Fast DNA Serotyping and Antimicrobial Resistance Gene Determination of *Salmonella enterica* with an Oligonucleotide Microarray-Based Assay

Sascha D. Braun¹*, Albrecht Ziegler¹, Ulrich Methner², Peter Slickers¹, Silke Keiling², Stefan Monecke¹, Stefan Monecke¹, Ralf Ehrlich³

1 Alere Technologies GmbH, Jena, Germany, 2 Institute of Bacterial Infections and Zoonoses at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Jena, Germany, 3 Institute for Medical Microbiology and Hygiene, Technical University of Dresden, Dresden, Germany

Abstract

Salmonellosis caused by *Salmonella* (S.) belongs to the most prevalent food-borne zoonotic diseases throughout the world. Therefore, serotype identification for all culture-confirmed cases of *Salmonella* infection is important for epidemiological purposes. As a standard, the traditional culture method (ISO 6579:2002) is used to identify *Salmonella*. Classical serotyping takes 4–5 days to be completed, it is labor-intensive, expensive and more than 250 non-standardized sera are necessary to characterize more than 2,500 *Salmonella* serovars currently known. These technical difficulties could be overcome with modern molecular methods. We developed a microarray based serogenotyping assay for the most prevalent *Salmonella* serovars in Europe and North America. The current assay version could theoretically discriminate 28 O-antigens and 86 H-antigens. Additionally, we included 77 targets analyzing antimicrobial resistance genes. The *Salmonella* assay was evaluated with a set of 168 reference strains representing 132 serovars previously serotyped by conventional agglutination through various reference centers. 117 of 132 (81%) tested serovars showed an unique microarray pattern. 15 of 132 serovars generated a pattern which was shared by multiple serovars (e.g., S. ser. Enteritidis and S. ser. Nitra). These shared patterns mainly resulted from the high similarity of the genotypes of serogroup A and D1. Using patterns of the known reference strains, a database was build which represents the basis of a new PatternMatch software that can serotype unknown *Salmonella* isolates automatically. After assay verification, the *Salmonella* serogenotyping assay was used to identify a field panel of 105 *Salmonella* isolates. All were identified as *Salmonella* and 93 of 105 isolates (88.6%) were typed in full concordance with conventional serotyping. This microarray based assay is a powerful tool for serogenotyping.

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* E-mail: sascha.braun@clondiag.com

Introduction

Salmonellosis caused by salmonellae belongs to the most prevalent food-borne zoonotic diseases throughout the world [1]. Therefore, serotype identification for all culture-confirmed cases of *Salmonella* infection is important for epidemiological purposes. The genus *Salmonella* includes two species: *Salmonella* (S.) enterica and *Salmonella* bongori. The species *Salmonella enterica* is divided into the following six subspecies: S. enterica subsp. enterica (I), S. enterica subsp. salamae (II), S. enterica subsp. arizonae (IIIa), S. enterica subsp. dublinense (IIIb), S. enterica subsp. houtenae (IV) and S. enterica subsp. indica (VI) [2]. The subspecies *Salmonella enterica* subsp. *enterica* (I) includes the most relevant zoonotic pathogens with a global occurrence. A serotyping scheme, proposed by Kauffmann 1934 [3], divides all subspecies into serovars by immunologic analyses of two surface structures, O-polysacharide (O-antigen) and flagellin protein (H-antigen). The Kauffmann-White scheme was expanded from 44 serovars in 1934 to 2,587 serovars currently known [2].

Genes required for the biosynthesis of the O-antigen are organized in the *rfb* cluster [4,5]. Within this cluster, sequences of the sugar transferases are relatively conserved and two genes are responsible for most of the genotypic and phenotypic differences of the 46 *Salmonella* O-serogroups described in the Kauffmann-White scheme. The genes of the O-antigen flippase (*wzx*) and polymerase (*wzy*) are highly variable and specific for their respective serogroup [5,6]. The H-antigen used for serotyping is encoded by two flagellar structure genes; *flIC* (phase 1 flagellin) and *flfB* (phase 2 flagellin). Both genes are highly conserved at their 5' and 3' ends and variable in their central region [7,8]. Most *Salmonella* serovars are diphasic where *flIC* or *flfB* is expressed alternately. Serovars with only one H-phase are considered to be monophasic. Monophasic *Salmonella* could theoretically originate in two different ways. They either might represent ancestral forms which lack the second flagellar antigen and did not yet evolve the necessary switching mechanism. Alternatively, they could be deletion mutants of biphasic salmonellae that have lost either the switching mechanism or the ability to express the second flagellar...
antigen [9]. The genetic switching between these two phases is regulated by the hin gene, coding for a DNA-invertase [10]. This approximately 900-base pair (bp) DNA fragment adjacent to fljB, which specifies the synthesis of the H2 flagellar antigen, can exist in either orientation with respect to fljB. The orientation of the inversion region controls the expression of fljB, i.e., in one orientation the adjacent fljB is expressed and in the opposite orientation fljB is not expressed [11].

As a gold standard, the traditional culture method is used to detect *Salmonella* and, since 2002, ISO 6579 represents a legislative norm for the detection of *Salmonella* [12]. This method includes the non-selective pre-enrichment in buffered peptone water followed by selective enrichment and plating on two solid selective media. Colonies of interest are confirmed biochemically and serologically by agglutination with specific sera. However, the procedure according to ISO 6579:2002 takes 4–5 days to be completed. Additionally, classical serotyping is labor-intensive, expensive, requires highly experienced laboratory staff and more than 250 reagents [13] that are necessary to characterize more than 2,500 *Salmonella* serovars currently listed. Besides, commercially available sera are not standardized and their availability is often limited due to a lack of resources and funding. In contrast, genotyping methods use DNA sequence information for identification. Such sequence information is unique and techniques can easily be reproduced and standardized between different laboratories. For this reason, there is an increasing need for a simple genotyping method that does not require a stock of different sera, but can be performed automatically in high throughput and for which reagents are available worldwide. Different molecular typing systems have been developed to meet this demand, such as multiplex real time PCR [14,15], primer extension [16], microarrays [13,17], DNA sequence approaches [18], bead-based suspension arrays [19,20] and ligation based microarrays [21]. Some recent molecular techniques have the disadvantage that only a small subset of serotypes can be typed whereas other approaches do not provide an antigenic formula compatible with the Kauffmann-White scheme. Some techniques are too expensive and/or labor intensive to be implemented in public health or diagnostic laboratories.

Ballmer et al. (2007) proposed a genotyping microarray for *Escherichia (E.)* *coli* [6]. Using a comparable system we aim to develop a high throughput, economical, array-based system to serotype *Salmonella* via its genotype. The microarray includes 255 different targets to analyze O- and H-phases and assign the genotype to the serovar. The microarray includes 255 different serotyping probes were designed by analyzing all available annotated GenBank sequences (NCBI, http://www.ncbi.nlm.nih.gov/) related to the genes *wzy* and *wzz* as well as *fljC* and *fljB*. Additionally, the genes *manC* (O7, O11, O18, O40, O41), *wbhI* (O41, O62), *wbuR* (O66), and *fljB* (O4) were used to discriminate O-serotypes (Table 2). The probes immobilized on the current array version can discriminate 28 O-antigens: A (O2), B (O4), C1 (O7), C2–C3 (O8), D1 (O9), D2 (O9,46), E1/E4 (O3,10/O1,3,19), F (O11), G (O13), H (O6,14), I (O16), J (O17), K (O18), M (O28), N (O30), O (O35), P (O38), Q (O39), R (O40), S (O41), U (O43), W (O45), Z (O50), O55, O56, O58, O62 and O66. From this set, 19 serogroups (A, B, C1, C2–C3, D1, D2, E1, E4, F, G, H, I, K, M, N, O, P, T, U) were selected on the microarray using the 132 reference serovars (Table 1). Due to the high similarity between the serogroups A and D1, additional probes were designed to discriminate S. *ser. Nitrat* and S. *ser. Enteritidis*. For this purpose, specific probes located in the genes *lygA*, *lygD*, *fljA*, *sefA*, *sefB* and *sefC* were designed to specifically identify *S. ser. Enteritidis* (Table 2). In order to identify S. *ser. Paratyphi* A, probes were designed to target the integron region SSPI, a genomic island next to *cpA* [22]. For the discrimination of S. *ser. Dublin* from S. *ser. Dublin*, the genes *SeD_A1100*, *SeD_A1101* and *SeD_A1102* were used as they code for a conserved putative protein being specific for serovar *Dublin* [23].

