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Phage Display Selection of Specific Ligands for *Listeria monocytogenes*: Novel Tools for Diagnostic or Therapeutic Purposes

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ABSTRACT

The gram-positive bacterium *Listeria monocytogenes* is the agent of listeriosis, a hazardous foodborne infection. Foodborne listeriosis is a comparably rare disease, but the fatality rate is up to 30%, despite antibiotic treatment. Prevention of Listeria contamination is an important food safety challenge and the demand for rapid and specific *L. monocytogenes* detection systems, as well as antimicrobial agents, is steadily rising. However, the development of effective detection methods for *L. monocytogenes* was hampered by the lack of specific and highly affine antibodies. Antibody fragments or short synthetic peptides are interesting alternatives as probes for bacterial detection, as well as for novel therapeutic approaches. They can easily be selected by phage display. In this review, we report on recent achievements on the identification of specific ligands for *L. monocytogenes*

INTRODUCTION

The gram-positive bacterium *Listeria monocytogenes* is the agent of listeriosis, a hazardous foodborne infection. *L. monocytogenes* naturally occurs in agricultural environments, but is commonly found in processed food, especially in ready-to-eat meals and dairy products [1]. It can adapt to a wide range of food processing and storage conditions, including refrigeration temperatures and extreme pH conditions or salt contents [2]. Foodborne listeriosis is a comparably rare disease, but the fatality rate is about 30%, in spite of antibiotics treatment [3]. The European commission notification rate of listeriosis in 2015 was 0.44 cases per 100,000 populations, with steadily increasing tendency since 2012 (European Food Safety Authority & European Centre for Disease Prevention & Control, 2015). Prevention of Listeria contamination is an important food safety challenge and the demand for rapid and specific *L. monocytogenes* detection systems, as well as antimicrobial agents, is steadily rising.

The facultative intracellular bacterium crosses the intestinal barrier and can spread systemically. In addition, it can overcome other host barriers like the placental or the blood brain barrier, leading to fetal infections or meningitis as well as encephalitis [4,5]. The cell biology of *L. monocytogenes* infection was studied intensively in cell lines in recent years. *L. monocytogenes* enters non-phagocytotic cells via the action of its surface proteins called internalin A and internalin B, which are necessary for infection. Interaction with human cell surface proteins trigger internalization via vacuolization. Escape from the host vacuole is mediated by the pore forming activity of a secreted protein, listeriolysin O, and a phospholipase. Once *L. monocytogenes* has reached the cytosol, it can transfer into neighboring cells [6,7].

To reduce the risk of *L. monocytogenes* infection by contaminated food and to prevent food recalls, rapid and accurate methods for on-site detection need to be established. Species specific detection is also of great importance, as of the 17 species in the genus Listeria (comprises next to *L. monocytogenes* five close relatives: *L. innocua*, *L. ivanovii*, *L. marthii*, *L. grayi* and *L. seeligeri* [8]), only *L. monocytogenes* is pathogenic to humans [9]. *L. monocytogenes* comprises 13 serotypes, dependent on variation in the somatic and flagellar antigens, of which three (serotype 4b, 1/2a, 1/2b) are responsible for the majority of human infections [10,11]. Currently, conventional culture methods take 3 to 7 days, a time span too long to test fresh products before consumption. So far, the development of rapid detection methods for *L. monocytogenes* was hampered by the lack of highly affine and species specific antibodies. Despite intensive efforts, no polyclonal or monoclonal antibodies with sufficient selectivity and

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affinity for the detection of *L. monocytogenes* could be developed, and Bhunia suggested, that *L. monocytogenes* either lacks surface epitopes that are both unique and antigenic, or that the epitopes are not effectively processed during antibody production and maturation [12].

Stable antibody fragments, as robust and inexpensive alternative for antibodies, or short synthetic peptides, both selected by phage display, are an interesting alternative as probes for bacterial detection, as well as for novel therapeutic approaches. Antibody fragments or peptides can be easily immobilized on any biosensor surface and have been successfully used for the detection of *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus anthracis* [13-18].

