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Short communication

Evaluation of the Premi®Test *Salmonella*, a commercial low-density DNA microarray system intended for routine identification and typing of *Salmonella enterica*

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Abstract

A new commercial system based on genetic profiling and aimed at identifying *Salmonella enterica* serovars was evaluated by comparing its performance with classical serotyping on 443 strains. Within 62 serovars represented, 60 gave unique genetic profiles while 2 were undistinguishable. Results were obtained within 8 h, were reproducible and clear-cut. The system allowed single-tube processing of the samples and required no peculiar technical skill. It showed interesting potential for routine laboratory testing.
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1. Introduction

Nontyphoidal salmonellae are a leading cause of reportable foodborne infection in industrialized countries. This bacterium typically causes intestinal infections accompanied by fever, abdominal cramps and diarrhea with symptoms lasting over 1 week (Hohmann, 2001). Wild and domestic animals act as reservoirs and transmission to humans occurs generally through consumption of contaminated food. Therefore, monitoring of the food chain by means of large surveillance programs has been implemented in many countries. *Salmonella enterica* isolates can be discriminated on the basis of O (surface polysaccharide) and H (flagellar) antigenic properties, the latest being expressed in two separate phases. Currently, more than 150 different O and H antigens are used for the characterization of over 2500 *Salmonella* serovars (Bopp et al., 2003; Perch et al., 2003). At least three antibody-antigen reactions are required to indentify a particular *Salmonella* serovar while uncommon serovars usually require further tests to be correctly identified. The scoring of antigenic formulae uses the Kaufmann–White scheme which is annually updated by the World Health Organization (Bopp et al., 2003; Popoff et al., 2004). Serotyping has clear deficiencies inherent to the analysis time and to the limited applicability of the methodology. It often takes 3 or more days to generate a result and only 92 to 95% of the isolates can be fully serotyped. Typing failure is caused by the lack of surface O antigens exposure in certain mucoid and “rough” strains (Bopp et al., 2003), while just partial typing can be performed in non-motile isolates (no expression of flagella). For such strains, additional biochemical is required. Phage sensitivity tests can be used to further discriminate strains with identical serotypes. Further problems with serotyping are due to (i) prolonged subculturing that can affect the antigenic properties of the strain, (ii) the technical expertise required, (iii) the costs issuing from specific antisera production and validation. All together, these

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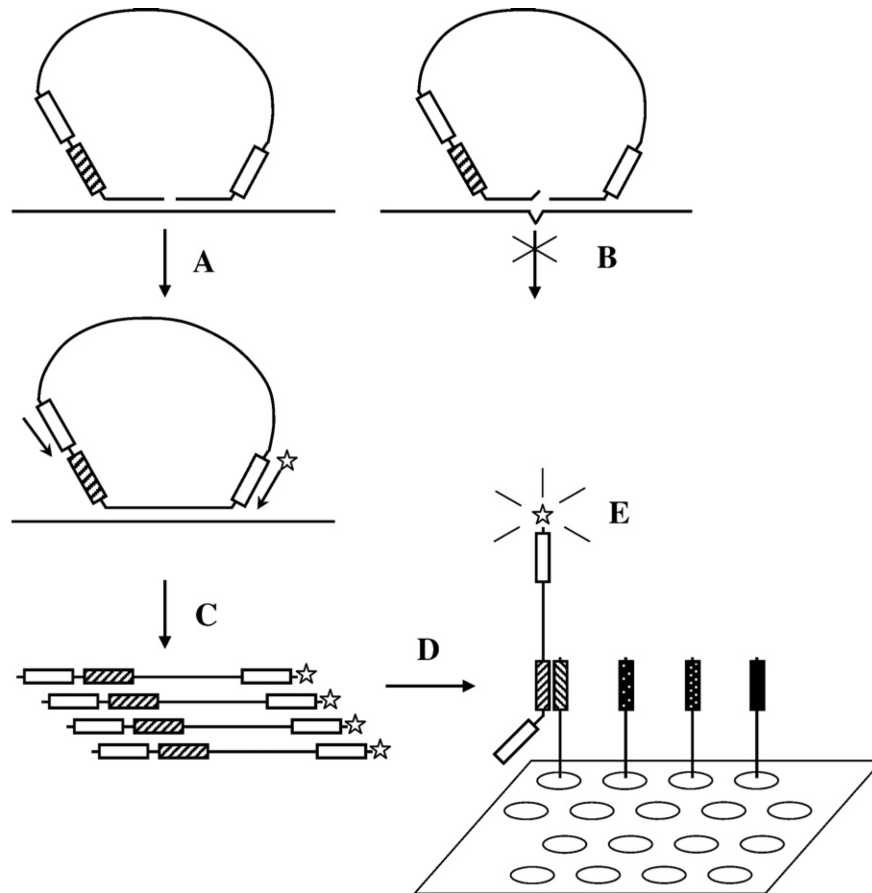