Flagellar probes were designed using distinctive antigenic sequences within phase 1 (*fljC*) and phase 2 (*fljB*) genes (Table 2). The following flagellar antigens can be identified on the array: a, b; c, d; e, h; c,n,x,z15; f,g,m,n,t; fgs; h; l,g,F; l; g, s, t; g, m, p; s; g, m, t; g, m, q; g, m, s; g, m, t, s; g, m, p, t; g, m, s, t; g, m, p, t, u; m, f, r, l, n; y, l; z, m, z, z10; z19; z23; z36; z36,38; z38; z39; z4,23; z4,23,25,32; z4,24; z4,24; z4,24; z4,32; z4; z44; z47; z52; z56; z6; z65; z69; z81; z91; 1.11 (AY353292); 1.16 (AY353263); 1.2; 1.4; 1.7; 1.8; 1.9; 1.5; 1.5, 1.5, 1.5, 1.5, 1.7; 1.7; 1.7; 1.7; 1.7; 1.7; c,n,x,z15, c,n,x,z15; k, l; l;z,13,23; z10; z33; z39; z41; z50 and z6. Additionally, probes specifying *inv1* [24], *galf* (this study) and *manC* (this study) that were introduced to confirm the identity of *Salmonella* and to serve as controls. These controls were always positive.
| Species         | Serovar       | Strain          | Results of classical Serotyping | Results of microarray based Serotyping | Unique Pattern | Pattern similar to Serovars |
|-----------------|---------------|-----------------|---------------------------------|----------------------------------------|----------------|----------------------------|
| S.e. enterica   | Paratyphi A   | CDC1             | A (O:2) 1,2,12:a[1,5]           | A (O:2)                                 | +/+           | Yes                        |
| S.e. enterica   | Nitra         | CDC1280          | A (O:2) 2,12g,m,-               | A (O:2)                                 | +/+           | No                         |
| S.e. enterica   | Kiel          | CDC09-1879; CDC674 | A (O:2) 1,2,12,g,p,-            | A (O:2)                                 | +/+           | No Dublin, Naestved, Moscow |
| S.e. enterica   | Koessen       | CDC2417          | A (O:2) 2,12,l,v:1,5            | A (O:2)                                 | +/+           | No Panama                  |
| S.e. enterica   | Abony         | CDC102; DSM4224  | B (O:4) 1,4,[5],12,27,b:e,n,x   | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Paratyphi B   | CDC3             | B (O:4) 1,4,[5],12,b:1,2        | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Wien          | SGSC2528         | B (O:4) 1,4,12,27,b,l,w         | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Jericho       | CDC621           | B (O:4) 1,4,12,27,c:e,n,z15     | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Duisburg      | SGSC2472         | B (O:4) 1,4,12,27,d,e,n,z15     | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Schwarzengrund | CDC1629; SGSC2514 | B (O:4) 1,4,12,27,d:1,7        | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Stanley       | CDC000477; SGSC2517 | B (O:4) 1,4,[5],12,27,d:1,2    | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Chester       | CDC17            | B (O:4) 1,4,[5],12, e,h,e,n,x   | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Reading       | CDC19; SGSC2510  | B (O:4) 1,4,[5],12, e,h:1,5     | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Saintpaul     | CDC108           | B (O:4) 1,4,[5],12, e,h:1,2     | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Sandiego      | CDC18            | B (O:4) 1,4,[5],12, e,h,e,n,z15 | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Derby         | CDC20            | B (O:4) 1,4,[5],12, f,g:1,2     | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Agona         | CDC1636          | B (O:4) 1,4,[5],12, f,g,s:1,2   | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | California    | CDC1109          | B (O:4) 4,12, g,m,t:z67        | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Budapest      | CDC23            | B (O:4) 1,4,12,27,g,-          | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Travis        | CDC990318        | B (O:4) 4,12, g,z51:1,7        | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | 1,4,[5],12:z- | CDCQA126; NRL688; NRL813 | B (O:4) 1,4,[5],12:z-       | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Agama         | CDC513           | B (O:4) 4,12:z:1,6             | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Gloucester    | CDC443           | B (O:4) 1,4,12,27:z,l,w        | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Typhimurium   | CDC14; DSM10506; DSM17058; DSM17058; DSM19387; DSM354; LT2 | B (O:4) 1,4,[5],12:z:1,2 | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Brandenburg   | CDC2519; SGSC2460 | B (O:4) 4,12, z:1,2, v:e,n,z15 | B (O:4)                                 | +/+           | Yes                        |
| S.e. enterica   | Bredeney      | CDC112           | B (O:4) 1,4,12,27:l,v:1,7      | B (O:4)                                 | +/+           | Yes                        |
| Species     | Serovar     | Strain         | Results of classical Serotyping | Results of microarray based Serotyping | Unique Pattern | Pattern similar to Serovars |
|-------------|-------------|----------------|-------------------------------|----------------------------------------|---------------|-----------------------------|
|             |             |                | Serogroup | Antigenic Formula | Serogroup | invA/alg/limC |                  |                          |
| S.e. enterica | Heidelberg | CDC 16; DSM9379 | B (O:4) | 1,4,[5],12:r;1,2 | B (O:4) | +/+/+ | Yes               |
| S.e. enterica | Indiana    | CDC 377; SGGC 482 | B (O:4) | 1,4,12:r;1,7 | B (O:4) | +/+/+ | No | Yes | Kiambu | Indiana |
| S.e. enterica | Kiambu     | CDC 399         | B (O:4) | 1,4,12:r;1,7 | B (O:4) | +/+/+ | No |
| S.e. enterica | Haifa      | SGGC 479        | B (O:4) | 1,4,[5],12:r;1,2 | B (O:4) | +/+/+ | Yes |
| S.e. enterica | Stanleyville | CDC 223; SGGC 2518 | B (O:4) | 1,4,[5],12;[7];1223[1,2] | B (O:4) | +/+/+ | Yes |
| S.e. enterica | Maska      | CDC 349         | B (O:4) | 1,4,12;7;24,1,7;2515 | B (O:4) | +/+/+ | Yes |
| S.e. enterica | Ohio       | CDC 710         | C1 (O:7) | 6,7,14:b,1,l,w | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Choleraesuis | CDC 34; DSM 14846 | C1 (O:7) | 6,7:c,1,5 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Paratyphi C | CDC 33; SGGSC 392 | C1 (O:7) | 6,7,14:6,1;5 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Typhimurium | SGGC 2527       | C1 (O:7) | 6,7,c,1,5 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Kambale    | CDC 1863        | C1 (O:7) | 6,7:d;1,2,1,7 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Livingston | NNR 720         | C1 (O:7) | 6,7,14/d;1,l,w | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Baenderup  | CDC 49          | C1 (O:7) | 6,7,14,e,1,7 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Nola       | CDC 2026        | C1 (O:7) | 6,7,e,h:1,7 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Rissen     | CDC 955         | C1 (O:7) | 6,7,14,f,g,| C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Montevideo | CDC 1904        | C1 (O:7) | 6,7,14,g,m,l;1,2,7 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Singapore  | CDC 010011      | C1 (O:7) | 6,7,k,e,nx | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Thompson   | CDC 00342       | C1 (O:7) | 6,7,14,k,1,5 | C1 (O:7) | +/+/+ | Yes |
| S.e. dianense | Bonn       | CDC 344         | C1 (O:7) | 6,7,14,v,e,nx | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Potsdam    | CDC 876         | C1 (O:7) | 6,7,14,14,v,e,n,1,5 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Kenya      | CDC 497         | C1 (O:7) | 6,7,14,13,e,n,x | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Haelingborg| CDC 586         | C1 (O:7) | 6,7,14,p,1,13 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Oranienburg| CDC 1271        | C1 (O:7) | 6,7,14,m,l;1,57 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Infantis   | CDC 1428        | C1 (O:7) | 6,7,14,r,1,5 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Virchow    | CDC 2688        | C1 (O:7) | 6,7,14,r,1,2 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Bareilly   | NNR 608         | C1 (O:7) | 6,7,14,r,1,5 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Mbundaka   | CDC 1906        | C1 (O:7) | 6,7,14,12,10,e,n,1,5 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Tennessee  | CDC 155         | C1 (O:7) | 6,7,14,22,1,2,7 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Tienba     | CDC 2425        | C1 (O:7) | 6,7,23,5,1,6 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Lille      | CDC 354         | C1 (O:7) | 6,7,14,23,8 | C1 (O:7) | +/+/+ | Yes |
| S.e. enterica | Manhattan  | CDC 122         | C2–C3 (O:8) | 6,8,1,5 | C2–C3 (O:8) | +/+/+ | Yes |
| S.e. enterica | Muenchen   | CDC 54; SGGC 243 | C2–C3 (O:8) | 6,8,1,5 | C2–C3 (O:8) | +/+/+ | Yes |
| Species    | Serovar | Strain                      | Results of classical Serotyping | Results of microarray based Serotyping |
|------------|---------|-----------------------------|-------------------------------|---------------------------------------|
|            |         |                             | Serogroup Antigenic Formula  | Serogroup invAgalRimanC Unique Pattern Pattern similar to Serovars |
| S. enterica | Virginia | CDC189                      | C2–C3 (O:8) 8:d:1,2           | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Kottbus  | CDC52                       | C2–C3 (O:8) 6,8:h:1,5         | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Newport  | CDC2343                     | C2–C3 (O:8) 6,8:20:e,h:1,2    | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Emek     | SGSC2477                    | C2–C3 (O:8) 8:20:g,m:1         | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Kentucky | CDC2590; Eng196b            | C2–C3 (O:8) 8:20:i:z6          | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Lindenburg | CDC334                 | C2–C3 (O:8) 6:8:1:2           | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Blockley | CDC448; Eng23b; Eng24b     | C2–C3 (O:8) 6:8:k:1,5          | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Litchfield | CDC000462              | C2–C3 (O:8) 8:6:1:2           | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Manchester | Eng205b                 | C2–C3 (O:8) 8:6:1:7           | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Breukelen | CDC1699                   | C2–C3 (O:8) 6:8:13:20:8:α,ν,τ:15 | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Goldcoast | NRI852                    | C2–C3 (O:8) 6:8:1:α,w         | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Bovismorbificans | CDC2201              | C2–C3 (O:8) 6:8:20:α,β:1,5    | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Hidalgo | CDC2359                    | C2–C3 (O:8) 6:8:20:α,ν,τ:15   | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Hadar    | CDC347;                   | C2–C3 (O:8) 6:8:20:α,ν,x      | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Istanbul | CDC1466                    | C2–C3 (O:8) 8:20:1:α,ν,x      | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Uno      | CDC1697                    | C2–C3 (O:8) 6:8:20:2:α,ν,τ:15 | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Corvalis | CDC1770                    | C2–C3 (O:8) 8:20:2:23:26      | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Düsseldorf | CDC130                 | C2–C3 (O:8) 6:8:2:24          | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Tallahassee | CDC196                | C2–C3 (O:8) 6:8:2:23:24       | C2–C3 (O:8) +/+/+ Yes                  |
| S. enterica | Gallinarum | CDC74; DSM13674           | D1 (O:9) 1:9,1:2:α            | D1 (O:9) +/+/+ Yes                     |
| S. enterica | Berta    | CDC69                      | D1 (O:9) 1:9,1:2:α,γ,δ:1:5:α | D1 (O:9) +/+/+ Yes                     |
| S. enterica | Miami    | CDC198; SGSC248           | D1 (O:9) 1:9,1:2:α,γ:1:5:1:5 | D1 (O:9) +/+/+ Yes                     |
| S. enterica | Goteborg | CDC696                    | D1 (O:9) 9:12:1:5             | D1 (O:9) +/+/+ Yes                     |
| S. enterica | Typhi    | No. 1C                     | D1 (O:9) 9:12:1:γ:1:5          | D1 (O:9) +/+/+ Yes                     |
| S. enterica | Enteritidis | CDC64; DSM14221; DSM14720 | D1 (O:9) 1:9,12:γ,μ:1:5:α     | D1 (O:9) +/+/+ No Nitra, Blegdam      |
| S. enterica | Blegdam  | CDC90361; CDC68             | D1 (O:9) 9:12:γ,μ,τ:1:5:α     | D1 (O:9) +/+/+ No Nitra, Enteritidis  |
| S. enterica | Dublin   | CDC10-0635; CDC65           | D1 (O:9) 1:9,12:1:5:1:5:α:1:5 | D1 (O:9) +/+/+ No Kiel, Naestved, Moscow |
| S. enterica | Naestved | CDC559; SGSC3612          | D1 (O:9) 1:9,12:γ,ρ,τ:1:5:α     | D1 (O:9) +/+/+ No Kiel, Dublin, Moscow |
| S. enterica | Moscow  | CDC67                      | D1 (O:9) 1:9,12:γ:1:5          | D1 (O:9) +/+/+ No Kiel, Dublin, Naestved |
| Species          | Serovar | Strain   | Results of classical Serotyping | Results of microarray based Serotyping |
|------------------|---------|----------|---------------------------------|---------------------------------------|
| **S. enterica**  | Panama  | CDC73;  | D1 (O:9) 1,9,12l,v:1,5          | D1 (O:9) +/+ +                       |
|                  |         | SGSC2496 |                                 |                                       |
|                  | salamae | DSM9220  | D1 (O:9) 9l,w,e,n,x              |                                       |
|                  | Javiana | CDC146   | D1 (O:9) 1,9,12l:28:1,5          |                                       |
|                  | Ottawa  | CDC1934  | D1 (O:9) 1,9,12z41:1,5           |                                       |
|                  | Franken | CDC2570  | D1 (O:9) 9,12:z6:z67             |                                       |
|                  | Fresno  | CDC1412  | D1 (O:9,46) 9,46:z38:-           |                                       |
|                  | Anatum  | CDC78    | E1 (O:3,10) 3,10(15,15,34):e,h:1,6 | E1 (O:3,10) +/+ +                     |
|                  | Meleagris | NRL737   | E1 (O:3,10) 3,10(15,15,34):e,h:l,w | E1 (O:3,10) +/+ +                     |
|                  | Muengster | CDC79   | E1 (O:3,10) 3,10(15,15,34):e,h:1,5 | E1 (O:3,10) +/+ +                     |
|                  | Amsterdam | CDC07056 | E1 (O:3,10) 3,10(15,15,34):g,m,s:- | E1 (O:3,10) +/+ +                     |
|                  | Westhampton | CDC326 | E1 (O:3,10) 3,10(15,15,34):g,s,t:- | E1 (O:3,10) +/+ +                     |
|                  | Westhampton | CDC326 | E1 (O:3,10) 3,10(15,15,34):g,s,t:- | E1 (O:3,10) +/+ +                     |
|                  | Senftenberg | CDC87; DSM10062 | E1 (O:1,3,19) 1,3,19g,s,t:- | E1 (O:1,3,19) +/+ +                     |
|                  | Westerstede | CDC607 | E1 (O:1,3,19) 1,3,19l,z13:1,2 | E1 (O:1,3,19) +/+ +                     |
|                  | Missouri | CDC2039  | F (O:11) 11g,s,t:-              | F (O:11) +/+ +                       |
|                  | Conneccticut | CDC2392 | F (O:11) 11l.12z3,28:1,5         | F (O:11) +/+ +                       |
|                  | Rubislaw | CDC102; SGSC2511 | F (O:11) 11r:e,n,x | F (O:11) +/+ +                       |
|                  | Mississippi | CDC154 | G (O:13) 1,13,23:b:1,5            | G (O:13) +/+ +                       |
|                  | Havana  | NRL607   | G (O:13) 1,13,23g,s,t:-           | G (O:13) +/+ +                       |
|                  | Idikan  | CDC1690  | G (O:13) 1,13,23:z:1,5            | G (O:13) +/+ +                       |
|                  | Kedougou | CDC1523  | G (O:13) 1,13,23:z:1,6            | G (O:13) +/+ +                       |
|                  | Poona   | CDC1243  | G (O:13) 1,13,23:z:1,6            | G (O:13) +/+ +                       |
|                  | Cubanua | CDC207   | G (O:13) 1,13,23:z:29:-           | G (O:13) +/+ +                       |
|                  | Ajibo   | CDC527   | G (O:13) 1,13,23:z:23:-           | G (O:13) +/+ +                       |
|                  | Indica  | DSM14848 | G (O:13) 6,14:z:6:z:1,5          | G (O:13) +/+ +                       |
|                  | Blijdorp | CDC765   | H (O:6,14) 1,6,14:25c:1,5        | H (O:6,14) +/+ +                     |
|                  | Carrau  | CDC93    | H (O:6,14) 6,14:24:y:1,7         | H (O:6,14) +/+ +                     |
|                  | Granicanaria | CDC2506 | I (O:16) 16z:39:1,6              | I (O:16) +/+ +                       |
| Species | Serovar | Strain | Results of classical Serotyping | Results of microarray based Serotyping |
|---------|---------|--------|-------------------------------|--------------------------------------|
|         |         |        | Serogroup Antigenic Formula    | Serogroup invA galF manC Unique Pattern |
| S.e. enterica | Cerro | CDC90087 | K (O:18) 6,14,18,82,23 (1,5) | K (O:18) +/+/+ Yes |
| S.e. enterica | Pomona | CDC2473A | M (O:28) 28y:1.7 | M (O:28) +/+/+ Yes |
| S.e. enterica | Morocco | CDC694 | N (O:30) 30;1,3z,28e,n;2,15 | N (O:30) +/+/+ Yes |
| S.e. enterica | Ealing | CDC745 | O (O:35) 35g,m,s,- | O (O:35) +/+/+ Yes |
| S.e. enterica | Alachua | CDC352 | O (O:35) 35z,4z,23,- | O (O:35) +/+/+ Yes |
| S.e. enterica | Kasenyi | NRL878 | P (O:38) 38e,h;1.5 | P (O:38) +/+/+ Yes |
| S.e. enterica | Lansing | CDC634 | P (O:38) 38i;1.5 | P (O:38) +/+/+ Yes |
| S.e. enterica | Inverness | CDC171 | P (O:38) 38k;1.6 | P (O:38) +/+/+ Yes |
| S.e. enterica | Gera | CDC1316 | T (O:42) 1,42-z,4,23,1,6 | T (O:42) +/+/+ Yes |
| S.e. enterica | Niederodernitz | CDC2579 | U (O:43) 43,b,- | U (O:43) +/+/+ Yes |
| S. bongori | 66z41; | DSM13774 | O:66 66z41; | O:66 +/+/+ Yes |