Phages display does, in contrast to antibody production, not rely on antigen processing and presentation. The method was invented in 1985 [19] and has been successfully applied to many areas of research for the identification of small peptide ligands and antibodies with therapeutic and diagnostic purposes [20].

Phage display technology is based on the construction of a polypeptide library fused to a bacteriophage coat protein. Hundreds of libraries where already constructed by different research groups, but the use of commercial libraries, e.g. the New England Biolabs (NEB) Ph.D. phage display libraries, are also common. The phage selection procedure, also referred as biopanning, contains the incubation of the target molecules (that often are immobilized on a solid surface) with a phage display peptide library, the washing of the unbound phages, the elution of the bound phages and the amplification of the eluted phages. Phage particles that bind to the target are captured and enriched in three to four rounds of selection. Rounds of counter- or subtractive selections, e.g. against related bacterial species, can increase the specificity of the resulting peptides, which can be synthesized synthetically after the phage DNA has been sequenced [21-23].

In this review, we focus on the phage display selection of antibody fragments or peptides binding selectively to *L. monocytogenes*. An overview of phage display selected probes is given in **(Table 1)**. The application of oligopeptide M13 phage display in pathogen research and the development of anti-infectives against bacteria, viruses and parasites were already reviewed elsewhere and are not focus of this review [23,24].

Table 1. Overview of *Listeria monocytogenes* specific antibody fragments and peptides.

Title of publication	Target for selection	Phage display library	Species specificity	Selected probe / peptide sequence(s)	Reference
Single-chain Fv antibody with specificity for *Listeria monocytogenes*	Intact live *L. monocytogenes* strain ATCC 19115, subtractive panning with *L. ivanovii* and *L. innocua*	Griffin.1 antibody phage display library	Species specific, detected six of eight strain of *L. monocytogenes*, did not detect any of 15 other bacterial strains	scFv LmP4:A8, sequence not given	[25-29]
Identification and characterization of species-specific nanobodies for the detection of *Listeria monocytogenes* in milk	Intact dead *L. monocytogenes* cells of serotype 4b, subtractive panning with a mixture of eight bacterial species	Naive VHH library, containing 10^7 independent clones, constructed from non-immune alpaca	Recognize three *L. monocytogenes* serotypes (1/2a, 1/2b, 4b), cross reactivity low	Nanobody / VHH antibody, clones L5-79 and L5-79, sequence not given	[30]
Identification of the insulin-like growth factor II receptor as a novel receptor for binding and invasion by *Listeria monocytogenes*	Whole cell cocktails of different serotypes, subtractive selection on *L. innocua* and *L. ivanovii* cocktail	NEB Ph.D. 12mer random phage display peptide library	Specific for *Listeria* spp.	TTSPLSQGSSYI	[31]
Recombinant phage probes for *Listeria monocytogenes*	*L. monocytogenes* strain ATCC 7644	pVIII-9aa and pVIII-12aa: phages display random 9- and 12-mer peptides, fused to the major coat protein pVIII [33]	Not tested	NKKALSPPR QRKLAAKLT RKVALPASK GKKIYMQANFDM MKKVTQVQQVLG RKLYALVPPPAP	[32]
Phage display-derived binders able to distinguish *Listeria monocytogenes* from other *Listeria* species*	Gamma irradiated *L. monocytogenes* strain NCTC 4885 (serovar 4b), subtractive selection with *L. innocua* NCTC 11288	NEB Ph.D. 12mer random phage display peptide library	Specificity described for several clones, tested for *L. innocua*, *Salmonella* spp., *Escherichia coli* and *Campylobacter jejuni*.	GVIYDKPA-KLH GPLATLHLPHKT GPIRDIGPVMDH GRIADLPPLKPN	[34]

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Inhibition of multidrug resistant *Listeria monocytogenes* by peptides isolated from combinatorial phage display libraries	Multidrug resistant strain isolated from cerebrospinal fluid	NEB Ph.D. 12mer random phage display peptide library	Not tested	DQFVHDVKGTKH NSWIQAPDTKSI NHLSTPVWSITG	[35]

*Only the peptides with the highest specificity for *L. monocytogenes* are listed. scFv: single chain variable fragment. VHH: Variable Domain of Heavy Chain Antibodies.

