02670079             | Heidelberg                       |
| flagellar basal body P-ring protein                                    | no                 | 60                      | ZP_03371027             | Typhi                            |
| hht-type transcriptional regulator                                     | no                 | 710                     | ZP_02698922             | Newport                          |
| DNA mismatch repair protein                                            | yes                | 122                     | YP_002638485            | Paratyphi C                      |
| colanic acid biosynthesis protein WcaK                                 | no                 | 270                     | ZP_02830210             | Weltevreden                      |
| outer membrane fimbrial usher protein                                  | yes                | 171                     | ZP_03336471             | Typhi                            |
| uroporphyrinogen-III synthase                                          | yes                | 49                      | ZP_03385917             | Gallinarium                      |
| ATP-dependent metalloprotease                                          | yes                | 130                     | ZP_03385917             | Typhi                            |
| aminoglycoside/multidrug efflux system                                 | yes                | 67                      | ZP_04657455             | Tennessee                        |
| penicillin-binding protein                                             | no                 | 56                      | ACN4754                 | Paratyphi C                      |
| C32 rRNA thiolase                                                     | yes                | 123                     | ZP_03365647             | Typhi                            |
| hypothetical protein SG0660                                            | no                 | 324                     | YP_002225743            | Gallinarium                      |
| putative LysR-family transcriptional regulator                         | no                 | 48                      | AET5395                 | Gallinarium                      |
| 23 S rRNA methyluridine methyltransferase                              | yes                | 214                     | ZP_03356931             | Typhi                            |
| hypothetical protein SeHA_C2934                                       | yes                | 55                      | YP_002046718            | Heidelberg                       |
| putative transport protein                                             | yes                | 55                      | ACN44236                | Paratyphi C                      |
| hypothetical protein SPC_0823                                          | yes                | 61                      | ZP_03365355             | Typhi                            |
| nitrate reductase, alpha subunit                                       | yes                | 298                     | ZP_03217505             | Virchow                          |

### Table 2 Selected immunogenic *Salmonella* Typhimurium proteins with out of frame with gIII fragments in pHORF3

| Protein                                                                 | pHORF3 insert size [bp] | NCBI reference sequence |
|------------------------------------------------------------------------|--------------------------|-------------------------|
| maltose ABC transporter periplasmic260                                  | NP_463094                |                         |
display technology can expand the identification of immunogenic proteins compared to 2D-PAGE followed by mass spectrometry or microsequencing [32,38,48,50]. The identification of immunogenic proteins via oligopeptide phage display is independent of the natural expression rate of the immunogenic protein, which also allows the identification of low abundant proteins or proteins only produced in host-pathogen interactions. A disadvantage could be that only oligopeptides can be selected which can be secreted by the SEC pathway [38]. Interestingly, when using sonicated S. Typhimurium DNA, the transformation rates were in the range of $10^2 - 10^4$ clones per transformation. This is very low compared to the transformation rates of $10^5$ for sonicated genomic DNA of *Mycoplasma hyopneumoniae* or $10^6$ clones for *E. coli* [38]. Hence, the sonication method appeared to be not applicable for some bacteria species or strains when constructing genomic libraries.

| Protein                                | pHORF3 insert size [bp] | NCBI reference sequence | Molecular mass complete protein [kDa] |
|----------------------------------------|------------------------|------------------------|-------------------------------------|
| putative dihydroxyacid dehydratase     | 118                    | NP_462432              | 62                                  |
| putative electron transfer protein alpha| 58                     | NP_459833              | 33                                  |
| 2,4-dienoyl-CoA-reductase              | 52                     | NP_460213              | 73                                  |
| phage tail-like protein                | 70                     | NP_461635              | 23                                  |
| putative dimethyl sulfoxide reductase  | 148                    | NP_460459              | 90                                  |
| hypothetical protein STM14             | 58                     | ACY86745               | 7                                   |
| putative carbohydrate kinase           | 40                     | CBG27384               | 72                                  |

**Table 3 Selected immunogenic *Salmonella Typhimurium* proteins**

**Figure 3** A agarose gel of seven PCR amplified identified genes. M: marker; 1: putative dihydroxyacid dehydratase; 2: putative electron transfer protein alpha; 3: 2,4-dienoyl-CoA reductase; 4: phage tail like protein; 5: putative dimethyl sulfoxide reductase; 6: hypothetical protein STM14. B: SDS-PAGE (12%) of five μL elution fraction of six IMAC purified immunogenic proteins. M: marker; 1: putative dihydroxyacid dehydratase; 2: putative electron transfer protein alpha; 3: 2,4-dienoyl-CoA reductase; 4: phage tail like protein; 5: putative dimethyl sulfoxide reductase.
Figure 4 ELISA for analysis of identified immunogenic *Salmonella* Typhimurium proteins with defined pig sera. 1: putative dihydroxyacid dehydratase; 2: putative electron transfer protein alpha; 3: 2,4-dienyl-CoA reductase, 4: phage tail like protein; 5: putative dimethyl sulphoxide reductase. The sera from *Salmonella*-positive pigs (according to “Pig Screen” ELISA) are marked dark blue, the sera from *Salmonella*-negative pigs (according to “Pig Screen” ELISA) are marked orange, the mixture of positive sera used for the selection of immunogenic proteins is marked green, the piglet serum is marked dark red and the detection system control (only detection antibodies) is marked in light blue. For the panel of positive and negative sera arithmetic mean and standard deviation are given as black lines. 1 μg purified antigens were coated. The antigens were detected with diluted swine sera (1:200 in 2% MPBST) and goat anti-swine IgG HRP conjugate (1:10,000).
Table 4 Characterisation of antibody fragments generated against Salmonella Typhimurium antigens. n.d. = EC50 not determined