*invA, galF and manC are species marker for Salmonella.
*generous gift of Paul Barrow, University of Nottingham Sutton Bonington Campus, UK.
*only genomic DNA of Salmonella Typhi, generous gift of Rene S. Hendrickson, DTU Food, Denmark.
Strains were classically serotyped by the CDC, Centers of Disease Control and Prevention, Atlanta, USA, DSMZ (German Collection of Microorganism and Cell Cultures, Brunswick, Germany), SGSC (Salmonella Genetic Stock Center, Calgary, Canada) and FLI (National Reference Laboratory for Salmonellosis in cattle at the Friedrich-Loeffler-Institute, Jena, Germany).
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Table 2. Summary of the potential function(s) on the basis of classical serotyping of each probe immobilized on the microarray.

| Probe | Potential Function | Probe | Potential Function | Probe | Potential Function |
|-------|-------------------|-------|-------------------|-------|-------------------|
| hp-3001-FL-e,n,x | e,n,x; e,n,x,z15; z6 | hp-3117-FL-l+z39+z52 | z39 | hp-3219-wzx_O35 | O35 |
| hp-3003-FL-e,n,x | 1,5; 1,6; e,n,x; e,n,x,z15; z6 | hp-3118-FL-l+z39+z52 | z52 | hp-3220-wzx_O4 | O4 |
| hp-3004-FL-e,n,x | 1,11,16; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; z | hp-3120-FL-g,z51 | g,z51 | hp-3221-wzx_O4 | O4 |
| hp-3005-FL-e,n,x | 1,11,16; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; z | hp-3121-FL-g,z51 | g,z51 | hp-3222-wzx_O4 | O4 |
| hp-3006-FL-e,n,x | 1,11,16; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; z | hp-3124-FL-e,n,x | e,n,x; e,n,x,z15 | hp-3223-wzx_O4,1+62 | O4,1; O62 |
| hp-3007-FL-e,n,x | 1,5; 1,6 | hp-3125-FL-b | b; z91 | hp-3224-wzx_O4,1+62 | O4,1; O62 |
| hp-3008-FL-e,n,x | 1,5; 1,6 | hp-3126-FL-b | b; z91 | hp-3225-wzx_O50 | O50 |
| hp-3009-FL-e,n,x | 1,5; 1,6 | hp-3127-FL-g,z51 | g,z51 | hp-3226-wzx_O50 | O50 |
| hp-3012-FL-e,n,x | 1,11,16; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; e,n,x; e,n,x,z15; z6 | hp-3129-FL-b | b; z91 | hp-3227-wzx_O55 | O55 |
| hp-3013-FL-e,n,x | 1,11,16; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; e,n,x; e,n,x,z15; z6 | hp-3130-FL-b | b; z91 | hp-3228-wzx_O55 | O55 |
| hp-3014-FL-e,n,x | 1,11,16; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; e,n,x; e,n,x,z15; z6 | hp-3134-FL-z | z6 | hp-3229-wzx_O56 | O56 |
| hp-3015-FL-e,n,x | 1,11,16; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; e,n,x; e,n,x,z15; z6 | hp-3135-FL-z | z6 | hp-3230-wzx_O56 | O56 |
| hp-3016-FL-c | c | hp-3136-FL-z | z69 | hp-3231-wzx_O58 | O58 |
| hp-3017-FL-c | c | hp-3138-FL-z | z | hp-3232-wzx_O58 | O58 |
| hp-3018-FL-d+j | d | hp-3139-FL-z | z | hp-3233-wzx_O6,14 | O6,14 |
| hp-3019-FL-d+j | d | hp-3140-FL-z | z | hp-3234-wzx_O6,14 | O6,14 |
| hp-3020-FL-d+j | d | hp-3141-FL-z | z50 | hp-3235-wzx_O66 | O66 |
| hp-3021-FL-d+j | d; j | hp-3142-FL-z | z; z35 | hp-3236-wzx_O66 | O66 |
| hp-3022-FL-d+j | d | hp-3144-FL-z | z50 | hp-3237-wzx_O7 | O7 |
| hp-3023-FL-d+j | d; j | hp-3145-FL-z | z | hp-3238-wzx_O7 | O7 |
| hp-3024-FL-e,h | e,h | hp-3146-FL-z | z | hp-3239-wzx_O7 | O7 |
| hp-3025-FL-e,h | e,h | hp-3149-FL-l+z39+z52 | z39 | hp-3240-wzx_O8 | O8 |
| hp-3026-FL-e,n,x | e,n,x,z15 | hp-3150-FL-z | z | hp-3241-wzx_O8 | O8 |
| hp-3027-FL-e,n,x | e,n,x; e,n,x,z15 | hp-3152-FL-i | i | hp-3242-wzy_O13 | O13 |
| hp-3029-FL-g,z51 | g,z51 | hp-3153-FL-I+z39+z52 | z; z; z35; z39; z65 | hp-3243-wzy_O18,28_Dakar | O13,28 |
| hp-3032-FL-i+r | i | hp-3154-FL-k+z | z10 | hp-3244-wzy_O16 | O16 |
| hp-3033-FL-i+r | i | hp-3155-FL-z4 | z4,223; z4,224; z4,232 | hp-3245-wzy_O16 | O16 |
| hp-3034-FL-i+r | i | hp-3157-FL-1-e,n,x | 1,11,16; 1,12; 1,2; 1,2,7; 1,5; 1,5,7; 1,5,7; 1,6; 1,7; e,n,x; e,n,x,z15; z6 | hp-3246-wzy_O17 | O17 |
| hp-3035-FL-i+r | r | hp-3158-FL-1-e,n,x | 1,11,16; 1,12; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; e,n,x; e,n,x,z15; z6 | hp-3247-wzy_O17 | O17 |
| hp-3036-FL-i+r | r | hp-3161-FL-1-e,n,x | 1,5; 1,6 | hp-3248-wzy_O18 | O18 |
| hp-3038-FL-k+z | k; z44; z58 | hp-3163-FL-1-e,n,x | 1,11,16; 1,12; 1,2; 1,2,7; 1,5; 1,5,7; 1,6; 1,7; z | hp-3250-wzy_O28_Dakar | O28 serovar Dakar |
| hp-3039-FL-k+z | l,v; z10; z35; z39; z65 | hp-3165-manC | species marker | hp-3251-wzy_O28_Dakar | O28 serovar Dakar |
| hp-3040-FL-k+z | z35 | hp-3166-wbyJ | O41 | hp-3252-wzy_O28_Pomona | O28 serovar Pomona |
| hp-3041-FL-k+z | k | hp-3167-wbyJ | O41 | hp-3253-wzy_O28_Pomona | O28 serovar Pomona |
| hp-3042-FL-k+z | k; z41 | hp-3168-manC-O16+39 | O16; O39 | hp-3254-wzy_O3,10+9,46 | O3,10,9,46 |
| hp-3043-FL-k+z | (k) | hp-3169-manC-O16+39 | O16; O39 | hp-3255-wzy_O3,10+9,46 | O3,10,9,46 |
| Probe   | Potential Function | Probe   | Potential Function | Probe   | Potential function |
|---------|--------------------|---------|--------------------|---------|--------------------|
| hp-3044-FL-z | z41                | hp-3170-manC-O7 | O7               | hp-3256-wzy, O3,10+9,46 | O3,10; O9,46 |
| hp-3045-FL-k-z | z10               | hp-3171-manC-O7 | O7               | hp-3257-wzy, O30          | O30            |
| hp-3046-FL-k-z | z10               | hp-3172-manC-O11 | O11             | hp-3258-wzy, O30          | O30            |
| hp-3047-FL-k-z | z10               | hp-3173-manC-O11 | O11             | hp-3259-wzy, O35          | O35            |
| hp-3048-FL-k-z | a,z10             | hp-3174-manC-O18 | O18             | hp-3260-wzy, O35          | O35            |
| hp-3049-FL-k-z | z10               | hp-3175-manC-O18 | O18             | hp-3261-wzy, O38          | O38            |
| hp-3050-FL-k-z | k, z10            | hp-3176-manC-O41 | O41             | hp-3262-wzy, O38          | O38            |
| hp-3051-FL-k-z | e, z10            | hp-3177-manC-O41 | O41             | hp-3263-wzy, O41+62      | O41; O62       |
| hp-3052-FL-z | z41               | hp-3178-manC-O41 | O41             | hp-3264-wzy, O41+62      | O41; O62       |
| hp-3053-FL-k-z | z10               | hp-3179-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3265-wzy, O50          | O50            |
| hp-3054-FL-k-z | z35               | hp-3180-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3266-wzy, O50          | O50            |
| hp-3055-FL-k-z | z10               | hp-3181-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3267-wzy, O55          | O55            |
| hp-3056-FL-k-z | z35               | hp-3182-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3268-wzy, O55          | O55            |
| hp-3057-FL-k-z | (k)               | hp-3183-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3269-wzy, O56          | O56            |
| hp-3058-FL-k-z | z10               | hp-3184-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3270-wzy, O56          | O56            |
| hp-3060-FL-k-z | k, z41            | hp-3185-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3271-wzy, O58          | O58            |
| hp-3061-FL-k-z | (k)               | hp-3186-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3272-wzy, O58          | O58            |
| hp-3062-FL-l+ | z39               | hp-3187-manC-O13+O30+O43+O45+O50 | O13; O30; O43; O45; O50 | hp-3273-wzy, O6,14        | O6,14          |
| hp-3063-FL-l+ | z39               | hp-3188-manC-O2+4+9+3,10 | O2; O4; O9; O3,10 | hp-3274-wzy, O6,14        | O6,14          |
| hp-3064-FL-l+ | z39               | hp-3189-manC-O2+4+9+3,10 | O2; O4; O9; O3,10 | hp-3275-wzy, O7           | O7             |
| hp-3066-FL-y | y                 | hp-3190-manC-O40 | O40             | hp-3276-wzy, O7           | O7             |
| hp-3067-FL-y | y                 | hp-3191-manC-O40 | O40             | hp-3277-wzy, O8           | O8             |
| hp-3068-FL-y | y                 | hp-3192-rfbV-O2+9+9,46 | O2; O9; O9,46 | hp-3278-wzy, O8           | O8             |
| hp-3069-FL-z29 | z29               | hp-3193-rfbV-O2+9+9,46 | O2; O9; O9,46 | hp-3279-wzy, O18          | O18            |
| hp-3070-FL-z29 | z29               | hp-3194-rfbV-O4   | O4              | hp-3280-SSPAI Paratyphi A |                |
| hp-3071-FL-z | z38               | hp-3195-rfbV-O4   | O4              | hp-3281-SSPAI Paratyphi A |                |
| hp-3072-FL-z | z36; z38          | hp-3196-wpuH-O41+62 | O41; O62 | hp-3282-Q8ZK10 Typhimurum |                |
| hp-3073-FL-z | z36; z38          | hp-3197-wpuH-O41+62 | O41; O62 | hp-3287-lygA Enteritidis |                |
| hp-3074-FL-z | z36; z38          | hp-3198-weib-O66 | O66             | hp-3288-lygD Enteritidis |                |
| hp-3075-FL-z | z36; z38, z38     | hp-3199-weib-O66 | O66             | hp-3289-Q8ZK15 Typhimurium |                |
| hp-3076-FL-z4 | z4,22; z4,22,23,32; z4,22,4; z4,23,2 | hp-3200-wzy, O13 | O13             | hp-3290-tviA plasmid Vi   |                |
| hp-3077-FL-z4 | z4,22             | hp-3201-wzy, O13 | O13             | hp-3292-tviA plasmid Vi   |                |
| hp-3078-FL-z4 | z4,22,3,22         | hp-3202-wzy, O16 | O16             | hp-3293-stgA Typhi        |                |
| hp-3080-FL-z65 | z65               | hp-3203-wzy, O16 | O16             | hp-3294-stgA Typhi        |                |
| hp-3085-FL-g | f,g,t; f,g,t; g,m,p,q; g,m,t; g,z2       | hp-3204-wzy, O17 | O17             | hp-3297-sefB Enteritidis |                |
| hp-3086-FL-g | f,g,t; f,g,t; g,m,p,q; g,m,s; g,m,t; g,t; g,z2 | hp-3205-wzy, O17 | O17             | hp-3298-sefA Enteritidis |                |
if *Salmonella* isolates were tested (Table 1). Further, two probes for the Vi capsular antigen (Table 2) were included and were partly positive for *S. ser.* Paratyphi C and always positive for *S. ser.* Typhi. All tested *S. ser.* Dublin strains were negative for the Vi capsular antigen.