MAIN TEXT

The first report on species specific selection of Single Chain Variable Fragments (scFvs) by phage display was published in 2004 by Paoli et al. A scFv is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids [36]. A pool of random scFvs expressed on the surface of filamentous bacteriophages was used for a solution biopanning with *L. monocytogenes* ATCC 19115 as a target. Strains of *L. ivanovii* and *L. innocua* were used for subtractive rounds of selection. A single phage clone, LmP4:A8, was selected using two independent panning schemes, one with and one without negative selection. The LmP4:A8 clone as well as a collection of other clones were tested for their binding specificity against eight strains of *L. monocytogenes*, the other six closely related species of Listeria and nine other bacterial species relevant in food microbiology by an Enzyme-Linked Immunosorbent Assay (ELISA), performed with the scFv fragment representing phages. For several of the clones, including LmP4:A8, binding to one or more strains of *L. monocytogenes* without cross-reactivity towards any other species in the panel could be demonstrated [25].

In 2007, a study was published in which the specificity of the phage displayed scFv LmP4:A8 was characterized in more detail. An ELISA, again performed with the scFv fragment representing phages, was used to investigate the binding selectivity for 26 strains of *L. monocytogenes* and 15 other Listeria spp. The phage displayed scFv showed absolute specificity for *L. monocytogenes*, and 21 of 26 *L. monocytogenes* strains were detected. To ensure that the surface target of LmP4:A8 was also expressed in other media than Brain-Heart Infusion Medium (BHI), which was used for selection, the binding of LmP4:A8 in other media was investigated for 21 Listeria strains. The specificity for *L. monocytogenes* was maintained, but the detection of *L. monocytogenes* strains varied significantly in different media.

To test whether the antigen for LmP4:A8 was a protein or any other macromolecule, whole-cell extracts of *L. monocytogenes* were subjected to SDS-PAGE and Western blot analysis. Identification of the protein antigen would allow more directed approaches to select antibodies or other binding probes. LmP4:A8 detected a protein with an apparent mass of about 90 kDa. Investigation of subcellular fractions showed that the respective protein was found at the cell wall as well as in the culture supernatant [27]. In a later publication, the protein was revealed to be ActA, an *L. monocytogenes* virulence factor which is expressed on the bacterial cell surface and plays an important role in cell-to-cell movement via actin polymerization [26].

In their next publication, the authors reported on the construction of a plasmid vector that allowed the expression of histidine-tagged biotinylated *L. monocytogenes* specific scFvs (clone LmP4:A8) in *Escherichia coli*. The scFvs were immobilized on streptavidin-coupled Immunomagnetic Beads (IMBs), and their ability to capture and detect *L. monocytogenes* were tested and compared to commercially available *L. monocytogenes* IMBs. Specificity for *L. monocytogenes* as well as capture efficiency was higher for the scFv-IMBs than for the commercially available anti-Listeria-IMBs [28].

Although the expression of *L. monocytogenes* specific scFvs was described in the publication of 2007, Brewster et al. used phages which express the scFv clone LmP4:A8 to develop an *L. monocytogenes* specific biosensor system, based on Surface Plasmon Resonance (SPR). Here, a SPREETA sensor was used with a simple fluidics system to characterize the interaction between by adsorption immobilized phage clones LmP4:A8 and Act A and to detect whole cells of *L. monocytogenes* [29]. SPREETA sensors are compact and inexpensive disposable sensors, which only require a fluidic system and interface electronics. They have already been used successfully for the detection of other bacterial species or their enterotoxins [37,38].