Fig. 1. Principle of the Premi®Test *Salmonella* typing system. (A) When properly hybridized to a target sequence, the nick lying between two adjacent LDR probe arms is ligated, so that a single circular fragment is generated. (B) Critical mismatches in the target sequence will cause ligation to fail, leaving the probe ends apart. (C) Successful ligation products are amplified by PCR using a single pair of amplimers annealing to complementary sequences included in the LDR probes (white boxes). (D) Unique ZIP codes (hashed box) assigned to each LDR probe will be specifically captured by complementary oligonucleotides (cZIP codes, reverse-hashed box) spotted on the microarray. (E) Detection occurs thanks to a biotin label incorporated at the 5′-end of one of the PCR primer. The system can be multiplexed with many different LDR probes, each bearing a unique ZIP code (black-filled boxes). The successive reactions are processed in a single tube.

difficulties hinder the response to an outbreak and/or its epidemiologic surveillance. Over the last 20 years, alternative strategies aimed at replacing or complementing traditional serotyping methods have been proposed. These include PCR/real-time PCR specific identification and typing methods (Esteban et al., 1993; Hoorfar et al., 2000; Jensen Hubner, 1996; Nair et al., 2002; Shangkuan Lin, 1998; Torpdahl Ahrens, 2004; Uzzau et al., 1999), multiplex PCR (Alvarez et al., 2004; Kim et al., 2006), DNA sequencing (Mortimer et al., 2004), and DNA microarrays (Chan et al., 2003; Porwollik et al., 2004). Protein-based approaches have been developed as well (Cai et al., 2005; Wilkes et al., 2005). The problems associated with these strategies include reproducibility of the results obtained in different laboratories, requirement of specialized equipment, high cost per sample analysis and highly trained staff. In this work, we evaluated the efficiency of the Premi®Test *Salmonella*,[1] a new commercial DNA-based test aimed at identifying common serovars of *S. enterica* (Andreoli et al., 2004). A total of 443 *Salmonella* strains belonging to 62 different serovars were tested in parallel using both Premi®Test and classical serotyping methods. In addition, 43 non-*Salmonella* strains were tested to assess the specificity of the system.

2. Materials and methods

2.1. Principle of the method

The Premi®Test *Salmonella* system uses a methodology called multiplex ligation detection reaction (LDR) to generate a collection of circular DNA molecules that are subsequently PCR amplified by means of a single pair of amplimers (Schouten et al., 2002; van Eijk et al., 2004). The PCR products are next sorted by hybridization to a low-density DNA microarray. Positive hybridization is detected using a biotin label incorporated in one of the PCR primer (Fig. 1). A set of genetic markers has been selected with the purpose of yielding unique microarray hybridization profiles to identify and discriminate *S. enterica* serovars (Andreoli et al., 2004; Baner et al., 2003). The test allows single-tube processing, which simplifies the technical work associated with strain typing.

[1] The Premi®Test *Salmonella* is a trademark of DSM Nutritional Products.