Probes and primers for AMR genotyping of *Salmonella* serovars were derived from a genotyping microarray for *E. coli* that was previously developed, validated and described ([25], http://alere-technologies.com/fileadmin/Media/Paper/Ecoli/Supplement_Geue__layout_E_coli.xlsx).

Table 2. Cont.

| Probe          | Potential Function     | Probe          | Potential Function     | Probe          | Potential Function     |
|----------------|------------------------|----------------|------------------------|----------------|------------------------|
| hp-3087-fb-flg| g: t; g: t; g: m: p: q; | hp-3026-wzx_018| O18                    | hp-3299-sefC   | Entertidis              |
|                | g: m: t; g: t; g: t; g: |                |                        |                |                        |
| hp-3089-flg   | g: t; g: t; g: m: p: q; | hp-3027-wzx_018| O18                    | hp-3300-galF   | species marker         |
|                | g: m: t; g: m: t; g: t; |                |                        |                |                        |
| hp-3090-flg   | g: t; g: t; g: m: p: q; | hp-3028-wzx_029| O2; O9                 | hp-3304-B5G7V7| Dublin                  |
|                | g: m: t; g: m: t; g: t; |                |                        |                |                        |
| hp-3091-flg   | g: t; g: t; g: m: p: q; | hp-3029-wzx_029| O2; O9                 | hp-3302-B5R5L5|                        |
|                | g: m: t; g: m: t; g: t; |                |                        |                |                        |
| hp-3092-flg   | g: t; g: t; g: m: p: q; | hp-3100-wzx_028| O28 serovar Dakar      | hp-3306-B5R7B6| Gallinarum,             |
|                | g: m: t; g: m: t; g: t; |                |                        |                | Weltevreden             |
| hp-3103-flg   | g: t; g: t; g: m: p: q; | hp-3111-wzx_028| O28 serovar Dakar      | hp-3307-B5R7C1|                        |
|                | g: m: t; g: m: t; g: t; |                |                        |                |                        |
| hp-3104-flg   | g: t; g: t; g: m: p: q; | hp-3121-wzx_028| O28 serovar Pomona     | hp-3308-ISR1   | Infantis                |
|                | g: m: t; g: m: t; g: t; |                |                        |                |                        |
| hp-3105-flg   | g: t; g: t; g: m: p: q; | hp-3131-wzx_028| O28 serovar Pomona     | hp-3310-ISR1   | Infantis                |
|                | g: m: t; g: m: t; g: t; |                |                        |                |                        |
| hp-3106-flg   | g: t; g: t; g: m: p: q; | hp-3134-wzx_0310| O3; O10                | hp-3311-Q57Q4  | Choleraeus              |
|                | g: m: t; g: m: t; g: t; |                |                        |                |                        |
| hp-3107-flg   | m: t                    | hp-3125-wzx_0310| O3; O10                | hp-3312-Q57Q4  | Choleraeus              |
| hp-3108-flg   | m: t                    | hp-3126-wzx_0300| O30                   | hp-3314-invA   | species marker         |
| hp-3109-flg   | m: t                    | hp-3127-wzx_0300| O30                   | hp-3315-invA   | species marker         |
| hp-3113-flg+  | z39                    | hp-3128-wzx_0350| O35                   | hp-3316-invA   | species marker         |
| hp-3113-flg+ | z39+z52                |                |                        |                |                        |

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Multiplex linear DNA amplification and labeling for hybridization to prepared ArrayStrips

For multiplex linear DNA amplification, a set of 292 primers (220 serotyping primer and 72 AMR primer, synthesized by Metabion, Martinsried, Germany) was used. These primers are located on the complementary strand, downstream of the sequence of the covalently immobilized oligonucleotide detection probes (the number of probes and primers do not need to be identical, a primer can target a consensus region, while probes might bind to more variable parts close by, which allows discerning different alleles of one gene). The labeling of the genomic DNA was accomplished during the linear amplification step by using dUTP linked biotin as a marker, thereby allowing site-specific internal labeling of the corresponding target region (Fig. 1a). Using the HybPlus Kit (Alere Technologies, Germany), at least 0.5 μg genomic DNA were labeled according to the manufacturer’s instructions. The linear amplification steps included 5 min of initial denaturation at 96 °C, followed by 50 cycles with 20 s of annealing at 50 °C, 40 s of elongation at 72 °C, and 60 s of denaturation at 96 °C. This reaction results in a multitude of specifically amplified, single-stranded, biotin-labeled DNA molecules for subsequent hybridization to the corresponding DNA microarray.

Hybridization of the ArrayStrips

For the hybridization procedures, the HybPlus Kit (Alere Technologies, Germany) was used according to the manufacturer’s instructions with an adapted protocol. This included hybridization buffer C1, washing buffer C2, peroxidase-streptavidin conjugate C3, conjugation buffer C4, washing buffer C5 and peroxidase substrate D1.
First, ArrayStrips were placed in a thermomixer (Quantifoil Instruments, Jena, Germany) and subsequently washed with 200 ml of de-ionized water for 5 min at 55°C/550 rpm and with 100 ml hybridization buffer C1 for 5 min at 55°C/550 rpm. All liquids were always completely removed with a soft plastic pipette to avoid scratching of the chip surface. In a separate tube, 10 ml of the labeled, single-stranded DNA were dissolved in 90 ml hybridization buffer C1. The hybridization was carried out at 55°C, shaking at 550 rpm for 1 h. After hybridization, the ArrayStrips were washed two times for 5 min with 200 ml washing buffer C2 at 45°C, shaking at 550 rpm. Peroxidase-streptavidin conjugate C3 was diluted 1:100 in buffer C4. A total of 100 ml of this mixture were added to each slot of the ArrayStrip, and subsequently incubated for 10 min at 30°C and 550 rpm. Afterwards, washing was carried out two times at 550 rpm with 200 µl C5 washing buffer at 30°C, with each step performed for 5 min. The visualization was achieved by adding 100 µl of peroxidase substrate D1 to the ArrayStrips, and signals were detected with the ArrayMate device (Alere Technologies, Jena, Germany) (Fig. 1b–c).

The described, final protocol was achieved by optimizing hybridization conditions (45°C–58°C) and washing temperatures (45°C–58°C) whereas the concentration of substances and incubation periods for each step were always constant. For this procedure, only strains were used for which published genome sequences (NCBI genome database) allowed to theoretically