| scFv       | Target                                | VH          | VL            | Yield [mg/L] | EC50 [nM] | EC 50 [μg/mL] | Immuno-blot |
|------------|---------------------------------------|-------------|---------------|--------------|-----------|---------------|-------------|
| TM228.2.3-B1 | putative dihydroxyacid dehydratase    | IGHV3-48*03| IGLV3-19*01   | 3.8          | 40        | 1.2           | yes         |
| TM228.2.3-D9 | putative dihydroxyacid dehydratase    | IGHV3-23*01| IGLV1-50*01   | 8.4          | 160       | 4.6           | yes         |
| TM228.2.3-H7 | putative dihydroxyacid dehydratase    | IGHV1-18*01| IGLV3-19*01   | 12.0         | 310       | 9.4           | yes         |
| TM228.3.3-A5 | putative electron transfer protein alpha | IGHV1-46*01| IGLV7         | 4.3          | 78        | 2.3           | yes         |
| TM228.3.3-C5 | putative electron transfer protein alpha | -          | IGLV3-21*02   | 7.9          | n.d.      | n.d.          | no          |
| TM228.3.3-D3 | putative electron transfer protein alpha | IGHV5-51*01| IGLV3-1*01    | 7.6          | n.d.      | n.d.          | no          |
| TM228.3.3-F10 | putative electron transfer protein alpha | IGHV1-46*01| IGLV3-19*01   | 0.5          | n.d.      | n.d.          | no          |
| TM228.4.3-A4 | 2,4-dienoyl-CoA-reductase             | IGHV3-15*01| IGLV3-19*01   | 1.3          | 310       | 9.4           | no          |
| TM228.5.3-G7 | phage tail-like protein               | IGHV1-2*02  | IGLV1-47*01   | 7.4          | 310       | 9.4           | yes         |
| TM228.6.3-A12 | putative dimethyl sulfoxide reductase | IGHV1-69*01| IGLV3-19*01   | 1.1          | n.d.      | n.d.          | yes         |
| TM228.6.3-C5 | putative dimethyl sulfoxide reductase | IGHV4-31*03| IGLV1-44*01   | 1.5          | 625       | 18.8          | yes         |
| TM228.6.3-H2 | putative dimethyl sulfoxide reductase | IGHV4-59*01| IGLV1-44*01   | 2.8          | 625       | 18.8          | yes         |

The V genes are given according to VBASE2 (www.vbase2.org).

In this work, 58 different oligopeptides were bound by convalescent serum from pigs infected with Salmonella Typhimurium. Interestingly, many of the encoding gene fragments were not in frame with gIII and therefore, in theory, should not result in the production of functional oligopeptide-pIII fusion proteins. However, similar observations, that gene fragments encoding oligo- or polypeptides frequently contain frameshifts, have been described previously for selections by phage display [37,51]. For +1 frameshifts it is reported that oligo- or polypeptides are still displayed on phage particles with the same amino acid sequence as the corresponding constructs without a frameshift. One suggested explanation of this effect was the occurrence of RNA secondary structures. A second explanation could be the selection pressure against oligo- or polypeptides which are toxic for E. coli and thus may lead to a negative selection against these potential toxic proteins [52].

The most frequently identified oligopeptides did not show the best match with the Salmonella serovar Typhimurium (NCBI taxonomy IDs: 99287, 588858 and 568708), but instead with other Salmonella serovars. These antigens with a higher homology to Salmonella serovars, could be interesting for further analyses. However in this work, we focused on the seven antigens with the highest homology to Salmonella Typhimurium. In contrast to former selections of immunogenic proteins using the pHORF system, where both new and known immunogenic proteins were selected [32,38], these seven antigens have not been described as immunogenic before. So far, five immunogenic proteins of S. Typhimurium were found using 2D-PAGE [53]. Putative dihydroxyacid dehydratase, putative dimethyl sulfoxide reductase and hypothetical protein STM14 of Salmonella Typhimurium have not been described as immunogenic before. The putative electron transfer protein alpha [54] is located on a pathogenicity island [55]. To date, 2,4-dienoyl-CoA-reductase of S. Typhimurium has not been identified as immunogenic, but interestingly, humans exhibiting anti-mitochondrial autoantibodies (AMA-positive), have also antibodies against the human 2,4-dienoyl-CoA-reductase [56]. For the phage tail-like protein, a bactericidal activity is described for some bacteria, e.g. Pseudomonas [57]. Immunogenic proteins from S. Typhimurium, which are used for diagnostics, are only rarely described in the literature. Described are OmpD [58] and a preparation of flagellates [59] for ELISA diagnostics. For nanobead based assays polyclonal antibodies against Salmonella were used whereupon the detailed antigens are unknown [14]. The V genes of the selected scFv against the five immunogenic genes are mainly derived from the HV families 1 and 3 and from the LV families 1 and 3. Member of these gene families are preferentially selected from naive scFv libraries [60,61]. Only scFv with a lambda VL but no kappa VL were selected. Interestingly, also one VL domain only binder was selected. This is an artefact from library cloning since the insert rate of HAL7 is not 100% [39]. Functional VL domain dAbs have been described before [62].