In 2015, Tu et al. described the identification and characterization of *L. monocytogenes* species-specific nanobodies, which were subsequently used for the detection of *L. monocytogenes* in milk. Two novel *L. monocytogenes* specific clones, designated L5-78 and L5-79, were isolated from a phage display antibody library, derived from the variable domain of heavy-chain antibodies (VHHs) of non-immunized alpaca. VHHs are antibody fragments consisting of a single monomeric variable antibody domain, able to bind selectively to a specific antigen. With molecular weights of 12–15 kDa, single-domain antibodies are much smaller than common antibodies. DNA encoding for VHH can easily be manipulated for cloning and expression, whereas the resulting proteins are more heat-resistant and stable towards detergents and high concentrations of urea than regular antibodies [39]. The phage library was first subjected to four rounds of panning on 96-well strip plates, four rounds of panning against heat-killed *L. monocytogenes* cells (serotype 4b) and one round of negative selection, performed on a mixture of eight different bacterial species from other genera. 96 randomly picked clones from the last round were subjected to indirect phage ELISA. The DNA of 38

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clones binding to *L. monocytogenes* was sequenced, and 10 distinct phage clones were tested for their binding activity against *Listeria spp*. and other bacterial genera. The phage clones L5-78 and L5-79 bound to various serotypes of *L. monocytogenes*, but to a much lesser extent to bacteria of other genera. The corresponding coding sequences were subcloned, expressed in *E. coli* and purified. Then, the thermal and urea stability, as well as the pH tolerance were characterized. As the recombinant VHH L5-79 showed more suitable properties, it was used to develop a VHH based *L. monocytogenes* specific sandwich ELISA with a detection limit of 1 x 10^4 CFU/ml [30].

In 2005, Gasanov et al. employed random peptide phage display to identify novel surface antigens for *Listeria spp*. and *L. monocytogenes*. The commercially available Ph.D.-12mer random peptide phage display library was used on immobilized *L. monocytogenes* cells. Unspecific phages were removed by absorbing the phage library on uncoated and BSA-blocked wells. Subtractive selections were performed by the sequential application on immobilized *L. innocua* and *L. ivanovii* cell cocktails. After two rounds of panning, the DNA of 10 single phage clones was extracted. The amino acid sequence for nine of the 10 clones was: TTSPLSQGSSYI. The identified peptide had significant homology with a peptide sequence found within the human Insulin-Like Growth Factor II Receptor (IGFIIR) protein, also known as cation-independent mannose 6-phosphate receptor, indicating that a new receptor for the binding and invasion of Listeria spp. might have been identified. The Fluorescein Isothiocyanate (FITC)-labelled synthetic peptide TTSPLSQGSSYI was bound by *Listeria spp*., but not by bacterial species of other genera, as confirmed by fluorescence spectrometry and FACS studies. Binding seemed to be independent of the two invasion factors InlA and InlB, whereas invasion of MSII and MS9 cells by *L. monocytogenes* deficient in both invasion factors was significantly reduced. Binding of IGFIIR by *Listeria spp.* was additionally confirmed by binding the cells or whole cell extract to magnetic beads, followed by subsequent affinity purification of the native receptor from fetal calf serum. Adherence to and invasion of mammalian cells by *L. monocytogenes* was significantly inhibited by the synthetic peptide and mannose 6-phosphate, which naturally binds to the IGFIIR receptor at the same epitope as the selected peptide [31].

Carnazza et al. made use of two phage displayed random peptide libraries (containing random nonapeptides and dodecapeptides) to select peptide ligands for the cell surface of *L. monocytogenes*. The phage clones were bound to the cells in suspension in four rounds of affinity selection, and phage clone pools from each round were analyzed for their relative binding to *L. monocytogenes* by ELISA. Selected clones from the pVIII-9aa library were found to bind *L. monocytogenes* to a relatively higher degree as from the pVIII-12aa library. The results were confirmed by co-precipitation assays. Eight clones reacted strongly with *L. monocytogenes* and their DNA was sequenced to reveal the amino acid sequences of the displayed peptides. The phages LN6 (RKLYALVPPPAP) and L16 (amino acid sequence not given) reacted strongly with *L. monocytogenes* in the co-precipitation assay and were used to test the possibility for immobilization of the *L. monocytogenes* affine phages to a putative biosensor surface. Both LN6 and LN16 could be immobilized to quartz, with LN6 appeared to bind more efficiently. The successful binding of *L. monocytogenes* to the immobilized phages could be demonstrated by high power optical phase contrast microscopy [32].

