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DEVELOPMENT OF A LATERAL FLOW ASSAY FOR THE DETECTION OF *Salmonella* BASED ON ANTIBODIES AGAINST PEPTIDES OF THE EFFECTOR PROTEIN SipC OF THE TYPE III SECRETION SYSTEM OF *Salmonella* spp.

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KEYWORDS

Salmonella

SipC

Effectors protein

translocation

Immunoassay

lateral flow assay

ABSTRACT

Present study was the first promising steps in developing a diagnostic procedure for detecting a *Salmonella* infection or contamination. The areas of application are human medicine, veterinary medicine and various industrial sectors. The development was based on the finding that high antigen areas are formed in the effectors protein SipC. These areas are used to generate antibodies against the synthetically produced peptides from 15-17 amino acids. By using a non-competitive lateral flow assay (FLA) developed under laboratory conditions, a *Salmonella* contamination could be successfully detected by the SipC protein released into the culture medium.

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1 Introduction

Salmonellosis are one of the most frequent bacterial infections worldwide and are currently the second most frequently reported zoonosis infection in humans in the European Union with a total of 95,548 confirmed illnesses from 27 member states (EFSA, 2013). Based on the number of illnesses cases as well as for reasons of consumer protection, there is still a great need for rapid and safe procedures to detect *Salmonella*. All test systems available in market till date, are associated with various prominent restrictions like the method cascade on classic microbiology, in case of doubt, additional serological differentiation in the form of slide agglutination is very time and material intensive. For alternative detection procedures, not all of the approximately 2,600 *Salmonella* serovars known to date can be reliably detected. Additional confirmations are sometimes necessary because some time testing procedure may produce false-positive or false-negative results, no reliable distinction can be made between an acute infection and one that has already been overcome, or there is only conditional practical suitability due to the high costs and limited possible uses by a low number of specialised laboratory service providers. (D'Aoust, 1981; Arvanitakis, 2010; Odumeru & León-Velarde, 2012).

The developed method should therefore detect the most *Salmonella* serovars possible whilst at the same time having a simpler, quicker and cost-effective application. The test system must be based on a property of *Salmonella* that is common to all serovars. *Salmonella* releases various proteins into the surrounding medium under in vitro culture conditions. One of these proteins is SipC protein, which in conjunction with the type III secretion system, acts both as membrane anchored translocation and as a translocated effector protein in the host cell (Scherer et al., 2000; Srikanth et al., 2011; Ziganirova et al., 2012). The nucleotide sequence of this SipC gene is well-known and also available at the gene database (Figure. 1). The type III secretion system used by various Gram-negative bacteria is similar in structure, but the effector proteins secreted are specific for each species. The sequence of the SipC protein is a highly conserved gene, i.e. the amino acid sequences of different *Salmonella* serovars have only slight differences. In addition, the genetic difference between the proteins of other species with similar function (e.g. the *Shigella* IpaC effector protein) is rather high and the sequence identity of the *Salmonella* Sip proteins with the *Shigella* Ipa proteins ranges from only 25% (SipA versus IpaA) to 40% (SipD versus IpaD, and intermediate among them SipC versus IpaC) (Galán, 1996; Suárez & Rüssmann, 1998).

Furthermore, *Salmonella* already forms the type III secretion system proteins at very early stage of infection. Collazo & Galán (1997) established that this secretion process is not necessarily linked to translocation, rather that SipC was also released into the surrounding medium, and discovered that only 10% of the SipC was transported into the host cell. The largest proportion of SipC had already been found in non-

attached bacteria (35%) and in the infection medium (37%). This result was confirmed by Daefler (1999) in his experiments on the study of the secretion of *Salmonella* ser. typhimurium in cell culture supernatants.

In present study, complete SipC protein was not discussed, but five partial sequences, individual peptides of this protein was assessed in present study. Due to the generation of antibodies against the selected sequences of the SipC protein, more options open up to develop a functional immunological detection system using a combination of individual antibodies.

2 Materials and Methods

2.1 Ethics statement

All animal experiments were performed according to guidelines of the German Law for Animal Protection and with the permission of the local ethics committee and the local authority LALLF (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern) under permission AZ LVL M-V/TSD/7221.3-2-018/04.