The gold standard for diagnostics of Salmonella infections is microbiological culture [18]. Currently, for high throughput detection of S. Typhimurium, ELISA is the ideal method [9,15,16,58]. The commercially available ELISA kits use a mixture of O-antigens (LPS) or total cell lysate of Salmonella enterica subspecies enterica serovars. This mixture of antigens causes, cross-reactions with other bacteria [9,18]. A
comparison of four different ELISA detection systems showed “both sample matrices, blood sera and meat juice, are suitable for antibody detection. However, the test sensitivity mainly depends on the respective cut-off used for the specific test” and “our findings indicate that the currently used LPS-ELISA systems have diagnostic uncertainties...” [9]. The use of one or a defined mixture of the selected immunogenic proteins and the corresponding antibody fragments will be useful to establish an ELISA based diagnostic kit with a higher specificity compared to the commercially available diagnostic kits.

Figure 5 Titration ELISA to analyse the selected anti-Salmonella Typhimurium antigens scFv. 1: putative dihydroxyacid dehydratase; 2: putative electron transfer protein alpha; 3: 2,4-dienyl-CoA reductase; 4: phage tail like protein; 5: putative dimethyl sulphoxide reductase. 1 μg purified antigens were coated and detected with a dilution series of purified scFv. Bound scFv fragments were detected with the anti myc 1-9E10 (1:500) and the goat-α-mouse-IgG (Fab spec.) HRP-conjugate (1:10,000).
Methods

Construction of the Salmonella Typhimurium genomic phage display library

Salmonella Typhimurium was cultivated in 2xTY medium [63] overnight at 34°C and 250 rpm. For isolation of genomic DNA, 6x 3 mL of the culture were used. The isolation was performed with the Quiaamp DNA Mini Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). After purification, the DNA was digested for 35 min using three different blunt end-cutting restriction endonucleases (Alul, Afel, DpnI) (NEB, Frankfurt, Germany). DNA fragments with a size up to 1200 bp, were used for cloning into the Pmel-restricted vector pHORF3 [38]. The ligated plasmids were transformed into E. coli Top10 F’ (Invitrogen, Karlsruhe, Germany) by electroporation.

Enrichment of ORFs using Hyperphage

The enrichment of ORFs in the S. Typhimurium genomic library requires the display of the corresponding polypeptides on phage particles for the panning. Therefore, the library was packaged using Hyperphage [40,41] as described previously [37,38].

Colony PCR

E. coli clones bearing pHORF3 were analysed by colony PCR using the primers MHLacZPro_f (5’ GGCTCGTATG TTGTGTGG 3’), MHgIII_r (5’ GGAAAGACGACAAAA CTTTAG 3’), and the following protocol: 94°C 1 min, 56°C 0.5 min, 72°C 1.5 min, 25 cycles. The DNA was separated by 1% Agarose gel electrophoresis.

Selection of immunogenic oligopeptides (Panning)