In 2013, Morten et al. used a combination of surface, solution and subtractive biopanning to select for *L. monocytogenes* specific binding peptides via phage display, using the NEB Ph.D. 12mer random phage display peptide library. The substraction biopanning was performed against *L. innocua*, the strain genetically closest to *L. monocytogenes* [40]. In the first three rounds, surface biopanning was performed against *L. monocytogenes*. Subsequently, in one of the schemes, subtractive surface biopanning against *L. innocua* was performed. For the other scheme, two rounds of solution biopanning followed, first against *L. innocua*, then against *L. monocytogenes*, respectively. DNA of the selected phages was sequenced after the third round of biopanning against immobilized *L. monocytogenes*, as well as after the last rounds of selection. Subsequent binding evaluations by phage clone ELISA revealed that all phage cloned tested from the pool obtained after the second round of biopanning in solution had higher specificity for *L. monocytogenes* 4b than for *L. innocua*, as well as for *Salmonella spp*., *Escherichia coli* and *Campylobacter jejuni*. Specificity testing of the four clones with highest affinity to *L. monocytogenes* with five other *Listeria spp*. could demonstrate that the phage clone expressing the peptide GRIADLPPLKPN was most specific for *L. monocytogenes*. Only one of the phage clones tested from the other selection pools showed higher relative binding to *L. monocytogenes* than to *L. innocua*. Interestingly, of the four phage clones which bound with highest affinity to *L. monocytogenes* 4b, only one was able to bind the other tested serotypes 1/2a and 1/2c. For some of the phage clones, the representing peptides were chemically synthesized and tested for their binding to *L. monocytogenes* and *L. innocua* by sandwich ELISA, or they were immobilized to dynabeads and tested by magnetic separation in combination with plate counts. All the peptides tested did not show any binding or were not anymore shown to be specific to *L. monocytogenes*.

Flachbartova et al. performed selection with the NEB Ph.D.-12mer phage display library against multidrug resistant *L. monocytogenes* with the aim to select peptides binding to the cell surface, in order to isolate and characterize novel antimicrobial peptides. After four rounds of selection in solution, DNA was isolated from 20 phage clones and sequenced. Ten clones encoded the sequence NHLSTPVWSITG, referred to L1, four clones encoded the sequence DQFVHDVKGTKH, referred to L2, and six clones encoded NSWIQAPDTKSI, referred to L3.

Whereas L1 showed no effect, peptides L2 and L3 inhibited growth of *L. monocytogenes* at a concentration of 30 μM and

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had a bactericidal effect at concentrations of 200 μM. Both had a significant effect on energy metabolism, as they caused a significant decrease in ATP levels. Both peptides did not exhibit any toxic effect to eukaryotic cells, as measured by XTT assay, nor did they show any hemolytic activity. Determination of the physicochemical properties showed a positive net charge for L2, a neutral net charge for L3, and a total hydrophobic ratio of 25 and 33 %, respectively. A positive net charge for antimicrobial peptides is necessary for the binding of the negatively charged membrane [41,42]. Both peptides shared sequence similarities with other antimicrobial peptides (each about 35 %) [34].

CONCLUSION

Listeriosis represents a major public health problem. It is fatal in 30% of the reported cases despite treatment with antibiotics [3]. During recent years, the incidence of Listeriosis is steadily increasing [43] and in addition, multidrug resistant strains of *L. monocytogenes*, the agent of Listeriosis, were described [44-47]. Microbial testing plays an important role in ensuring food safety. The rapid and species specific monitoring of foodborne pathogens is the most effective way to prevent human infections [48]. Currently, time consuming culture-based methods are the gold standard, but lots of efforts are spent on the development of rapid detection methods, suitable for on-site detection. More advanced methods include Polymerase Chain Reaction (PCR), DNA hybridization or methods with rely on bioselective probes to capture and detect bacteria or other indicating biomolecules, e.g. ELISA, immunomagnetic separation or biosensor techniques [48].