2.2. Microarray setup

The microarray setup is depicted in Table 1. It includes a number of controls assessing the success of each critical step in the procedure including ligation specificity and efficiency, PCR amplification, hybridization efficiency, label detection and label quality. Moreover, the use of *Salmonella* general LDR probes ensures that the investigated strain indeed belongs to the expected species. Serovar identification relies on a series of 14 LDR probes targeting DNA markers whose sequence vary within the different *Salmonella* serovars. Each LDR probe carries a ZIP code that is complementary to a unique oligonucleotide (cZIP) immobilized on the microarray. The detailed DNA sequences of the LDR probes were not released by the manufacturer. Their selection was based on nucleotide sequence comparisons performed at 30 random genomic loci on twenty *S. enterica* subsp. *enterica* strains, each belonging to one of the following serovars: Agona, Anatum, Bovismorbificans, Braenderup, Brandenburg, Dublin, Enteritidis, Goldcoast, Hadar, Heidelberg, Infantis, Livingstone, München, Newport, Oranienburg, Panama, Saint-Paul, Typhimurium, Typhimurium DT104, Virchow. For each locus, 400 to 600 bp of sequence information was generated in all 20 serovars and multiple alignments were carried out to identify SNPs. Each SNP was assessed for three criteria, namely (i) an allele frequency between 30 and 70%, (ii) the absence of additional SNPs adjacent to the investigated SNP, (iii) a GC% in the range of 40–60% up and downstream from the SNP locus.

2.3. Microarray reading

Microarray results were read on a single-channel ATR03 reader consisting basically in a CCD-based transmission detector (Clondiag, Jena, Germany). The reader was connected to a standard computer and the data were processed by a customized software supplied by the manufacturer (Check-Points, Wageningen, The Netherlands). The software automatically translated the *Salmonella* microarray data into a serovar name.

2.4. Premi®Test Salmonella procedure

Reagents and procedures were supplied in a kit provided by the manufacturer Check-Points (Wageningen, The Netherlands). Briefly, 10 µl of a 0.1 ng μl^{-1} solution of genomic DNA purified on a dedicated mini-column (Qiagen, Valencia, CA) was added to 8 µl of a freshly prepared proprietary mix containing ligation probes and thermostable DNA ligase. Alternatively, 10 µl of a crude bacterial extract (approximately 2×10^6 CFU μl^{-1}) obtained by boiling a water suspension for 10 min, was used as template material. The sample was heated in a MyCycler PCR instrument (Biorad, La Jolla, CA) during 3 min at 95 °C followed by 24 cycles of 0.5 min at 95 °C and 5 min at 65 °C followed by a final denaturation at 98 °C for 2 min. Next, 15 µl of a second proprietary exonuclease mix was added. The sample was incubated for 45 min at 37 °C and subsequently for 10 min at 95 °C to remove non-ligated LDR probes. Next, 15 µl of a third proprietary mix containing PCR primers, deoxynucleoside triphosphates and thermostable polymerase was added. The sample was heated during 10 min at 95 °C followed by 30 cycles of 0.5 min at 95 °C, 0.5 min at 55 °C, 1 min at 72 °C, and a final denaturation step of 2 min at 98 °C. The amplified ligation products (final volume 48 µl) were then subjected to DNA hybridization in a customized 1.5-ml ArrayTube® (Clondiag, Jena, Germany) containing a 2×2 mm microarray spotted with 100 DNA cZIP-code oligonucleotides complementary to a set of unique sequences included in the LDR probes. Hybridization was performed using 10 µl of each amplified reaction supplemented with 300 µl of pre-heated hybridization buffer and lasted for 30 min at 50 °C under rotational shaking (400 rpm) in a thermo-mixer (Eppendorf, Hamburg, Germany). Thanks to the use of 3