The panning was performed by following the protocol described before [38] with modifications. Six wells of a MaxiSorp® 96-well microtitre plate (MTP; Nunc, Wiesbaden, Germany) were coated with 150 μL 5 μg/mL goat anti-swine IgG in PBS [63] overnight. The wells were washed with phosphate buffered saline (PBS) supplemented with 0.1% Tween20 (PBST) (Roth, Karlsruhe, Germany). Afterwards, they were blocked with PBST supplemented with 2% (w/v) skim milk powder (2% MPBST) for 1 h. In parallel, several wells of a MaxiSorp® plate were coated with 150 μL 1 x 10^11 cfu/mL Hyperphage in PBS overnight and blocked with 2% MPBST for 1 h. All washing steps were performed three times using PBST buffer and an enzyme-linked immunosorbent assay (ELISA) washer (Tecan Columbus, Cralshelm, Germany). A swine serum mixture (obtained from pigs after infection with S. Typhimurium and field sera) was diluted 1:10 in 2% MPBST and pre-incubated on MaxiSorp® MTP wells coated with Hyperphage for 1 h at RT, to remove serum IgG binding to the helper-phage. After pre-incubation, the swine serum was incubated in goat anti-swine IgG-coated MTP wells for 2 h. After washing, 5 x 10^10 cfu polypeptide phage particles of the Hyperphage-packaged Salmonella Typhimurium genomic library were incubated on the captured swine IgGs for 2 h. For the following panning rounds, 100 μL of amplified phage of the previous panning round were used. The non-binding polypeptide phage particles were removed by ten stringent washing steps. In the second and third panning round, the number of washing steps was increased to 20 and 30, respectively. Elution of bound phage particles was performed using 200 μL of 10 μg/mL trypsin (10 μg/mL trypsin in PBS) for 30 min at 37°C. Ten microlitres of the eluted phage solution were used for titration. Twenty millilitres of the E. coli TOP10 F’ cells were grown to an OD_600 of 0.4 - 0.5 which were then infected with the remaining 190 μL of the eluted phage solution and incubated for 30 min at 37°C. Afterwards, the cells were harvested by centrifugation for 10 min at 3.220 x g. The bacterial pellet was resuspended in 250 μL 2 x TY medium (1.6% [w/v] tryptone, 1% [w/v] yeast, 0.5%[w/v] NaCl) containing 100 mM glucose and 100 μg/mL ampicillin (2 x TY-GA), plated onto 15 cm 2 x TY-GA agar plates and incubated overnight at 37°C. Grown colonies were harvested in 5 mL 2 x TY-GA medium using a Drigalsky spatula. Fifty millilitres of 2 x TY-GA medium were inoculated with 200 μL bacteria culture and grown to an OD_600 of 0.4-0.5 at 37°C and 250 rpm in a shaking incubator. Five millilitres of bacterial culture corresponding to about ~2.5 x 10^9 cells were infected with 5 x 10^10 cfu Hyperphage, incubated at 37°C for 30 min without shaking and another 30 min with shaking at 250 rpm. The infected cells were harvested by centrifugation for 10 min at 3.220xg. The pellet was resuspended in 30 mL 2xTY medium containing 100 μg/mL ampicillin and 50 μg/mL kanamycin (2 x TY-AK). Phage particles were produced at 30°C and 250 rpm overnight. On the following day, the supernatant containing phage particles was collected.

Production of individual oligopeptide phage clones for screening

For phage production, polypropylene 96-well U bottom plates (Greiner bio-one, Frickenhausen, Germany) containing 175 μL 2XTY-GA per well were inoculated with single E. coli colonies from the phage titration plates of the third panning round and incubated at 37°C with constant shaking at 850 rpm (thermo shaker PST60-HL4, lab4you, Berlin, Germany) overnight. A new plate with 165 μL 2XTY-GA per well was inoculated with 10 μL of the overnight cultures and incubated at 37°C and 850 rpm for 2 h. Afterwards, the bacteria were infected with 5 x 10^9 cfu Hyperphage/well and incubated at 37°C without shaking for 30 min, followed by shaking at 850 rpm for 30 min. The MTP plate was centrifuged at
3,220xg for 10 min and the supernatants were discarded. Afterwards, the bacterial pellets were resuspended in 175 µL/well 2xTY containing 100 mg/mL ampicillin and 30 µg/mL kanamycin (2xTY-AK) and incubated at 30°C at 850 rpm overnight for phage production. The bacteria were pelleted again and the supernatants were transferred to a new plate. The phage were precipitated with 1/5 volume 20% PEG/2.5 M NaCl solution at 4°C for 1 h and centrifuged at 3,220xg for 1 h. The phage pellet was dissolved in 150 µL PBS and residual bacteria were removed by another centrifugation at 3,220xg for 5 min. The phage containing supernatants were stored at 4°C or directly used for ELISA.

Screening of individual oligopeptide phage clones

For phage ELISA, the produced polypeptide phage particles were captured. Here, 100 µL of 250 ng/mL mouse anti-M13 (B62-FE2, Progen, Freiburg, Germany) in PBS were coated at 4°C overnight. After coating the wells were washed three-times with PBST using anti-M13 (B62-FE2, Progen, Freiburg, Germany) in PBS and centrifuged at 3,220xg for 1 h. The phage containing supernatants were stored at 4°C overnight. After coating, the wells were washed seven-times with PBST using 1/10 volume 1/100 serum was diluted 1:200 in 2% MPBST supplemented with FastFlow Sepharose (GE Healthcare) loaded with nickel. The Sepharose was washed with 10 mM, 30 mM and 60 mM imidazole (20 mM Na2HPO4 0.5 M NaCl, 10 mM Imidazol). For elution, 5 mL 100 mM EDTA in PBS supplemented with 8 M urea was used.

Production of the immunogenic proteins

Five hundred mL 2xTY-GA medium were inoculated with 5 mL overnight culture and cultivated to an O.D.600 of 1.0 at 37°C and 250 rpm. The expression was induced with 1 mM IPTG (final concentration) overnight. Cells were harvested by centrifugation at 7,500 xg for 15 min. Lysis was performed with 1 mg/mL lysozyme and 5 µg/mL DNaseI in 15 mL His-tag binding buffer pH7.4 (20 mM Na2HPO4, 0.5 M NaCl, 10 mM Imidazol). For Isolation of inclusion bodies 8 M Urea was added. The purification was performed under denaturing conditions with FastFlow Sepharose (GE Healthcare) loaded with nickel. The Sepharose was washed with 10 mM, 30 mM and 60 mM imidazole (20 mM Na2HPO4 0.5 M NaCl, 10, 30 or 60 mM Imidazol). For elution, 5 mL 100 mM EDTA in PBS supplemented with 8 M urea were used.