**Table 3. Summary of probes detecting antibiotic resistance genes and virulence factors.**

| Probe       | Potential Function        | Probe       | Potential function        |
|-------------|---------------------------|-------------|---------------------------|
| hp_armA_611 | aminoglycoside resistance | hp_ble_611  | bleomycin resistance      |
| prob_aac3la_1 | aminoglycoside resistance | prob_catA1_11 | chloramphenicol resistance |
| hp_aac3_611 | aminoglycoside resistance | prob_catB3_11 | chloramphenicol resistance |
| prob_aac6b_1 | aminoglycoside resistance | prob_catB8_12 | chloramphenicol resistance |
| prob_aadA1_1 | aminoglycoside resistance | prob_cmlA1_11 | chloramphenicol resistance |
| prob_aadA2_1 | aminoglycoside resistance | prob_floR_11 | florfenicol and chloramphenicol resistance |
| prob_aadA4_1 | aminoglycoside resistance | hp_mphA_611 | erthymycin and roxythromycin resistance |
| prob_ant2la_1 | aminoglycoside resistance | hp_ereA_611 | erthymycin resistance      |
| hp_aac6_612 | aminoglycoside resistance | prob_qnrB_12 | fluoroquinolone resistance |
| hp_aac6_615 | aminoglycoside resistance | hp_kpc4_611 | imipenem resistance       |
| hp_aac6_618 | aminoglycoside resistance | hp_qnrD_611 | quinolone resistance       |
| hp_aadB_611 | aminoglycoside resistance | prob_qnr_12 | quinolone resistance       |
| hp_aadB_2_611 | aminoglycoside resistance | prob_qnrS_11 | quinolone resistance       |
| hp_spH_611 | aminoglycoside resistance | prob_sul1_11 | sulfonamide resistance     |
| prob_strA_611 | aminoglycoside resistance | prob_sul2_11 | sulfonamide resistance     |
| prob_strB_611 | aminoglycoside resistance | prob_sul3_11 | sulfonamide resistance     |
| hp_aac3_614 | aminoglycoside resistance | prob_tetA_1 | tetracycline resistance    |
| hp_apHA_611 | aminoglycoside resistance | prob_tetB_1 | tetracycline resistance    |
| hp_blaCMY_611 | beta-lactam resistance | prob_tetC_1 | tetracycline resistance    |
| hp_per2_611 | beta-lactam resistance | prob_tetD_1 | tetracycline resistance    |
| prob_acc1_11 | beta-lactam resistance | prob_tetG_1 | tetracycline resistance    |
| prob_acc2_11 | beta-lactam resistance | prob_dfr12_1 | trimethoprim resistance   |
| prob_cmy_11 | beta-lactam resistance | prob_dfr13_1 | trimethoprim resistance   |
| prob_ctxM1_11 | beta-lactam resistance | prob_dfrA1_21 | trimethoprim resistance  |
| prob_ctxM2_11 | beta-lactam resistance | prob_dfrA1_22 | trimethoprim resistance  |
| prob_ctxM26_11 | beta-lactam resistance | prob_dfrA14_21 | trimethoprim resistance  |
| prob_ctxM9_11 | beta-lactam resistance | prob_dfrA15_1 | trimethoprim resistance  |
| prob_dha1_1 | beta-lactam resistance | prob_dfrA17_1 | trimethoprim resistance  |
| prob_oxa1_21 | beta-lactam resistance | prob_dfrA19_1 | trimethoprim resistance  |
| prob_oxa2_11 | beta-lactam resistance | prob_dfrA7_1 | trimethoprim resistance  |
| prob_oxa7_11 | beta-lactam resistance | prob_dfrA7_12 | trimethoprim resistance  |
| prob_per2_1 | beta-lactam resistance | prob_dfrV_21 | trimethoprim resistance  |
| prob_pse1_1pm | beta-lactam resistance | prob_intl1_1 | integrases                 |
| prob_shv1_11 | beta-lactam resistance | prob_intl2_11 | integrases                 |
| prob_tem1_1 | beta-lactam resistance |                 |                           |

Table: Table 3. Summary of probes detecting antibiotic resistance genes and virulence factors.

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predict hybridization patterns (see Result part). These were strains \( \text{S. ser. Agona (SL483), S. ser. Choleraesuis (SGSA50, SC-B67), S. ser. Dublin (SD3246, CT02021853), S. ser. Enteritis (P125109), S. ser. Gallinarum (287/91, SG9), S. ser. Heidelberg (SL476), S. ser. Infectis (SIN), S. ser. Newport (SL254), S. ser. Paratyphi A (AKU12601, ATCC9150), S. ser. Paratyphi B (SPB7), S. ser. Paratyphi C (RKS4594), S. ser. Schwarzengrund (CV19633), S. ser. Typhii (CT18, Ty2), S. ser. Typhihimurium (1402S8, 27120, D23580, SL1344, T000240, UK-1, LT2) and S. ser. Weltevreden (2007-60-3289-1). These predictions were subsequently compared with the results of real hybridization experiments. The absence and presence of signals at different hybridization temperatures were monitored and the final protocol as described above based on the experiments in which the best accordance between predictions and real hybridizations was observed.

**Processing data using PatternMatch algorithm**

Hybridization signals were processed using the IconoClust software, version 3.2r1 (Fig. 1d). All spots were normalized automatically by the software according to the quotation

\[ NI = 1 - \left( \frac{M}{BG} \right) \]

where \( NI \) is the normalized intensity, \( M \) the average intensity of the automatically recognized spot, and \( BG \) the intensity of the local background. The output range of the signals were between 0 and 1 with 0 being negative and 1 being the maximal possible signal value. A probe-matching matrix was used to construct the theoretical hybridization pattern of the fully sequenced strains listed in NCBI database (Table S1). The definition of the theoretical signal intensity was 0.9 for perfect match, 0.6 for 1 mismatch, 0.3 for 2 mismatches, 0.1 and below for 3 mismatches and no signal for more mismatches. For each of these sequenced strains, at least one reference strain was used to assign the expected pattern with the pattern of the real hybridization experiments. For this operation, the PatternMatch algorithm was used [29]. The final numerical output was given as the matching score (MS), which represents the overall sum of all differences between corresponding signal intensities of theoretical and real hybridization experiments. Thus, the MS value is a measure of overall similarity/dissimilarity between two hybridization patterns. An ideal match of two patterns based on the same set of oligonucleotide probes will yield MS = 0, whereas values above MS = 6.5 require critical scrutiny because they may indicate a poor match. The Delta MS value, defined as the arithmetic difference between best and second best match, served as measure for the accuracy of species identification. A Delta MS higher than 1.5 was considered to be sufficient for an unambiguous distinction between two patterns.

Calculation of similarities was carried out by comparing signals for all 255 probes between theoretical predictions and real experiments. Signals with intensities higher than 0.3, were considered positive and set as "1". Signals lower than 0.3, were regarded negative and set as "0". The number of probe differences was summarized and the percentage was calculated. In order to assess the reproducibility, eight experiments were performed under identical conditions. All experiments were compared to each other using the PatternMatch algorithm and the mean, maximum and minimum MS were calculated.

**Figure 1. Multiplex linear DNA amplification, labeling and hybridization of the ArrayStrips.** (a) Linear Multiplex Amplification starting from clonal RNA free genomic DNA, extracted DNA is internally labeled with biotin (Label [L]) and amplified in a linear multiplex PCR reaction; (b) Hybridization: the biotin labeled, single-stranded DNA product hybridizes specifically under stringent conditions to the corresponding probes. The resulting duplex is detected using a horse-radish peroxidase (Enzyme [E]) – streptavidin conjugate, which converts the substrate (Serumun green [S]) into a colored local precipitate. (c) Detection: the ArrayMate™ Reader (or ArrayTube™ Reader ATR 03) enables the visualization and subsequent automated analysis of the array image. The presence of a dark precipitated spot indicates successful hybridization; (d) Analysis: the assay specific software analysis script, supplied with the ArrayMate™ Reader (or ArrayTube™ Reader ATR 03), measures the signal intensity of each probe and determines with an assay specific algorithm which genes/alleles are present in the sample. (e) Genotype analysis: the PatternMatching software supplied with the ArrayMate™ Reader (or ArrayTube™ Reader ATR 03) is comparing the resulting pattern with a local database including 132 reference serovars previously sero- and genotyped, finally a report is given to which serovar the sample strain belongs with regard to the Kauffman-White Scheme.

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Antimicrobial resistance

All isolates in which AMR genes were detected, a total of 34 Salmonella isolates belonging to 18 serovars, were tested for their phenotypic antimicrobial resistance. This was carried out using the VITEK 2 system with the AST-N111 test panel (bioMerieux Deutschland GmbH, Nürtingen, Germany). Additionally, chloramphenicol (30 μg), kanamycin (30 μg) and streptomycin (10 μg) were tested by disk diffusion assay. This assay was performed using CLSI.

Verification of the assay and database building for PatternMatch

A set of 168 Salmonella strains representing 132 different serovars were used to evaluate the probes printed on the array, the primers in the labeling mixture, and to build a database for identification of the globally most prominent Salmonella serovars. Comparison of predicted and real hybridization results was performed for strains with fully sequenced genomes (see Materials and Methods and Table 4). The similarity between the predicted and real hybridization results of the serogenotyping array was more than 99 percent (Table 4). Because both, the full sequence information of the genome and the antigenic formula of S. ser. Typhimurium strain LT2, were available, an exact comparison of predicted and actual experimental hybridization pattern was possible (Fig. 2). It showed a 100% identity when regarding just positive and negative signals. A more detailed analysis, also considering signal intensities, showed a high degree of similarity between theoretical predictions and actual experiments with exceptions at probe hp-3221-wzx_O4 (signal intensity increased about 42% as predicted by the theoretical experiment) and hp-3292-Q8ZK10 (signal intensity decreased about 43% as predicted by the theoretical experiment). The highest discrepancy was found for S. ser. Paratyphi A and S. ser. Paratyphi B. Analysis of the results of S. ser. Paratyphi B showed that two probes were negative in actual hybridizations compared to the theoretical predictions (Fig. 2). However, the missing probes were redundant for one target gene (e.g., S. ser. Paratyphi B fliC-H1:b), so that this issue did not influence the identification. Because of the high correlation between theoretical predictions and actual experiments, as well as the high similarity of Tm of all 255 serotyping probes, it is assumed that the detection efficiency with other Salmonella serovars will also be comparably precise under the same conditions. Furthermore, the results of these theoretical experiments were used to find an optimal protocol (data not shown) for the hybridization of the Salmonella array so that an optimal, stringent hybridization and washing temperature could be defined (see Methods part).

Using this optimized protocol (as described in Materials and Methods), strains of all 132 Salmonella serovars were analyzed. Each serovar was tested at least three times using the Salmonella array to ensure consistent results and the identification of the unique and reproducible serovar-specific probe patterns. These unique patterns were used to build a PatternMatch database consisting of data from real experiments instead of theoretical experiments from defined strains. A manual serotyping using the probe-function table (Table 2) was restricted by the resolution of probes identify the H2-phase. This phase was mainly a combination of different probes, e.g. H2:1,5 of different serovars was always a combination of different “FL-1+e,n,x” probes (Table 2). Nevertheless it was possible to estimate the Salmonella serotype at least for the serogroup and in most cases for the phase H1. In the end the probe-function table served as a control for classical serotyped Salmonella before they were used in the PatterMatch database.
Table 4. Comparison of theoretical predictions and real hybridization patterns for the *Salmonella* array and previously typed strains (CDC, DSMZ).