Despite intensive efforts [49-53], no polyclonal or monoclonal antibodies with sufficient selectivity and sensitivity for the detection of *L. monocytogenes* could be developed. High sensitivity is obligate because the number of bacteria likely to be present in food is very low, specificity is critical because bacteria of the genus Listeria are widely distributed in the environment, but only *L. monocytogenes* is pathogenic to humans [9,27]. Antibody fragments and specific peptides, possibly presented on phage clones, can be an attractive alternative for antibodies and offer durability, stability, and low-cost standardized production [54-56].

Both, antibody fragments as well as peptides, can be easily selected via phage display, and the method has been successfully applied in many different areas of research, including cancer research, Alzheimer's research and infectious diseases [23,57,58].

With respect to the selection of antibody fragments or peptides to bind to *L. monocytogenes*, in all the articles reviewed here whole cells were used as targets. Cell surfaces are composed of a complex mixture of lipids, carbohydrates and proteins [59,60], and *L. monocytogenes* comprises 13 serotypes, dependent on variation in the somatic and flagellar antigens. To ensure specificity of the resulting probes, counter- or subtractive selection was applied in nearly all phages display schemes described in the literature reviewed here [25,30,31,33]. In the articles published by Carnazza and colleagues and Flachbartova at al., no subtractive rounds of selection were performed, nor were the resulting probes tested for their specificity [32,34]. Although biopanning is a simple process, the outcome is strongly dependent on multiple factors like counter selection, nature of the target, composition of the library and experimental parameters [23]. The resulting probes have to be carefully tested for their specificity, as phage libraries contain a large population of phages, which bind to different materials used in the panning procedure, like plastic, streptavidin, bovine serum albumin or other blocking materials etc., and false-positives need to be eliminated strictly. One point of importance is the affinity of the resulting probes. In tendency, peptides are less affine than antibodies, their KD within the micromolar range. As long as the probes are represented by phages, the avidity effect plays a role, as the probes are presented more than once, depending on the phage protein they are fused to. In the NEB Ph.D. peptide libraries, the peptides are fused to the phage coat protein III and are represented 5-fold. In the library used by Carnazza et al., the peptides are fused to the coat protein pVIII and are represented in 150 to 300 copies. If the represented peptides are synthesized, the avidity effect gets lost and the affinity can be significantly reduced. Interestingly, in the majority of articles reviewed here binding characterization of synthesized peptides is not demonstrated.

L. monocytogenes affects preferentially individuals with deficient immune system, pregnant woman, the youngest and the elderly, with fatal consequences in 30% of the cases [3]. The upcoming of multidrug resistant clinical and food borne isolates enforces the need of novel therapeutic strategies. *L. monocytogenes* is highly susceptible to antimicrobial peptides, which paves the way for the development of new therapeutic strategies or food preservatives. Growth of the bacteria is strongly inhibited by human defensins as well as some plant derived peptides [61]. Antimicrobial peptides have a non-specific mechanism of action, often resulting in cell wall disruption or inhibition of cell wall related processes, or inhibition of nucleic acid production, protein production or enzymes [62-65]. Some antimicrobial peptides, e.g. Leucocin A, which belongs to the class IIa bacteriocins, bind *L. monocytogenes* with higher affinity than other gram-positive bacteria, and where already used as bio-recognition elements in biosensor platforms for real-time bacterial detection [66].

Flachbartova et al. used combinatorial phage display to isolate peptide binding to the surface of a *L. monocytogenes* multi-resistant clinical isolate. Surprisingly, despite selection only for surface binding, two of three selected peptides, which do not show any clear consensus sequence pattern amongst each other, showed antimicrobial activity. The mode of action of the peptides as well as the binding epitope on the cell surface would be highly interesting, but are not revealed yet.

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