2.2 Antibodies (Abs)

To detect SipC from culture supernatants, Enzyme Linked Immunosorbent Assay (ELISA) and a polyclonal antibody (pAb) methods are available for research purposes and acted as the primary antibody against SipC (antibodies-online GmbH), and anti-Rabbit-IgG (Sigma Aldrich Co. LLC.) was used as a secondary AB. Monoclonal antibodies (mAb) for the lateral flow assay were produced in collaboration with Biometec GmbH, Greifswald. The antibodies which used in present study are mAb P1, mAb P2, mAb P3, mAb total SipC. In addition, the following commercially available antibodies were included for the lateral flow assay: mAb against the total SipC (antibodies-online GmbH), anti-Mouse-IgG (Sigma Aldrich). The antibodies were used to form the test line (T) and were also conjugated with 40nm colloidal gold particles (OD 50). The control line (C) was generated using anti-Mouse-IgG.

2.3 Bacterial strains and pre-enrichment

Seven liquid standard culture media for the pre-enrichment of *Salmonella* i.e. lactose broth (LB), casein-soya-peptone broth (Caso), nutrient broth (NB), buffered peptone water (BPW), Ringer's solution with Brilliant Green (RiB), Brilliant Green with water (WB), skimmed milk with Brilliant Green (SMB) were used to test the ideal secretion of SipC (D'Aoust, 1981). The *Salmonella* test strains used for this experiment were adapted to hygienically relevant serovars. Furthermore, additional representatives of the *Enterobacteriaceae* family as well as non-*Enterobacteriaceae* strains (Table 1) were selected which likewise form a type III secretion system to some extent (*Yersinia*, *Shigella*, *Escherichia*, *Pseudomonas*).

Table 1 selected test strains for the detection of the secretion of SipC in pre-enrichment media.

Serial Number	Serovar (ser.) or species	Name of the strain
<i>Salmonella</i>		
1	<i>Salmonella enterica</i> ser. Gallinarum	ATCC 9184, DSM 4883
2	<i>Salmonella enterica</i> ser. Choleraesuis	ATCC 13312, DSM 14846
3	<i>Salmonella enterica</i> ser. Typhimurium	ATCC 14028, DSM 19587
4	<i>Salmonella enterica</i> ser. Senftenberg	ATCC 43845, DSM 10062
5	<i>Salmonella enterica</i> ser. Enteritidis	ATCC 13076, DSM 17420
6	<i>Salmonella enterica</i> ser. Heidelberg	ATCC 8326, DSM 9379
7	<i>Salmonella arizonae</i> ser. 51:Z ₄ ,Z ₂₃ :-	ATCC 13314, DSM 9386
Other <i>Enterobacteriaceae</i>		
8	<i>Escherichia coli</i>	ATCC 25922, DSM 1103
9	<i>Citrobacter freundii</i>	ATCC 8090, DSM 30039
10	<i>Shigella sonnei</i>	ATCC 29930, DSM 5570
11	<i>Yersinia pseudotuberculosis</i>	ATCC 29833, DSM 8992
Non <i>Enterobacteriaceae</i>		
12*	<i>Pseudomonas aeruginosa</i>	ATCC 9027, DSM 1128
13**	<i>Staphyococcus aureus</i>	ATCC 25923, DSM 1104

ATCC = American Type Culture Collection, DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, * no application in the LFA, **no application in the ELISA

An initial bacterial count of approximately 50 CFU was transferred into a 20ml pre-enrichment medium and incubated with agitation for up to 20 hours at 37°C (150 rpm). The culture supernatants were removed at different times, beginning with after 8 hours, centrifuged off (3500 x g, 30 minutes, 4°C) and each 50µl of the sterile-filtered supernatant bound on an ELISA plate.

The pre-enrichment of the culture for use in the lateral flow assay took place exclusively in casein-soya-peptone broth over 20 hours, in keeping with the aforementioned conditions.

However, the broth was not centrifuged or sterile-filtered but was directly applied. As the LFA test was carried out over various days, the broth was stored at -18°C until use where necessary.

2.4 ELISA for the detection of the secretion of SipC in pre-enrichment media

The wells of a microtitre plate were coated with 50µl of the undiluted culture supernatant and incubated overnight at 4°C (approx. 14 hours).