| Serovar     | Strain   | Accession No. | Serogroup | Antigenic Formula | Correct Antigenic Formula Designation | Serovar     | Reference Strain | Correct Serovar Designation | Number of Probe Differences between Virtual and Real Hybridization | Similarity in % |
|-------------|----------|---------------|-----------|-------------------|---------------------------------------|-------------|------------------|-----------------------------|---------------------------------------------------------------|----------------|
| Agona       | SL483    | NC_011149.1   | B (O:4)   | 1,4,[5],12,g,gs:{1,2} | YES                                   | Agona       | CDC1636          | YES                         | 0/255                                                          | 100.0          |
| Choleraesuis| SGSA30   | CM001062.1    | C1 (O:7)  | 6,7,c:1,5         | YES                                   | Choleraesuis| DSM14846         | YES                         | 0/255                                                          | 100.0          |
| Choleraesuis| SC-867   | NC_006905.1   | C1 (O:7)  | 6,7,c:1,5         | YES                                   | Choleraesuis| DSM14846         | YES                         | 0/255                                                          | 100.0          |
| Dublin      | SD3246   | CM001151.1    | D1 (O:9)  | 1,9,12[v]:g,p:-   | YES                                   | Dublin      | CDC10-0635       | YES                         | 1/255                                                          | 99.6           |
| Dublin      | CT02021853| NC_011205.1   | D1 (O:9)  | 1,9,12[v]:g,p:-   | YES                                   | Dublin      | CDC10-0636       | YES                         | 1/255                                                          | 99.6           |
| Enteritidis | P125109  | NC_011294.1   | D1 (O:9)  | 1,9,12,g,m:-      | YES                                   | Enteritidis| DSM17420         | YES                         | 0/255                                                          | 100.0          |
| Gallinarum  | 287/91   | NC_011274.1   | D1 (O:9)  | 1,9,12:-:-        | YES                                   | Gallinarum  | CDC74            | YES                         | 1/255                                                          | 99.6           |
| Gallinarum  | SG9      | CM001153.1    | D1 (O:9)  | 1,9,12:-:-        | YES                                   | Gallinarum  | CDC74            | YES                         | 1/255                                                          | 99.6           |
| Heidelberg  | SL476    | NC_011083.1   | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Heidelberg  | CDC16            | YES                         | 0/255                                                          | 100.0          |
| Infantis    | SIN      | sanger.ac.ukb| C1 (O:7)  | 6,7,14,r:1,5      | YES                                   | Infantis    | CDC1428          | YES                         | 0/255                                                          | 100.0          |
| Newport     | SL254    | NC_011080.1   | C2–C3 (O:8)| 6,8,20,e,h:1,12   | YES                                   | Newport     | CDC2434          | YES                         | 0/255                                                          | 100.0          |
| Paratyphi A | AKU_12601| NC_011147.1   | A (O:2)   | 1,2,12:a:[1,5]    | YES                                   | Paratyphi A| CDC1            | YES                         | 2/255                                                          | 99.2           |
| Paratyphi A | ATC C9150| NC_006511.1   | A (O:2)   | 1,2,12:a:[1,5]    | YES                                   | Paratyphi A| CDC1            | YES                         | 2/255                                                          | 99.2           |
| Paratyphi B | SP87     | NC_010102.1   | B (O:4)   | 1,4,[5],12,b:1,2  | YES                                   | Paratyphi B| CDC3            | YES                         | 2/255                                                          | 99.2           |
| Paratyphi C | RKO4594  | NC_012125.1   | C1 (O:7)  | 6,7,[V]:c,1,5     | YES                                   | Paratyphi C| CDC3            | YES                         | 0/255                                                          | 100.0          |
| Schwarzengrund| CVM19633| NC_011094.1   | B (O:4)   | 1,4,12,27:d,[1,7] | YES                                   | Schwarzengrund| CDC1629       | YES                         | 1/255                                                          | 99.6           |
| Typhi       | CT18     | NC_003198.1   | D1 (O:9)  | 9,12,[v]:d:-      | YES                                   | Typhi       | No. 1c           | YES                         | 0/255                                                          | 100.0          |
| Typhi       | Ty2      | NC_004631.1   | D1 (O:9)  | 9,12,[v]:d:-      | YES                                   | Typhi       | No. 1c           | YES                         | 0/255                                                          | 100.0          |
| Typhimurium | I40285   | NC_016856.1   | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Typhimurium| CDC14          | YES                         | 0/255                                                          | 100.0          |
| Typhimurium | Z7120    | NC_016857.1   | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Typhimurium| CDC14          | YES                         | 0/255                                                          | 100.0          |
| Typhimurium | D23580   | FN424405.1    | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Typhimurium| CDC14          | YES                         | 0/255                                                          | 100.0          |
| Typhimurium | LT2      | NC_003197.1   | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Typhimurium| LT2            | YES                         | 0/255                                                          | 100.0          |
| Typhimurium | SL1344   | NC_016810.1   | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Typhimurium| CDC14          | YES                         | 0/255                                                          | 100.0          |
| Typhimurium | T0002-40 | NC_016860.1   | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Typhimurium| CDC14          | YES                         | 0/255                                                          | 100.0          |
| Typhimurium | UK-1     | NC_016863.1   | B (O:4)   | 1,4,[5],12,r:1,2  | YES                                   | Typhimurium| CDC14          | YES                         | 0/255                                                          | 100.0          |
| Weltevreden | 2007–60–3289-1| FR75255.1 | E1 (O:3,10)| 3,(10,15):r:z:6   | YES                                   | Weltevreden| CDC147        | YES                         | 0/255                                                          | 100.0          |

*a* maximal difference of serogenotyping probes at a signal threshold of 0.3.

*b* ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/SG.dbs.

*only genomic DNA of *Salmonella* Typhi, courtesy of Rene S. Hendriksen, DTU Food, Denmark.

Calculation of similarities was carried out by comparing predictions to measured signals for all 255 probes. Signals with intensities higher than 0.3 were considered positive and set as “1”. Signals lower than 0.3 were regarded negative and set as “0”. The number of probes which differ was summarized and the percentage was calculated.

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Detection software

Using the described PatternMatch module, a software package was developed to analyze Salmonella serovars directly at the ArrayMate device directly after scanning and calculating signals of the stained arrays (IconoClust Software version 3.2r1) (Fig. 1e). The detection software used the same database comprising 168 reference Salmonella strains (representing 132 Salmonella serovars) which were classically serotyped. Patterns of unknown Salmonella were compared to the whole database and the two best hits were given in a result sheet (Fig. 3). Prior to PatternMatching, all calculated signals were normalized within a range of 0 and 1. Briefly, the mean of valid signals was calculated and subsequently, the formula

\[ S_n = \frac{(S_m - \text{min})}{(\text{max} - \text{min})} \]

\[ S_n = \text{normalized signal, } S_m = \text{mean of signal, } \text{min = Minimum of all signals, max = maximum of all signals} \]

was used to normalize the mean of valid values. Due to the normalization procedure, experiments with very low signal intensity could also be analyzed and subsequently compared with the database. This method guaranteed a correct assignment to the reference pattern within the provided database. Furthermore, different parameters were requested by the software: a) two biotin marker spots as positive staining controls, b) spotting buffer as a negative control and c) marker for detection of Salmonella. These results were included in the result sheet (Fig. 3). Additionally, the report contains the genotyping results of all AMR genes. The software tool was evaluated using all reference strains included in the database. All 168 reference strains were perfectly identified even if the experiment showed weak signals (data not presented here). A multiple PatternMatch analysis of eight identical hybridization experiments with the same genomic DNA isolated from S. ser. Typhimurium DSM5569 showed a mean matching score (MS) of 2.12±0.65 with a maximum MS of 3.32 and a minimum MS of 1.15. The mean and maximum MS were significantly (t-test, p<0.05) lower than the MS value for poor matches (MS> 6.5). These results showed the high reproducibility of this assay described in this study.

Antibiotic resistance

In a panel of 34 Salmonella strains 26 different AMR genes were detected and subsequently compared with the AMR phenotype of these strains (Table 5, detailed view in Table S2). A high correlation was observed for all detected genes relating to the AMR phenotype.

An extended-spectrum beta-lactamase (ESBL) gene, ctxM1, was detected once, in an isolate of S. ser. Anatum AMR07. This strain was resistant against ceftazidime and cefpodoxime, both members of third generation beta lactams.

AMR phenotypes for which no corresponding AMR genotype were detected included streptomycin resistance in two isolates (S. ser. Saintpaul and S. ser. 1,4,[5],12:i:-) and ampicillin resistance in one S. ser. Bredeney isolate. The latter isolate yielded a positive signal in a nitrocefin assay (BBL DrySlide Nitrocefin, Becton Dickinson).

No assessment was possible for resistance genes sul1 and sul2 that should cause isolated resistance to sulfonamides because
Table 5. Comparison of antimicrobial resistance (AMR) genotype and AMR phenotype.

| AMR Genes | Genbank No. | AMR Family | Antibiotics Tested to AMR Phenotype | Gene detected | Resistance detected | Sensitivity detected | Correlation (%) |
|-----------|-------------|------------|-------------------------------------|--------------|---------------------|----------------------|-----------------|
| aac6II    | AY123251.1  | Aminoglycoside | Gentamicin, Tobramycin              | 1            | 1                   | 0                    | 100             |
| aadA1     | AB126599.1  | Aminoglycoside | Streptomycin                        | 14           | 14                  | 0                    | 100             |
| aadA2     | AB126602.1  | Aminoglycoside | Streptomycin                        | 5            | 5                   | 0                    | 100             |
| aphA1     | AB366440.1  | Aminoglycoside | Kanamycin                           | 5            | 5                   | 0                    | 100             |
| sph       | AB366441.1  | Aminoglycoside | Streptomycin                        | 1            | 1                   | 0                    | 100             |
| strA      | AB366442.1  | Aminoglycoside | Streptomycin                        | 10           | 10                  | 0                    | 100             |
| strB      | AB366440.1  | Aminoglycoside | Streptomycin                        | 14           | 14                  | 0                    | 100             |
| catA1     | AB366440.1  | Chloramphenicol| Chloramphenicol                     | 5            | 5                   | 0                    | 100             |
| cmlA      | AJ487033.2  | Chloramphenicol| Chloramphenicol                     | 4            | 4                   | 0                    | 100             |
| floR      | AF181870.1  | Chloramphenicol| Chloramphenicol                     | 2            | 2                   | 0                    | 100             |
| sul1      | AF261825.2  | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 8  | 1                   | 7                    | -^a            |
| sul2      | AB366440.1  | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 3  | 2                   | 1                    | -^a            |
| sul1, sul2 | AF261825.2, AB366440.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 0                   | 1                    | -^a            |
| sul1, dA1  | AF261825.2, AF203818.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul1, dA15 | AF261825.2, AF203818.1, AJ867237.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul1, dA12 | AF261825.2, AB366440.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul1, dA13 | AF261825.2, AM932669.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul1, d8V | AF261825.2, DQ133140.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul1, sul2, dA1 | AF261825.2, AB366440.1, AF203818.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 2  | 2                   | 0                    | 100            |
| sul1, sul2, dA12 | AF261825.2, AB366440.1, AB366440.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul2, dA14 | AB366440.1, DQ388123.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul3, dA1  | AY162033.1, AF203818.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 1  | 1                   | 0                    | 100            |
| sul3, dA12 | AY162033.1, AB366440.1 | Sulfonamide/Trimethoprim | Co-trimoxazol (Sulfamethoxazol/Trimethoprim) | 3  | 3                   | 0                    | 100            |
testing was performed only for co-trimoxazole only.). A gene mphpA mediating erythromycin resistance was found in S. ser. Anatum strain AMR05 (Table S2), but erythromycin susceptibility was not tested using a panel for gram-negatives on the VITEK 2 system.

**Field study**

After assay verification, the *Salmonella* serogenotyping assay was used to identify a field panel of 105 *Salmonella* isolates (Table 6) sampled and serotyped by the National Reference Laboratory for Salmonellosis in Cattle at the Friedrich-Loeffler-Institute (FLI, Jena, Germany). All tested isolates were identified as *Salmonella* and, out of 105 isolates, 93 were typed correctly (88.6%, Table 6). The limitation of the actual assay was that certain strains yielded identical patterns on the current array thus prohibiting further differentiation (Table 6). Such limitations occurred for S. ser. Enteritidis, which actually cannot be discriminated from S. ser. Nitra and S. ser. Bledgdam. Furthermore, the pattern of S. ser. Dublin was identical to S. serovars Naestved, Moscow and Kiel. A discrimination of S. ser. Dublin and S. ser. Kiel was impossible as probes representing the genes SeD_A1100, SeD_A1101 and SeD_A1102 were positive for both serovars. Similar limitations were also observed for S. ser. Panama (identical pattern as S. ser. Koessen), S. ser. Indiana (identical pattern as S. ser. Klambu) and S. ser. Senftenberg (identical pattern as S. ser. Westhampton). A monophasic S. ser. Typhimurium isolate (1,4,[5],12:i:-) was identified correctly. *Salmonella* ser. Typhimurium var. Copenhagen (1,4,12:i:1,2) was assigned to S. ser. Typhimurium (1,4,[5],12:i:1,2) and a rough form of S. ser. Infantis was assigned to non-rough S. ser. Infantis. These limitations were evaluated as minor mistakes and subsequently regarded as correct hits.

**Discussion**

The microarray for *Salmonella* serogenotyping was validated against the gold standard and was evaluated as an economical, fast, accurate and easy-to-use diagnostic tool with a high potential for standardization and automated high throughput use. For identification of *Salmonella* using serogenotyping assays, several studies have already been published [13,19,20,21]. The results of these publications showed high correlation of genotypic and phenotypic characterizations for genus *Salmonella*. Similar studies serogenotyping *Esherichia coli* [6,30,31] or *Chlamydia* [29,32] also found a direct correlation of geno- and phenotype.