SDS-PAGE

Antigens were analysed by 12% SDS-PAGE using a Protein II Minigel system (BioRad Inc, München, Germany) according to [63]. Protein gels were stained with coomassie blue.

Enzyme linked immunosorbent assay (ELISA) for verification of immunogenic proteins

One µg of antigen was coated to 96 well microtitre plates (MaxiSorp, Nunc) in 50 mM NaHCO3 pH 9.6 overnight at 4°C. After coating, the wells were washed three times with PBST and blocked with 2% MPBST for
1.5 h at RT, followed by three washing steps with PBST. For serum ELISA, sera were diluted 1:200 in 100 μL 2% MPBST and incubated in the antigen coated plates for 1.5 h at RT, followed by three PBST washing cycles. Bound pig IgGs were detected with goat anti-swine IgG HRP conjugate (1:10,000) (Dianova, Hamburg, Germany). The visualisation was performed with TMB (3,3’,5,5’-tetramethylbenzidine) as a substrate and the staining reaction was stopped by adding 100 μL 1 N sulphuric acid. Absorbance at 450 nm was measured by using a SUNRISE™ microtitre plate reader (Tecan, Crailshelm, Germany).

**Identification of monoclonal scFv using ELISA**

Antigen coating was performed as described above (Enzyme linked immunosorbent assay (ELISA) for verification of immunogenic proteins). For identification of binders, supernatants containing monoclonal scFv were incubated in the antigen coated plates for 1.5 h at RT followed by three PBST washing cycles. Bound scFv were detected using murine mAb 9E10 which recognises the C-terminal c-myc tag and a goat anti-mouse serum conjugated with horseradish peroxidase (HRP) (Sigma; 1:10,000).

The detection was performed as described above.

**Production of scFv in the LEX system**

The large-scale expression system (LEX) (Harbinger Biotech, Toronto, Canada) was used for production of scFv. *E. coli* (XL1-Blue-MRF') was cultivated in 2 L glass bottles up to a cultivation volume of 1.5 L. To obtain sufficient oxygenation and mixing of the culture, the bottles were connected to an air manifold, which allows a general air flow rate of 4–6 L/min. A thermostat-controlled water bath was used for regulating the temperature of the cultivation. 50 ml TB supplemented with 100 μg/mL ampicillin, 100 mM sucrose an 50 μM isopropyl-beta D thiogalactopyranoside (IPTG) and incubated at 30°C and 800 rpm overnight. Bacteria were pelleted by centrifugation for 10 min at 3,220 g and 4°C. The scFv-containing supernatant was transferred to a new PP-MTP and stored at 4°C before analysis.
ampicillin were inoculated with a glycerol stock of each scFv clone and the culture was grown over night at 37°C. Glass bottles with 1.5 L TB supplemented with 100 μg/mL ampicillin and 500 μL antifoam 204 (Sigma, München, Germany) were inoculated with the overnight culture. The O.D.600 was adjusted to 0.1 and incubated at 37°C until an O.D.600 of 1.5 to 5 was reached. The temperature of the water bath was then reduced to 25°C. After 1 h, scFv expression was induced by addition of 50 μM IPTG. The cultivation was continued for 3 h resulting in a final O.D.600 of 2 to 7 depending on the antibody clone. E. coli cells were harvested by centrifugation at 4,400g (Sorvall Zentrifuge RC6 Plus, Rotor F9S-4x1000Y) for 10 min at 4°C. The pellet was resuspended in 60 mL ice-cold PE-buffer pH 8 (20% (w/v) sucrose, 50 mM Tris, 1 mM EDTA) and was incubated on ice for 20 min while shaking. Afterwards the sample was centrifuged at 20,000g and 4°C for 30 min (Sorvall Zentrifuge RC6 Plus, Rotor F12-6x500y). The supernatant (periplasmatic preparation) was filled into a fresh glass bottle and kept on ice. The pellet was resuspended in 60 mL ice-cold OS-buffer (5 mM MgSO4 in dH2O) and was incubated on ice while shaking. After 20 min the preparation was centrifuged at 20,000g and 4°C for 30 min (Sorvall Zentrifuge RC6 Plus, Rotor F12-6x500y). The supernatant (osmotic shock fraction) was combined with the periplasmatic preparation and was used for protein purification.

IMAC purification of scFv
Antibody fragments were purified by affinity chromatography using IMAC. Chromatography using Profinia (BioRad) and 1 mL FF-crude column (GE Healthcare, München, Germany) was performed according to the manufacturer’s instruction. The protein solution was adjusted to 10 mM imidazol containing buffer (20 mM Na2HPO4, 500 mM NaCl, 10 mM imidazol) for loading. The column was washed one time with 10 mM imidazol buffer (20 mM Na2HPO4, 500 mM NaCl, 10 mM imida zol). Five hundred mM imidazol was used for elution, followed by desalting and storage in PBS.