Table 1
Probe types and capture position on the DNA microarray

Probe target	Panel	Array-tube	Comments
Detection controls	None [a]	A1 A12 C1 C12 D1 D12 E12 F12 G12 H12 J1 J12	Biotinylated oligonucleotides spotted on the microarray and used as control spots for the staining process. Reference spots for image analysis software.
Hybridization control probes	None [a]	A2 D5 F2 I5	Biotinylated probe with blocked 3′-end complementary to spotted oligonucleotide at indicated array positions
Salmonella general LDR probes	Panel I	B7 B10 C3	These LDR probes bind to ubiquitous *Salmonella* sequences such as *invA* and other highly-conserved sequences (2).
	Panel II	E10 F4 F7	
	Panel III	I3 I7 I10	
Negative LDR controls	Panel I	B9 C2 C5	These LDR probes match the *Salmonella* general LDR probes but contain annealing mismatches aimed at adjusting the sensitivity threshold.
	Panel II	F3 F6 F9	
	Panel III	I6 I9 J2	
DNA controls	Panel I	B8 B11 C4	These LDR probes bind to a control (non-*Salmonella*) sequence spiked at suboptimal concentration in the ligation mix and yield detectable signals only in case of insufficient genomic DNA concentration.
	Panel II	E11 F5 F8	
	Panel III	I4 I8 I11	
LDR typing probes	Panel I	A3-11 B2-6	These LDR probes bind to critical *Salmonella* markers used to infer the serovar signature (2).
	Panel II	D6-11 E2-9	
	Panel III	G9-11 H2-11 I2	

[a] Out of panel position.

Table 2
Premi®Test *Salmonella* results summary

	Serovar	Number of strains typed	Serovar identifiers		
1	Adelaide	3	14857		
2	Agona	9	07204		
3	Albany	4	04149		
4	Altona	4	07180		
5	Anatum	6	14574		
6	Bareilly	4	02073	02601	
7	Blockley	7	06465		
8	Bovismorbificans	5	02246		
9	Braenderup	7	00426		
10	Brandenburg	7	06255	14447	
11	Bredeney	5	14399		
12	Cerro	10	02733	10919	
13	Chandans	3	14379		
14	Cholerasuis	2	06856		
15	Cubana	3	05195		
16	Derby	8	04146		
17	Dublin	9	05016		
18	Duisburg	4	10435		
19	Enteritidis	35	13202	00429	
20	Gallinarum	9	13186		
21	Give	5	14375		
22	Goldcoast	4	05636		
23	Hadar	15	14724		
24	Havana	8	05196	04654	
25	Heidelberg	5	02523		
26	Hithergreen	3	14853		
27	Ibadan	3	03658		
28	Indiana	5	02607		
29	Infantis	17	06789	07813	
30	Isangi	5	04696		
31	Kedougou	5	07207		
32	Kentucky	4	15414		
33	Kottbus	5	00384	06376	
34	Lexington	5	07176	05128	
35	Liverpool	4	09859	13955	
36	Livingstone	13	10304	13449	10592
37	London	5	10567		
38	Manhattan	9	04530	04538	
39	Mbandaka	5	15364		
40	Meleagridis	5	04772		
41	Minnesota	5	14373		
42	Montevideo	5	12335		
43	Munchen	3	12934		
44	Munster	4	12399 [a]		
45	Newport	6	00645	01701	14991
46	Ohio	4	02253		
47	Oranienburg	8	05193	14909	14383
48	Panama	5	14396		
49	Paratyphi B	3	02150		
50	Paratyphi B var. Java	9	08772	06246	
51	Poona	6	14381	14445	
52	Rissen	5	05126		
53	Sandiego	3	12399 [a]		
54	Saint-Paul	6	01438		
55	Schwarzengrund	7	14397	02634	12577
56	Senftenberg	26	04172	07245	
57	Tennessee	4	05149		
58	Thompson	6	02159		
59	Typhimurium	33	01039		
60	Virchow	11	15493		
61	Worthington	7	06145	07181	06144
62	Yoruba	4	02358		

[a] Identifiers associated with 2 different serovars.

independent probe sets, each ArrayTube® could detect 3 independent amplification reactions at once. Unbound DNA was washed away using two 5-min incubation steps with 300 µl blocking buffer in the same conditions. Biotin label detection was then performed with 150 µl of freshly prepared 0.125 µg ml^{-1} poly-horseradish peroxidase-conjugated streptavidin (Endogen, Rockford, IL) incubated for 15 min at 30 °C and shaken at 400 rpm. Excess conjugate was washed away by two 5-min incubation steps using 300 µl detection buffer in the same conditions. Finally, the detection reaction was initiated at room temperature by replacing the last washing solution by 150 µl of ready made TrueBlue™ peroxidase substrate (KPL, Gaithersburg, MD). DNA hybridization results were measured after 15 min on the reader.