10	20	30	40	50	60
MLISNVGINP	AAYLNNHVE	NSSQTASQSV	SAKDILNSIG	ISSSKVSDLG	LSPTLSAPAP
70	80	90	100	110	120
GVLQTPTGTI	TSFLKASIQN	TDMNQDLNAL	ANNVTTKANE	VVQTQLREQQ	AEVGKFFDIS
130	140	150	160	170	180
GMSSSAVALL	AAANTLMLTL	NQADSKLSGK	LSLVSFDAK	TTASSMMREG	MNALSGSISQ
190	200	210	220	230	240
SALQLGITGV	GAKLEYKGLQ	NERGALKHNA	AKIDKLTTES	HSIKNVNLNGQ	NSVKLGAEV
250	260	270	280	290	300
DSLKSLNMKK	TGTDATKNLN	DATLKSNAQT	SATESLGIKD	SNKQISPEHQ	AILSKRLESV
310	320	330	340	350	360
ESDIRLEQNT	MDMTRIDARK	MQMTGDLIMK	NSVTVGGIAG	ASGQYAATQE	RSEQQISQVN
370	380	390	400		
NRVASTASDE	ARESSRKSTS	LIQEMLKTME	SINQSKASAL	AAIAGNIRA	

Figure 1 Amino acid sequence of the SipC protein of *Salmonella* ser. Typhimurium (ATCC 700720), based on UniProt, accessed on 05.02.2015.

Subsequently, the plate was washed with 200µl of wash buffer in three separate wash cycles and blocked with 100µl 3% skimmed milk powder in PBS at room temperature for one hour. Three new wash cycles subsequently followed. The primary antibody used was pAb against SipC, 100µl of which was transferred into each well of the plate in a dilution of 1:2000 in PBS with 0.5% BSA. The incubation time was one hour at 37°C in a humidity chamber. Another three wash cycles (see above) followed to remove all unbound elements, if possible, from the wells of the microtitre plate. Subsequently, the secondary antibody, 50µl anti-rabbit-IgG-POD, was pipetted 1:2000 in PBS with 0.5% BSA.

This was again incubated with a subsequent washing process. In the next step, each deposit was filled with 100µl TMB solution and incubated for 20 minutes at room temperature. The enzyme-substrate reaction was stopped with 100µl 1 mol l⁻¹ H₂SO₄. The absorbance of the individual wells was measured at a wavelength of 450nm at a reference wavelength of 630nm on a standard fluorescence reader (Raem & Rauch, 2007).

2.5 Set-up of the lateral flow assay

The Abs were manually immobilised using a 1µl pipette onto a nitrocellulose membrane (MDI, CNPF-SN12 L2-H50, 10µm). The spacing between the test and control lines was approximately 5mm. The membrane was subsequently dried for 15 minutes at 37°C. On the upper strip of the plastic card, approximately 2mm was fixed overlapping the sample pad (MDI, GFB-R7, 0.6µm, glass fibre). The absorbent pad (MDI, AP-080, 0.8mm, cellulose) was attached to the lower adhesive surface. The membrane prepared in this way was cut into an approximately 5mm wide strip. The finished strip was subsequently inserted into a plastic cassette (MDI, device 3). To start the test, 100µl of the analyte was mixed in a micro-reaction vessel (Eppi) with 20µl gold-labelled antibodies and 100µl of this conjugate was transferred into the specimen well provided in the plastic cassette. The formation of the control line appeared shortly after it was crossed (after approx. 2 minutes). As a clear formation of the test line took different

lengths of time (2 to 10 minutes), the test result was read after 10 minutes had passes.

2.6 Statistics

The ELISA tests took place in a three-fold determination. The assessment was calculated by first calculating the mean value of X_N and the standard deviation SD of the negative controls. To classify a sample as positive, its value must be equal to or greater than the mean value of the negative controls plus the three-fold standard deviation (positive $\geq X_N + 3 \times SD$). A sample was classified as uncertain when its value was equal to or greater than the mean value of the negative controls plus the two-fold standard deviation (uncertain $\geq X_N + 2 \times SD$) and less than the mean value of the negative controls plus the three-fold standard deviation (uncertain $< X_N + 3 \times SD$). A sample was classified as negative when its value was less than the mean value of the negative controls plus the two-fold standard deviation (negative $\geq X_N + 2 \times SD$).

The LFA was set up and the test carried out in duplicate on different days and each showed the same results. The tests were assessed visually. The test was deemed functional when after 10 minutes a clear reddish-purple colour appeared at the height of the control line. A test was assessed as positive when within 10 minutes a clear reddish-purple colour appeared both at the height of the test line and at the height of the control line. A test was assessed as negative when no clear reddish-purple colour appeared at the height of the test line and a clear reddish-purple colour was indicated at the height of the control line within 10 minutes.