For *Salmonella*, at least four genes seem to be significant for specification of the genotype; *wzx* and *wzy* specify the O-serogroup, and the genes *fbC* and *fbB* specify the H antigens. To improve the correlation of geno-and phenotype, we analyzed fully sequenced *Salmonella* strains (Table 4) using theoretical hybridization with all probes on the microarray. The result was a similarity of over 99% between the phenotype represented by the antigenic formula and the genotype represented by the microarray based assay. Within the panel of theoretical reference experiments, strain S. ser. Typhimurium LT2 was the only one which was both fully sequenced and classically serotyped. Therefore, it was possible to compare the genotype represented through the NCBI database entry (NC_003197.1) with our theoretical experiments and subsequently with the real experiments using the same strain, S. ser. Typhimurium LT2. Theoretical and real experiments had a concordance of 100%. Even a deeper view of the signal-mismatch prediction from theoretical experiments resulted in a good correlation to the real experiment (Fig. 2). Only two probes showed signal strengths that differed from the results predicted by the theoretical experiment. Such discrepancies may occur due to secondary structure of the amplicon which decreases the binding

| Antibiotics Tested to AMR Phenotype | Genbank No. | AMR Genes | AMR Family | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-------------------------------------|------------|-----------|------------|----------------------|---------------------|-----------------|
| AMR Phenotype | Gene detected | Resistance detected | AMR Family | Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|----------------|----------------|-------------------|------------|------------|----------------|-------------------|-----------------|
| Ampicillin | ctxM1 | 110 | 1 | 0 | 0 | 0 | 100 |
| Ceftazidime | oxa1 | 2 | 2 | 0 | 100 |
| Ampicillin | ctxM1 | 15 | 15 | 0 | 100 |
| Aerobactin | tetA | 10 | 10 | 0 | 100 |
| Tetacycline | tetB | 13 | 13 | 0 | 100 |
| Tetracycline | tetG | 1 | 1 | 0 | 100 |

**Table 5.**

| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
| AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | AMR Genes Genbank No. | Sensitivity detected | Correlation (%) |
|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|-----------------|
Table 6. Field study with a blind panel of 105 isolates using the Salmonella serogenotyping assay.

| Species       | Serovar         | Strain  | Results of classical Serotyping | Results of microarray based Serotyping |
|---------------|-----------------|---------|---------------------------------|---------------------------------------|
|               |                 |         | Serogroup | Antigenic Formula | Unique Pattern | Serovar | Alternative Serovar* |
| S. enterica   | 1,4,5,12:i:-    | NRL688  | B (O:4) | 1,4,[5],12:i:- | yes | 1,4,[5],12:i:- |
| S. enterica   | 1,4,5,12:i:-    | NRL749  | B (O:4) | 1,4,[5],12:i:- | yes | 1,4,[5],12:i:- |
| S. enterica   | 1,4,5,12:i:-    | NRL813  | B (O:4) | 1,4,[5],12:i:- | yes | 1,4,[5],12:i:- |
| S. enterica   | 1,4,5,12:i:-    | NRL982  | B (O:4) | 1,4,[5],12:i:- | yes | 1,4,[5],12:i:- |
| S. enterica   | 1,4,5,12:i:-    | NRL1004 | B (O:4) | 1,4,[5],12:i:- | yes | 1,4,[5],12:i:- |
| S. enterica   | 1,4,5,12:i:-    | NRL1019 | B (O:4) | 1,4,[5],12:i:- | yes | 1,4,[5],12:i:- |
| S. enterica   | Abony           | NRL794  | B (O:4) | 1,4,[5],12,27:b:e,n,x | yes | Abony |
| S. enterica   | Agona           | FLI415  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Agona |
| S. enterica   | Agona           | FLI417  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Agona |
| S. enterica   | Agona           | FLI1157 | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Agona |
| S. enterica   | Agona           | FLI449  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Agona |
| S. enterica   | Agona           | FLI709  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Agona |
| S. enterica   | Agona           | FLI1027 | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Agona |
| S. enterica   | Anatum          | NRL939  | E1 (O:3,10) | 3,[10],[15],[15,34]:e,h:1,6 | yes | Anatum |
| S. enterica   | Anatum          | NRL946  | E1 (O:3,10) | 3,[10],[15],[15,34]:e,h:1,6 | yes | Anatum |
| S. enterica   | Anatum          | FLI452  | E1 (O:3,10) | 3,[10],[15],[15,34]:e,h:1,6 | yes | Anatum |
| S. enterica   | Anatum          | NRL1006 | E1 (O:3,10) | 3,[10],[15],[15,34]:e,h:1,6 | yes | Anatum |
| S. enterica   | Bareilly        | FLI608  | C1 (O:7) | 6,14,y:1,5 | yes | Bareilly |
| S. enterica   | Bovismorbificans| FLI466  | C2–C3 (O:8) | 6,8,20,r:1,1,5 | yes | Bovismorbificans |
| S. enterica   | Bovismorbificans| FLI525  | C2–C3 (O:8) | 6,8,20,r:1,1,5 | yes | Bovismorbificans |
| S. enterica   | Braenderup      | FLI544  | C1 (O:7) | 6,7,14:e,h:e,n,z15 | yes | Braenderup |
| S. enterica   | Brandenburg     | NRL796  | B (O:4) | 4,[5],[12]:e,v:e,n,z15 | yes | Brandenburg |
| S. enterica   | Brandenburg     | NRL869  | B (O:4) | 4,[5],[12]:e,v:e,n,z15 | yes | Brandenburg |
| S. enterica   | Brandenburg     | NRL892  | B (O:4) | 4,[5],[12]:e,v:e,n,z15 | yes | Brandenburg |
| S. enterica   | Brandenburg     | FLI419  | B (O:4) | 4,[5],[12]:e,v:e,n,z15 | yes | Brandenburg |
| S. enterica   | Cerro           | NRL721  | K (O:18) | 6,14,18,24,23:1,5 | yes | Cerro |
| S. enterica   | Choleraesuis    | FLI826  | C1 (O:7) | 6,8,c:1,6 | yes | Choleraesuis |
| S. enterica   | Choleraesuis    | FLI987  | C1 (O:7) | 6,7,c:1,5 | yes | Choleraesuis |
| S. enterica   | Derby           | FLI605  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Derby |
| S. enterica   | Derby           | FLI624  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Derby |
| S. enterica   | Derby           | NRL723  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Derby |
| S. enterica   | Derby           | NRL776  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Derby |
| S. enterica   | Derby           | NRL960  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Derby |
| S. enterica   | Derby           | FLI529  | B (O:4) | 1,4,[5],12,f:gs[1,2] | no | Derby |
| S. enterica   | Derby           | FLI624  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Derby |
| S. enterica   | Derby           | FLI666  | B (O:4) | 1,4,[5],12,f:gs[1,2] | yes | Derby |
| S. enterica   | Derby           | FLI1111 | B (O:4) | 1,4,[5],12,f:gs[1,2] | no | Derby |
| S. enterica   | Dublin          | NRL683  | D1 (O:9) | 1,9,12[V]:g,p: | no | Moscow Naestved |
| S. enterica   | Dublin          | NRL684  | D1 (O:9) | 1,9,12[V]:g,p: | no | Moscow Naestved |
| S. enterica   | Dublin          | NRL703  | D1 (O:9) | 1,9,12[V]:g,p: | no | Naestved Dublin |
| S. enterica   | Dublin          | NRL704  | D1 (O:9) | 1,9,12[V]:g,p: | no | Naestved Dublin |
| S. enterica   | Dublin          | NRL915  | D1 (O:9) | 1,9,12[V]:g,p: | no | Naestved Dublin |
| S. enterica   | Dublin (Bovisaloral) | NRL904 | D1 (O:9) | 1,9,12[V]:g,p: | no | Naestved Dublin |
| S. enterica   | Dublin (rough) | NRL787  | D1 (O:9) | 1,9,12[V]:g,p: | no | Naestved Dublin |
| S. enterica   | Enteritidis     | FLI95   | D1 (O:9) | 1,9,12,g,m: | no | Blegdam Enteritidis |
| S. enterica   | Enteritidis     | NRL685  | D1 (O:9) | 1,9,12,g,m: | no | Nitra Enteritidis |
| S. enterica   | Enteritidis     | NRL875  | D1 (O:9) | 1,9,12,g,m: | no | Belgdam Enteritidis |
| S. enterica   | Gallinarum      | FLI151  | D1 (O:9) | 1,9,12:--:-- | yes | Gallinarum |
| Species          | Serovar          | Strain     | Results of classical Serotyping | Results of microarray based Serotyping |
|------------------|------------------|------------|---------------------------------|----------------------------------------|
|                  |                  |            | Serogroup | Antigenic Formula | Unique Pattern | Serovar | Alternative Serovar¹ |
| S.e. enterica    | Gallinarum      | FL155      | D1 (O:9) | 1,9,12:-- | yes | Gallinarum       |
| S.e. enterica    | Gallinarum      | FL1969     | D1 (O:9) | 1,9,12:-- | yes | Gallinarum       |
| S.e. enterica    | Goldcoast       | NRL852     | C2–C3 (O:8) | 6,8:r,l,w | yes | Goldcoast       |
| S.e. enterica    | Goldcoast       | FL1990     | C2–C3 (O:8) | 6,8:r,l,w | yes | Goldcoast       |
| S.e. enterica    | Hadar            | FL1636     | C2–C3 (O:8) | 6,8:10:e,n,x | yes | Hadar           |
| S.e. enterica    | Hadar            | FL1638     | C2–C3 (O:8) | 6,8:10:e,n,x | yes | Hadar           |
| S.e. enterica    | Havana           | FL1607     | G (O:13) | 1,13,23:f,g,[l]:- | yes | Havana        |
| S.e. enterica    | Havana           | FL1755     | G (O:13) | 1,13,23:f,g,[l]:- | yes | Havana        |
| S.e. enterica    | Indiana          | NRL872     | B (O:4) | 1,4,12:z,1,7 | no | Kiambu         |
| S.e. enterica    | Indiana          | NRL1022    | G (O:13) | 1,13,23:i,l,w | yes | Kiambu         |
| S.e. enterica    | Infantis         | NRL718     | C1 (O:7) | 6,7,14:r,1,5 | yes | Infantis       |
| S.e. enterica    | Infantis         | NRL822     | C1 (O:7) | 6,7,14:r,1,5 | yes | Infantis       |
| S.e. enterica    | Infantis         | FL1630     | C1 (O:7) | 6,7,14:r,1,5 | yes | Infantis       |
| S.e. enterica    | Infantis         | FL1761     | C1 (O:7) | 6,7,14:r,1,5 | yes | Infantis       |
| S.e. enterica    | Infantis (R-form) | FL1546    | C1 (O:7) | 6,7,14:r,1,5 | yes | Infantis       |
| S.e. enterica    | Kasesenyi        | NRL878     | P (O:38) | 38:e,h:1,5 | yes | Kasesenyi     |
| S.e. enterica    | Kedougou         | FL1515     | G (O:13) | 1,13,23:i,l,w | yes | Kedougou       |
| S.e. enterica    | Kedougou         | NRL1022    | G (O:13) | 1,13,23:i,l,w | yes | Kedougou       |
| S.e. enterica    | Litchfield       | FL1218     | C2–C3 (O:8) | 6,8:1:2 | yes | Litchfield     |
| S.e. enterica    | Livingstone      | FL1720     | C1 (O:7) | 6,7,14:d,l,w | yes | Livingstone    |
| S.e. enterica    | London           | NRL700     | E1 (O:3,10) | 3,10(15,35):v:1,6 | yes | London         |
| S.e. enterica    | London           | NRL849     | E1 (O:3,10) | 3,10(15,35):v:1,6 | yes | London         |
| S.e. enterica    | Manhattan        | FL1662     | C2–C3 (O:8) | 6,8:1,5 | yes | Manhattan       |
| S.e. enterica    | Mbandaka         | FL1534     | C1 (O:7) | 6,7,14:10:e,n,z15 | yes | Mbandaka       |
| S.e. enterica    | Minnesota        | NRL814     | L (O:21) | 21:bre,n,x | yes | Minnesota       |
| S.e. enterica    | Minnesota        | NRL839     | L (O:21) | 21:bre,n,x | yes | Minnesota       |
| S.e. enterica    | Montevideo       | NRL930     | C1 (O:7) | 6,7,14:10:e,n,z15 | yes | Montevideo     |
| S.e. enterica    | Montevideo       | FL1552     | C1 (O:7) | 6,7,14:10:e,n,z15 | yes | Montevideo     |
| S.e. enterica    | Muenchen         | NRL801     | C2–C3 (O:8) | 6,8:1,2 | yes | Muenchen       |
| S.e. enterica    | Muenster         | FL1325     | E1 (O:3,10) | 3,10(15,35,45):e,h:1,5 | yes | Muenster       |
| S.e. enterica    | Ohio             | NRL882     | C1 (O:7) | 6,7,14:b,l,w | yes | Ohio           |
| S.e. enterica    | Oranienburg      | FL1429     | C1 (O:7) | 6,7,14:m,t(257) | yes | Oranienburg    |
| S.e. enterica    | Panama           | FL1604     | D1 (O:9) | 1,9,12:i,v,1,5 | no | Koessen        |
| S.e. enterica    | Panama           | FL1411     | D1 (O:9) | 1,9,12:i,v,1,5 | no | Panama         |
| S.e. enterica    | Panama           | FL1413     | D1 (O:9) | 1,9,12:i,v,1,5 | no | Panama         |
| S.e. enterica    | Paratyphi B      | FL1588     | B (O:4) | 1,4,5,12:b:1,2 | yes | Paratyphi B    |
| S.e. enterica    | Paratyphi B      | FL1590     | B (O:4) | 1,4,5,12:b:1,2 | yes | Paratyphi B    |
| S.e. enterica    | Pomona           | FL1700     | M (O:28) | 28:y:1,7 | yes | Pomona         |
| S.e. enterica    | Saintpaul        | FL1344     | B (O:4) | 1,4,5,12:e,h:1,2 | yes | Saintpaul      |
| S.e. enterica    | Saintpaul        | FL1423     | B (O:4) | 1,4,5,12:e,h:1,2 | yes | Saintpaul      |
| S.e. enterica    | Sandiego         | NRL987     | C1 (O:7) | 1,4,5,12:e,h,v,15 | yes | Sandiego       |
| S.e. enterica    | Senftenberg      | NRL682     | E4 (O:1,3,19) | 1,3,19:g,[s]:- | no | Westhampton    |
| S.e. enterica    | Tennessee        | FL1347     | C1 (O:7) | 6,7,14:229:1,2,7 | yes | Tennessee      |
| S.e. enterica    | Tennessee        | FL1606     | C1 (O:7) | 6,7,14:229:1,2,7 | yes | Tennessee      |
| S.e. enterica    | Thompson         | FL1658     | C1 (O:7) | 6,7,14:k,1,5 | yes | Thompson       |
| S.e. enterica    | Typhimurium      | FL1598     | B (O:4) | 1,4,5,12:i,1,2 | yes | Typhimurium    |
| S.e. enterica    | Typhimurium      | NRL990     | B (O:4) | 1,4,5,12:i,1,2 | yes | Typhimurium    |
A more recent method to identify Salmonella is a system using a microsphere-based liquid array [19,20]. This method uses a set of beads which are coupled with probes for one attribute within the antigenic formula of Salmonella serovars. While the method is highly sensitive and specific, a multitude of different beads is required for every attribute within the antigenic formula (e.g., O-antigen). Therefore, at least three reactions have to be performed before obtaining the antigenic formula. A drawback of the method is the multiplex PCR used to amplify short DNA fragments which are then hybridized to the probes on the beads. Due to the inherent disadvantages of any multiplex PCR [34,35], the options are limited for a further expansion of the assay beyond the serovars it currently recognizes.