Titration ELISA using scFv
For the scFv titration ELISA the antigen was coated as described above (Enzyme linked immunosorbent assay (ELISA) for verification of immunogenic proteins). The ELISA was performed as described above (Identification of monoclonal scFv using ELISA) with one modification: a dilution series of IMAC purified scFv was used instead of the scFv supernatant. The EC50 values (antibody concentration at the half maximal binding) are deduced from this titration ELISA.

Detection of the immunogenic proteins by immunostain using scFv
Purified immunogenic proteins were separated by 12% SDS-PAGE. Western Blotting on PVDF (Polyvinyliden-fluorid) membranes of gels was performed using the Mini Trans-Blot® system (BioRad). The membrane was blocked with 2% (w/v) skimmed milk powder in PBST over night.

The antigens were detected with 20 μg/mL scFv for 1 h at RT. The scFv myc-tag was detected with mouse anti myc-tag (9E10, Sigma, Taufkirchen, Germany) for 1 h, followed by goat anti-mouse (Fc specific) (Sigma) conjugated with alkaline phosphatase (1:20,000) for 1 h. The visualisation was performed by addition of BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium).

Conclusion
A “pipeline” from antigen identification to the generation of recombinant antibodies using phage display was shown. Here, novel immunogenic proteins of Salmonella Typhimurium were identified using phage display and validated using a panel of positive and negative sera. Afterwards, recombinant human antibody fragments were generated against these marker proteins.

Competing interests
The authors declare that they have no competing interests.

Acknowledgements
This project was supported by the BMBF (BioRegionN) and EFRE. Our special thanks go to Ronny Fischer from our EC office who helped with bureaucracy of EFRE/NBANK. We also thank to David Havlik, Jonas Zantow, Alex Pytka and David Becker for careful corrections on the manuscript.

Authors’ contributions
TM and SM performed most of the experiments and helped to draft the manuscript. TS and AF performed some of the experiments and participated in the design and coordination of the study. JSS, SD, GFG and KSM participated in the design and coordination of the study. All authors read and approved the final manuscript.

Received: 23 January 2012 Accepted: 25 May 2012
Published: 15 June 2012

References
1. Fookes M, Schroeder GN, Langridge GC, Blondel CJ, Mammina C, Connor TR, Seth-Smith H, Vernikos GS, Robinson KS, Sanders M, Petty NK, Kingsley RA, Baumler AJ, Nuccio S-P, Contreras I, Santiviago CA, Maskell D, Barrow P, Humphrey T, Nastasi A, Roberts M, Frankel G, Parkhill J, Dougan G, Thomson NR: Salmonella bongori provides insights into the evolution of the Salmonellae. PLoS Pathog 2011, 7:e1002191.
2. Guibourdenche M, Roggentin P, Mikolét M, Fields Pl, Bockemühl J, Grimont PAD, Weill F-X: Supplement 2003–2007 (No. 47) to the White-Kauffmann-Le Minor scheme. Res Microbiol 2010, 161:26–29.
1. van der Wolf PJ, Peperkamp NH: Salmonella (sero)types and their resistance patterns in pig faecal and post-mortem samples. Vet Q 2001, 23:175–181.

2. Jacobsen A, Hendriksen RS, Aaresturp FM, Ussery DW, Friis C: The Salmonella enterica pangenome. Microb Ecol 2011, 62:487–504.

3. European Food Safety Authority, European Centre for Disease Prevention and Control: The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2009. EFSA Journal 2011, 9:1–378.

4. Little CL, Richardson JF, Owen RJ, de Pinna E, Threlfall EJ: Campylobacter and Salmonella in raw red meats in the United Kingdom: prevalence, characterization and antimicrobial resistance pattern, 2003–2005. Food Microbiol 2008, 25:536–543.

5. Menez G, Aldred P, Vriesekoop F: Growth and survival of foodborne pathogens in beer. J Food Prot 2011, 74:1670–1675.

6. Bugarel M, Granier SA, Weill F-X, Fach P, Brisaibois A: A multiplex real-time PCR assay targeting virulence and resistance genes in Salmonella enterica serotype Typhimurium. BMC Microbiol 2011, 11:151.

7. Roels CJ, Szabo J, Matthews C, Albrecht K, Leffler M, Scheer K, Nöckler K, Lehmann J, Mether J, Hensel A, Truyen U: Comparison of validating four ELISA-systems for detection of Salmonella derby- and Salmonella infantis-infected pigs. Berl Munch Tierarztl Wochenschr 2011, 124:265–271.

8. Silverman AP, Kool ET: Quenched autoligation probes allow in rRNA. Nucleic Acids Res 1999, 27:442–458.

9. Gallo C, di Gaudio B, Garzotto P, Giannini P, Marchetti C: Comparison of CHROMagar Salmonella medium and hektoen enteric agar for isolation of salmonellae from stool samples. J Clin Microbiol 1999, 37:762–765.

10. Silverman AP, Kool ET: Quenched autoligation probes allow discrimination of live bacterial species by single nucleotide differences in rDNA. Proc Natl Acad Sci USA 2005, 102:4978–4986.