3 Results and Discussion

When considering the structural analysis of the amino acid sequence of the SipC protein, five high antigen areas were determined by using the numerical methods of Parker et al. (1986), Karplus & Schulz (1985) and Kyte & Doolittle (1982) (software: PCOM by CoshiSoft/PeptiSearch Arizona USA). The following amino acid sequences were identified:

Peptide 1 (P1):	Protein segment 363-378 with the sequence VASTASDEARESSRKS
Peptide 2 (P2):	Protein segment 15-30 with the sequence NNHSVENSSQTASQSV
Peptide 3 (P3):	Protein segment 343-357 with the sequence GQYAATQERSEQQIS
Peptide 4 (P4):	Protein segment 276-289 with the sequence LGIKDSNKQISPEH
Peptide 5 (P5):	Protein segment 246-260 with the sequence LNMKKTGTDATKNLN

Table 2 Detection of SipC from culture supernatants of different culture media; summarising presentation, derived from measured ELISA absorptions at a wavelength of 450nm/630nm (– no SipC can be detected, + three-fold absorption via the blank, ++ more than 10-fold absorption via the blank).

	LB	Caso	NB	BPW	RiB	WB	SMB
<i>Salmonella enterica</i> ser. Gallinarum	–	++ 20h	++ 20h	++ 20h	–	–	–
<i>Salmonella enterica</i> ser. Choleraesuis	+ 20h	+ 20h	+ 10h ++ 20h	+ 10h ++ 20h	–	–	–
<i>Salmonella enterica</i> ser. Typhimurium*	+ 12h	+ 11h ++ 20h	+ 12h ++ 13h	+ 13h ++ 20h	–	–	–
<i>Salmonella enterica</i> ser. Senftenberg	+ 20h	++ 20h	++ 10h	+ 10h ++ 20h	–	–	–
<i>Salmonella enterica</i> ser. Enteritidis	++ 20h	++ 20h	+ 10h ++ 20h	+ 9h ++ 10h	–	–	–
<i>Salmonella enterica</i> ser. Heidelberg	+ 20h	++ 20h	++ 10h	+ 10h ++ 20h	–	–	–
<i>Salmonella arizonae</i> ser. 51:z ₄ ,z ₂₃ :-	+ 20h	++ 20h	+ 9h ++ 10h	+ 10h ++ 20h	–	–	–
<i>Escherichia coli</i> *	+ 10h	–	+ 10h	+ 13h	–	–	–
<i>Citrobacter freundii</i>	–	–	–	–	–	–	–
<i>Shigella sonnei</i>	–	–	–	–	–	–	–
<i>Yersinia pseudotuberculosis</i>	–	–	–	–	–	–	–
<i>Pseudomonas aeruginosa</i>	–	–	–	–	–	–	–

*For these test strains, the absorptions were measured hourly (beginning after 8 hours, ending after 20 hours); for all other test strains, measurement was taken after 8 hours, 9 hours, 10 hours and 20 hours.

Using affinity chromatography, specific antibodies (Ab) against these peptides were isolated from rabbit serums and tested for their suitability using different methods e.g. ELISA. As a result, three of the five Abs (against P1, P2 and P3) were classified as highly promising. To be able to fall back on specific antibodies in a sufficient concentration over the course of development, monoclonal Abs (mAbs) were also produced.

In parallel, best suited media for *Salmonella* cultivating was also investigated with regard to an optimum secretion of SipC. In supernatants from buffered peptone water and nutrient broth, SipC with the highest concentration could be detected (Table 2). These nutrient-rich media support intensive *Salmonella* growth. In addition, bacteria damaging pH value fluctuations are stopped due to the presence of buffer substances. SipC could frequently be detected from this media after only 10 to 12 hours. The disadvantage of these media was that cross reactions with *Escherichia coli* (*E.coli*) appeared. Either the pAb used led to non-specific binding on the proteins contained in the supernatant or *E.coli* secretes a SipC-like protein into the surrounding environment. Homologies to date, however, have only been described on the effector proteins of the Gram-negative bacteria *Shigella* ssp. (IpaC) and *Yersinia* ssp. (YopQ) (Galán, 1996; Suárez & Rüßmann, 1998). Research using Basic Local Alignment Search Tool (BLAST), showed similarities to the invasion protein (31.8%) which, according to Isberg et al. (1987), is localised to the cell membrane, and to a non-defined protein (37.7%) from *E.Coli*.

However, the absorptions did not continue to increase over the test duration of 20 hours. It is possible that the pAb used against the SipC total protein allows for non-specific reactions due to similar epitopes. A possible strategy for avoiding cross reactions could lie in pre-enrichment with caso broth. In this medium, although the evidence of the SipC could only be provided at the end of the test period, no cross reactivity with *E.coli* could, however, also be established. Further tests should focus on the pre-enrichment times between 10 and 20 hours as it is in this time frame that the shift from "no detection" to "very high detection" lies.