The described microarray based serogenotyping assay for Salmonella overcomes most of these bottlenecks. It is easy-to-use, an unlimited expandability and fully automated data analysis, making it an attractive platform for a widespread application. The multiplex primer extension reaction used for labeling is highly specific, but exhibits low sensitivity, due to linear (non-exponential) amplification. However, for typing colony material of a fast growing organism, such as Salmonella, this is no issue. The use of colony material instead of original field samples allows both, to obtain the necessary amount of DNA and to ensure pureness and clonality of cultures to be genotyped. Besides, the limited amplification can prove to be an advantage under routine conditions as the assay becomes less susceptible to contamination. Using a classic multiplex PCR, the sensitivity is very high, but contaminants will also be amplified to a detectable level because of the near-exponential kinetics of a PCR. This fact might cause difficulties in high-throughput routine laboratories.

In our approach, primers and their respective probe binding sites are very close to each other. The probability of secondary structures (e.g., hairpins) forming in short generated fragments is lower than in long fragments and this may increase signal intensity. Additionally, the use of single stranded DNA prevents the competition between probe and antisense strand and increases the probability of the single stranded amplicon binding to the probe. Labeling methods using biotin attached to primers were often used [36], but we assumed that, due to cross hybridizations of biotin labeled primer which are in relatively high concentrations, false positive signals will occur more often. In this study,

### Table 6. Cont.

| Species           | Serovar               | Strain      | Results of classical Serotyping | Results of microarray based Serotyping |
|-------------------|-----------------------|-------------|---------------------------------|---------------------------------------|
|                   |                       |             | Serogroup | Antigenic Formula | Unique Pattern | Serovar | Alternative Serovar* |
| S. e. enterica    | Typhimurium           | NRI729      | B (O:4)   | 1,4,[5],12x1,2   | yes           | Typhimurium |                       |
| S. e. enterica    | Typhimurium           | NRI737      | B (O:4)   | 1,4,[5],12x1,2   | yes           | Typhimurium |                       |
| S. e. enterica    | Typhimurium           | FLI17       | B (O:4)   | 1,4,[5],12x1,2   | yes           | Typhimurium |                       |
| S. e. enterica    | Typhimurium           | NRI990      | B (O:4)   | 1,4,[5],12x1,2   | yes           | Typhimurium |                       |
| S. e. enterica    | Typhimurium           | NRI993      | B (O:4)   | 1,4,[5],12x1,2   | yes           | Typhimurium |                       |
| S. e. enterica    | Typhimurium var. Copenhagen | NRI797  | B (O:4)   | 1,4,12x1,2       | yes           | Typhimurium |                       |
| S. e. enterica    | Typhimurium var. Copenhagen | NRI912  | B (O:4)   | 1,4,12x1,2       | yes           | Typhimurium |                       |
| S. e. enterica    | Typhimurium var. Copenhagen | FLI1033 | B (O:4)   | 1,4,12x1,2       | yes           | Typhimurium |                       |
| S. e. enterica    | Virchow               | FLI640      | C1 (O:7)  | 6,7,14x1,2       | yes           | Virchow    |                       |
| S. e. enterica    | Virchow               | FLI649      | C1 (O:7)  | 6,7,14x1,2       | yes           | Virchow    |                       |

*tested isolate generated a serogenotyping pattern which is shared by multiple serovars.

Results were analyzed by the PatternMatch software and compared with the results of classical serotyping performed by the National Reference Laboratory for Salmonellosis in cattle at the Friedrich-Loeffler-Institute (NRL, FLI, Jena, Germany).

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Salmonella

Serogenotyping of Salmonella
biotin labeled dUTP was used for internal labeling of the multitude of single stranded amplicons. This method prevents false positive signals due to unused primer which bind on empty probes. Another significant advantage of the described serogenotyping method is the economical and ready-to-use availability of all components, even in large scales. For DNA isolation, we used standard DNA isolation kits from Roche or Qiagen. Furthermore, it is conceivable that, after heating at 100 °C and RNase treatment (assay sensitivity may decreases due to single stranded RNA which may trap primer used in the multiplex linear DNA amplification), the crude cell extract could be used directly with this assay. All substances for the linear multiplex PCR and the labeling process are available as HyBio Plus Kit (Alere Technologies, Germany). Due to the standardized availability of all components, this method can immediately be used for routine serogenotyping of Salmonella. Up to 96 samples can be analyzed simultaneously.

So far, the serogenotyping assay shows the limitation of the inability to discriminate between serogroup A (O:2) and serogroup D1 (O:9). This is due to the high sequence similarity within the rfb region between strains of both serogroups. Within the genome of serogroup A strains, the rfb region has been shown to be a minor modification of a serogroup D1 rfb region; it has a frameshift mutation that inactivates tev, a sugar biosynthesis gene required for the biosynthesis of tyvelose [37]. Serogroup D1 strains have tyvelose as their O-antigen side chain sugar, whereas serogroup A strains have paratose, the substrate for tyvelose, as its side chain sugar. Thus, a small genetic change is responsible for a substantial O-antigen difference. Additional probes, including lgyA, lgyD, sefA, sefB and sefC, which were only described for serovar Enteritidis [38,39], also give positive signals for serovar Nitra. Additionally, S. ser. Blegdams (O9:g,m,q-) showed an identical pattern on the microarray, but in this case the antigenic formula is highly similar to S. ser. Enteritidis (O9:g,m-). This result showed how closely related these serovars are to each other. A similar observation between the serogroups A and D1 were made for the serovars Dublin (O9:g:q-) and Kiel (O2:g:p-), where additional probes for SeD_A1100, SeD_A1101 and SeD_A1102 were also positive with serovar Kiel. This observation may indicate a high degree of relationship between these two serovars. Furthermore, we assume a high genome sequence similarity between Panama (O91,1:5) and Koessen (O2:1v:1,5) as the microarray pattern were also identical. Paratyphi A could be unambiguously identified due to the probes of intergenic region SSPAI. With the knowledge about the genotype of these described serovars a question arises: Is there a need to differentiate between serogroup D1 and A or between C and D1? However, all these ambiguous strains are very rare in a clinical environment, each being reported less than 10 times worldwide during the last 10 years ([1], www.cdc.gov/ncezid/dwesd/PDFs/SalmonellaAnnualSummaryTable2009.pdf), and additional probes can easily be introduced should a need arise, or should new sequence information become available.

Due to the absence of a probe which can determine the genetic loci of the O:5 epitope, the isolate S. ser. Typhimurium var. Copenhagen, which is O:5-negative by serotyping, was identified as S. ser. Typhimurium; this minor mistake was regarded as a correct hit.

Another minor limitation is that R- forms (rough forms) cannot be identified using the current array as observed in one isolate of Infantis. R- forms mainly result from mutations of genes within the lipopolysaccharide core [45]. Mutations within the genes rfa (glycosyltransferase), galE (UDP-galactose epimerase), or galF (UDP-glucose pyrophosphorylase) can cause an interruption of the biosynthesis of the lipopolysaccharides. No probes detecting such mutations were included to the array, and failure to identify R- forms regarded as minor issue.

The described assay for serogenotyping is the basis for a fast method to identify Salmonella serovars. We believe that the usage of this assay in a routine laboratory setting is warranted due to the high correlation between serotype and genotype. An advantage of the genotype as the basis for serovar identification is that phenotypic differences (e.g., R-forms that are difficult to analyze by classical serology) play no role. Furthermore, the serogenotyping assay could be used worldwide, where antisera are not available. In such areas, a Salmonella infection in livestock or

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Salmonella contamination in food could be identified very quickly. Salmonella outbreaks could consequently be retraced to their origin. This microarray-based assay is a powerful tool for epidemiological studies, as many samples can be analyzed rapidly and in parallel. For such cases, a point-of-care application represents an ideal standard.

During an outbreak situation, this assay could be extremely helpful to identify the outbreak isolate including AMR genotype within hours after they are obtained as clonal serovar. Extremely helpful to identify the outbreak isolate including AMR phenotype of 34 strains.

**Supporting information**

**Table S1** Probe-matching matrix used to construct the theoretical hybridization pattern of the fully sequenced strains listed in NCBI database. (XLSX)

**Table S2** Comparison of the AMR-genotype and AMR-phenotype of 34 Salmonella strains. (XLSX)

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**Author Contributions**

Performed the experiments: SDB SK. Analyzed the data: SDB AZ PS. Contributed reagents/materials/analysis tools: SDB AZ PS. Wrote the paper: SDB SM RE UM.
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