11. Albertz J, Sato M, Vivanco AB, Parales RJ, Estaba R, Farrantia A, Garaciz J: Development of a multiplex PCR technique for detection and epidemiological typing of salmonella in human clinical samples. J Clin Microbiol 2004, 42:1734–1738.

12. Hang W, Li Y, Wang A, Slavik M: Rapid, sensitive, and simultaneous detection of three foodborne pathogens using magnetic nanobead-based immunomagnetic separation and quantum dot based multiplex immunomassay. J Food Prot 2011, 74:2039–2047.

13. Nielsen B, Baggesen D, Bager F, Haugegaard J, Lind P: The serological response to Salmonella serovars typhimurium and infants in experimentally infected pigs. The time course followed with an indirect anti-LPS ELISA and bacteriological examinations. Vet Microbiol 1995, 47:205–218.

14. Steinbach G, Stadler C: Assessment of the Salmonella burden in slaughter pigs through the results of meat-juice-ELISA. Berl Munch Tierarztl Wochenschr 2001, 114:174–178.

15. van der Heiden HM: First international ring trial of ELISAs for Salmonella-antigen detection in swine. Berl Munch Tierarztl Wochenschr 2001, 114:389–392.

16. Kuhn KG, Falkenhorst G, Ceper TH, Dalby T, Ethelberg S, Mølbak K, Krogfelt KA: Detecting non-typhoid Salmonella in humans by ELISAs: a literature review. J Med Microbiol 2012, 61:1–7.

17. Delvecchio VG, Connolly JP, Allen E, Bresse L, Consurcin O, Zervinis G, Tilt K, Poulton B, Kaukonen A, Bide Patternis M, Wiersma EJ: Analysis of single chain Fv fragment production of single chain Fv fragments in Escherichia coli. J Biotechnol 2003, 104:424–428.

18. McCafferty J, Griffiths AD, Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR: Identification of immunogenic proteins in Treponema phagedenis-like strain V1 from dogs. J Biotechnol 1990, 21:85–90.

19. Jacobsen A, Hendriksen RS: Improved microtitre plate production of single chain Fv fragments in Escherichia coli. J Biotechnol 2000, 73:27–34.

20. Mei M, Li X, Zhang Y, Wang Y, Xu C: Identification of immunoreactive proteins of Brucella melitensis by mass spectrometry. J Biotechnol 2005, 114:178–187.

21. Kwon HS, Yoon J, Park JS, Bae OY, Lee HS, Lee JH, Bae YH, Lee HS: Characterization of a multiplex real-time PCR assay for detection of pathogenic Campylobacter. J Microbiol Biotechnol 2011, 21:763–770.

22. Marzec M, Wójcik M, Górecka M, Grzywacz M, Kornicki A, Płonka M, Kryński W, Smoluchowski M, Skrzypek M: Identification of immunogenic proteins from Mycoplasma mycoides subsp. mycoides small colony type. Vet Microbiol 2010, 142:285–292.

23. Kuhn KG, Falkenhorst G, Ceper TH, Dalby T, Ethelberg S, Mølbak K, Krogfelt KA: Detecting non-typhoid Salmonella in humans by ELISAs: a literature review. J Med Microbiol 2012, 61:1–7.

24. McCafferty J, Griffiths AD, Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR: Identification of immunogenic proteins in Treponema phagedenis-like strain V1 from dogs. J Biotechnol 1990, 21:85–90.

25. Smith GP: Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 1985, 228:1315–7.

26. Surn D, Stevens JA, Pausig MJ, Hutt M: Generating recombinant antibodies to the complete human proteome. Trends Biotechnol 2010, 28:333–339.

27. Hoogenboom HR: Selecting and screening recombinant antibody libraries. Nat Biotechnol 2005, 23:1105–1106.

28. Thie H, Meyer T, Schirrmann T, Hutt M, Düb S: Phage display derived therapeutic antibodies.Curr Pharm Biotechnol 2008, 9:439–446.

29. Winter G, Griffiths AD, Hawkes RE, Hoogenboom HR: Making antibodies by phage display technology. Annu Rev Immunol 1994, 12:433–55.

30. Crameri R, Kozlowski K, Khovrat A, Lehrich H, Baser KH, Walter G: Tapping allergen repertoires by advanced cloning technologies. Int Arch Allergy Immunol 2001, 124:43–7.

31. Govant S, Somers K, Hupperts R, Stouwen P, Somers W: Exploring cDNA phage display for autoantibody profiling in the serum of multiple sclerosis patients: optimization of the selection procedure. Ann N Y Acad Sci 2007, 1109:372–84.

32. Luscher A, Meens J, Jones J, Heller M, Düb S, Hutt M, Gerlach G-F: Phage display-based identification and potential diagnostic application of novel antigens from Mycoplasma mycoides subsp. mycoides small colony type. Vet Microbiol 2010, 142:285–292.