The pre-enrichment media without nutrients themselves delivered no detectable SipC after 20 hours and neither did the medium with skimmed milk. From the work by D'Aoust (1981), it emerges that the incubation duration for the isolation of *Salmonella* was of greater importance than the pre-enrichment medium used. It is possible that longer incubation would lead to the dispersement of SipC, which however is not relevant for the development of the most rapid possible test system and which thus was not further followed up on.

As a result, the observation of Srikanth et al. (2011) and Daefler (1999) that the secretion and translocation of *Salmonella* can appear independently of each other can be confirmed.

Test performed in present study moreover shows that *Salmonella* secretes the SipC protein into the surrounding medium even in the absence of host cells. Furthermore, it could be established that a selective enrichment following the pre-enrichment, as customary with many alternative *Salmonella* detection procedures, is not necessary.

In practice, a non-competitive LFA should be used to confirm the SipC protein in a sample as evidence of a *Salmonella* contamination. In this test format no special or expensive laboratory equipment are required for detection. LFAs are also easy to operate, easy to assess, have good storage stability and can be produced inexpensively. The limited capacity of LFAs to deliver quantitative results is of secondary importance with regard to rapid *Salmonella* diagnostics, as all *Salmonella* serovars are first of all considered potential pathogens, whereby a quantitative statement is not necessary; rather a "yes/no" answer is sufficient.

When setting up the LFA (see Table 3), the Ab against P3 as a detection Ab in combination with the Ab against P1 and the Ab against the total SipC as a capture Ab showed a clear formation of the test line with all tested *Salmonella* of the *enterica* subspecies (also named Group I) (Figure. 2) and showed no signal with the tested non-target germs.

Likewise, no SipC could be detected in the pre-enrichment with the *Salmonella* subspecies *arizonae* (also named Group IIIa) in Tests 2, 3, 7, 9, 10 and 12. This is presumably based on the fact that the protein was not actually secreted. *Salmonella* species of the *enterica* subspecies are very well researched with regard to their effector proteins and their secretion, whereas there is only little work on Group IIIa. *Salmonella* of Group IIIa are primarily found in cold-blooded and poikilothermic vertebrates and only sporadically lead to infections in mammals and birds. Katribe et al. (2007) found a possible explanation: although *Salmonella arizonae* forms on the intestine epithelial cells in the mouse model, it is unable to cause systematic infections involving SipC. Although scientists have also been able to detect SipC with a structural similarity of 94% in Group IIIa, the protein with corresponding antibodies was detected only in the cell lysate. Under in vitro conditions that allow for the secretion of Group I SipC, the secretion of the SipC from the tested *Salmonella arizonae* strains failed to materialise.

Depending on the selected antibody combinations, difficulties arose in detection in the remaining tests. The cause of the false positive and false negative test results can be traced back to the disruptive effects and must be further investigated in the course of assay optimisation. Due to diverging test signals and the fact that the same sample material with the same concentration was used for all test series, most of the interferences described in literature are excluded (Wood, 1991; Miller, 2004; Tate & Ward 2004). The mAb was produced in collaboration with an experienced manufacturer. The antibody qualities were also tested in advance and only the Abs with the highest affinity for the LFA set-up were used. However, there is a possibility that

interferences were caused due to the molecular-biological structure of the analyte. It is possible that the Ab combinations mAb P1 with mAb P2 may block one another in tests 1 and 4. The epitopes may possibly lie too closely in such a way that, although the detector Ab can bind the SipC from the sample, this antibody-antigen complex cannot however bind to the capture Ab, which results in false negative results from *Salmonella* samples. Peptide P1 and P2 are, however, separated in the primary structure by 332 amino acids, whereas the peptides of the Ab combination that delivered correct test results (mAb P1 with mAb P3) with a distance of only 5 amino acids clearly lie a lot more closely together. As the secondary, tertiary and quaternary structure of the SipC protein is unknown and the SipC has not yet been crystallised or fully analysed with regard to its spatial structure, it is not possible to make any statement on the accessibility of the epitopes in the folded protein. Moreover, the direction in which the ABs bind is unknown. Alpha-helix structures were predicted by Scherer et al. (2000) for amino acids 345 to 407 of the SipC protein, where the sequences for P1 and P3 are also found, using the PROSITE database. Although only few amino acids are separated in the primary structure, the spiral-shaped arrangement could in turn lead to these amino acid sequences lying sufficiently far apart for the binding to Abs P1 and P3. A conclusive analysis of this question is not possible at this time.

Rauch et al. (2005) describe cross reactions in connection with the use of Abs that are directed at a target with conserved amino acid sequences of a protein, the sequence motives of which are also found with other proteins. With regard to P1, a sequence comparison using BLAST showed a similarity with two *E.coli* proteins described, unspecified with regard to their functions, in the amount of 73.3%. They are proteins that had also been found against the total SipC protein. With P2, no conformities with the test strains selected in the test could be found, whereas with P3, homologies to the *E.coli* proteins already established with P1 can also be found (93.3%). The similarities in the peptide sequences, as described above, of the SipC proteins together with the *Shigella* invasion proteins could be a reason for the false-positive signal in *E.coli* in test 9 or in *Shigella sonnei* in test 10. However, as both exact measurement results were achieved without any indication to cross reactions with *Escherichia* and *Shigella* and false-positive and false-negative test signals were also generated, any indication to the cross reactions is not clear in this regard.

What is striking is that a non-specific background colour of the entire membrane appeared in all LFAs in this test series. A possible cause may be the lack of mobility of the labelled antibodies in the membrane or, according to Schneider et al. (2007), even a concentration of gold-labelled antibodies that is too high. Klewitz (2005) demonstrated in his tests that a high concentration of gold conjugate led to non-specific binding with the antibodies immobilised in the test line. This phenomenon is also raised by Rauch et al. (2005). The gold-labelled antibodies appear to bind to the Fc region of the capture Ab, resulting in positive test results even in the absence of the analyte.

Table 3 Set-up of an LFA with AB against P1, P2, P3 and total SipC.

Test	test line	Detector #	<i>Salmonella enterica</i> ser. Gallinarum	<i>Salmonella enterica</i> ser. Choleraesuis	<i>Salmonella enterica</i> ser. Typhimurium	<i>Salmonella enterica</i> ser. Senftenberg	<i>Salmonella enterica</i> ser. Enteritidis	<i>Salmonella enterica</i> ser. Heidelberg	<i>Salmonella arizonae</i> ser. 51:z4,z23:-	<i>Escherichia coli</i>	<i>Citrobacter freundii</i>	<i>Shigella sonnei</i>	<i>Yersinia pseudotuberculosis</i>	<i>Staphylococcus aureus</i>
1	mAB P1	mAB P2	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +
2	mAB P1	mAB P3	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +
3	mAB P1	mAB SipC	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +
4	mAB P2	mAB P1	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +
5	mAB P2	mAB P3	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +
6	mAB P2	mAB SipC	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +
7	mAB P3	mAB P1	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +
8	mAB P3	mAB P2	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +
9	mAB P3	mAB SipC	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: - C: +	T: + (weak) C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +
10	mAB SipC	mAB P1	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: - C: +	T: - C: +	T: - C: +	T: + (weak) C: +	T: - C: +	T: - C: +
11	mAB SipC	mAB P2	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +
12	mAB SipC	mAB P3	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: + C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +	T: - C: +

T = Test line, C = Control line, + = Positive test result, - = Negative test result, # - (AB gold complex, 40nm, OD 50)

Figure 2 LFA, tested with pre-enrichment broth of different bacteria; Ab gold complex (mAb against P3), test line (mAb against P1) and control line (Anti-mouse-IgG).

The view is also supported by manually mixing the analyte with the labelled Ab and the lack of the conjugate pad and could explain the false-positive signals (tests 5, 6 and 11). Moreover, non-specific effects caused by unbound gold particles that are preserved in porous solid phases like cellulose are discussed by Wood (1991). In the course of assay optimisation, the use of a conjugate pad should be considered, sample buffers used and the gold conjugate concentration reduced. However, this procedure requires the associated reduction of the test sensitivity to make detection of the analyte also possible.

Outlook

The LFA was produced manually under laboratory conditions, as was the agglutination of the membrane and pad materials, the cutting of the strip and the application of the test and control lines and the conjugate. The template LFAs do nonetheless offer a basis for further research work. The aim is to validate the LFA upon conclusion of the optimisation work that is still necessary in accordance with DIN EN ISO 16140:2011 by carrying out repeated studies on different real samples with different concentrations in order to determine the detection limits in this respect in comparison with the reference procedure of classic *Salmonella* diagnostics.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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