33. Kuhn KG, Falkenhorst G, Ceper TH, Dalby T, Ethelberg S, Mølbak K, Krogfelt KA: Detecting non-typhoid Salmonella in humans by ELISAs: a literature review. J Med Microbiol 2012, 61:1–7.
S: A roadmap to generate renewable protein binders to the human proteome. Nat Methods 2011, 8:551–558.
47. Bretting F, Dübel S, Seehaus T, Kiewinghaus J, Little M: A surface expression vector for antibody screening. Gene 1991, 104:147–53.
48. Mittadou CR, Mather A, Wei EM, Du Plessis DH: Identification of genes coding for B cell antigens of Mycoplasma mycoides subsp. mycoides Small Colony (MmmSc) by using phage display. BMC Microbiol 2009, 9:215.
49. Kodzius R, Rhyner C, Konthur Z, Buczek D, Lehrah H, Walter G, Cramer R: Rapid identification of allergen-encoding cDNA clones by phage display and high-density arrays. Comb Chem High Throughput Screen 2003, 6:147–54.
50. González E, Robles Y, Govezensky T, Bobes RJ, Gevorkian G, Manoutcharian K: Isolation of neurocysticercosis-related antigens from a genomic phage display library of Taenia solium. J Biornol Sci 2010, 15:1268–1273.
51. Cárcamo J, Ravera MW, Brissette R, Dedova O, Beasley JR, Alam-Moghé A, Miltiadou DR, Mather A, Vilei EM, Du Plessis DH: Identification of genes coding for B cell antigens of Mycoplasma mycoides subsp. mycoides Small Colony (MmmSc) by using phage display. BMC Microbiol 2009, 9:215.
52. Goldman E, Korus M, Mandecki W: Efficiencies of translation in three reading frames of unusual non-ORF sequences isolated from phage display. FASEB J 2000, 14:603–611.
53. Michels J, Geyer A, Mocanu V, Welte W, Burlingame AL, Przybylski M: Structure and functional characterization of the periplasmic N-terminal polypeptide domain of the sugarspecific ion channel protein (ScrY poin). Protein Sci 2002, 11:1565–1574.
54. Zhou D, Harth WD, Galán JE: Salmonella typhimurium encodes a putative iron transport system within the centisome 63 pathogenicity island. Infect Immun 1999, 67:1974–1981.
55. Michels J, Geyer A, Mocanu V, Welte W, Burlingame AL, Przybylski M: Structure and functional characterization of the periplasmic N-terminal polypeptide domain of the sugarspecific ion channel protein (ScrY poin). Protein Sci 2002, 11:1565–1574.
56. Rong G, Zhong R, Lleo A, Leung PSC, Bowlus CL, Yang G-X, Yang C-Y, Coppel RL, Ansari AA, Cuebas DA, Worman HJ, Invernizzi P, Gores GJ, Norman G, He X-S, Gershwin ME: Epithelial cell specificity and apoptosis recognition by serum autoantibodies in primary biliary cirrhosis. Hepatology 2011, 54:196–203.
57. Scholl D, Cooley M, Williams SR, Gebhart D, Martin D, Bates A, Mandrell R: An engineered R-type pyocin is a highly specific and sensitive bactericidal agent for the food-borne pathogen Escherichia coli O157:H7. Antimicrob Agents Chemother 2009, 53:3074–3080.
58. Meyer T, Straßmann-Selke J, Meers J, Schirmann T, Gerlach GF, Frank R, Dübel S, StrutzbergMinder K, Hust M: Isolation of scFv fragments specific to OmpD of Salmonella Typhimurium. Ver Microbiol 2011, 147:162–169.
59. Dalby T, Strid MA, Beyer NH, Blom J, Maltbak K, Kroghfelt KA: Rapid decay of Salmonella flagella antibodies during human gastroenteritis: a follow up study. J Microbiol Methods 2005, 62:233–243.
60. Schofield DJ, Pope AR, Clementel V, Buckel J, Chapelle SD, Clarke KF, Conquer JS, Crofts AM, Crowther SRE, Dyson MR, Flack G, Griffin GJ, Hooick V, Havat WJ, Kolb-Kokocinski A, Kunze S, Martin CD, Macken GL, Mitchell JN, O’Sullivan M, Perera RL, Roake W, Shadbolt SP, Vincent KJ, Warford A, Wilson WE, Xie J, Young JL, McCafferty J: Application of phage display to high throughput antibody generation and characterization. Genome Biol 2007, 8:R254.
61. Frenzel A, Fride D, Meyer T, Schirmann T, Hust M: Generating Recombinant Antibodies for Research, Diagnostics and Therapy Using Phage Display. Curr Bioch 2012, 1:33–41.
62. Pereira B, Benedict CR, Le A, Shapiro SS, Thangarajan P: Cardiolipin binding a light chain from lupus-prone mice. Biochemistry 1998, 37:1430–1437.
63. Sambrook J, Russell D: Molecular cloning: a laboratory manual. 3rd edition. New York: Cold Spring Harbor Laboratory Press; 2001.
64. Schirmann T, Hust M: Construction of human antibody gene libraries and selection of antibodies by phage display. Methods Mol Biol 2010, 651:177–209.