2.5. PCR primers and ligation probes

The PCR primers had the following raw sequences: 5′-Biotin-GTAGACTGCGTACCAATTC-3′ (primer Eco) and 5′-GACGATGAGTCCTGAGTAA-3′ (primer Mse). The ligation probes had the following generic sequence: P-5′-TSL-TTACTCAGGACTCATCGTC-$(N)_x$-GTAGACTGCGTACCAATTC-ZIP-TSR-3′-OH where TSL and TSR are target-specific left and right arms, respectively. Underlined nucleotides are the PCR priming sites. ZIP defines 20–25 nucleotide-long sequences matching complementary capture probes (cZIPs) on the microarray.

2.6. DNA strains

Salmonella and non-*Salmonella* strains are listed in the Supplementary material (Tables S3 and S4, respectively).

3. Results and discussion

The system was evaluated on a number of strains of both human and animal origin isolated mostly between 1997 and 2005 in Belgium and The Netherlands in course of routine laboratory typing. The typing results are summarized in Table 2. The combination of positive and negative hybridization reactions resulted in a 14-digit binary code matching a particular *Salmonella* serovar (Andreoli et al., 2004). For better convenience, this code was converted into a unique identifier calculated with the following formula

$$\sum_{x=1}^{14} 2^{x-1}k$$

where x is the spot number considered and k a multiplication factor equals to 1 for positive hybridizations or 0 for negative hybridizations. For instance, when applied to serovar Braenderup, the Premi®Test *Salmonella* gives the 14-digit code 01010101100000 converted into the unique identifier 426 with the above formula. Typical microarray results are shown in Fig. 2. The use of 3 independent sets of identical LDR probes differing only by their ZIP codes allowed for the simultaneous typing of 3 strains per microarray (Fig. 2, Panels I to III). In total, 443 *Salmonella* and 43 non-*Salmonella* strains were tested. Both common and less common serovars were each represented as far as possible

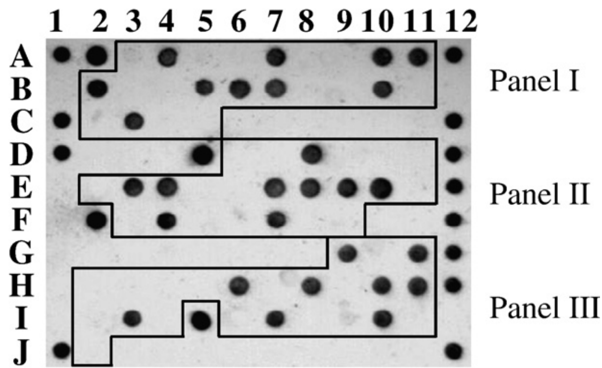

Fig. 2. Typical DNA microarray pictures obtained with the ArrayTube® 100-spot setup. This format uses a DNA microarray fixed at the bottom of a micro-reaction vial. The microarray consists in unique complementary (cZIP) oligonucleotides targeting individual LDR probes. When hybridization of the PCR-amplified ligation products to the microarray is complete, colorimetric detection of the positive reactions is initiated. Polygons delineate panels in the array. Each panel defines the typing results of one strain and consists in control spots and specific marker spots (listed in Table 1). The strains typed in this figure belong to serovar Enteritidis (Panel I), Hadar (Panel II) and Infantis (Panel III).

with several strains of different origin. Serovars associated with broiler chickens and regarded as important for human health, were the most represented. These included *S. enterica* sv. Enteritidis (35 strains), Hadar (15 strains), Infantis (17 strains), Typhimurium (33 strains) and Virchow (11 strains). Each strain was typed in parallel by slide agglutination using the classical serotyping scheme with antisera purchased from the Statens Serum Institute (Copenhagen, Denmark) and from BioRad (La Jolla, CA) according to the manufacturer's instructions. When required, additional biochemical tests were conducted to complete the identification. Phage typing (Ward et al., 1987) was performed on 17 out of the 35 strains of *S. enterica* sv. Enteritidis and 13 different phage types were found. Of the 443 *Salmonella* strains tested with the Premi®Test *Salmonella*, 84 unique profiles were identified compared to the 62 serovars found by classical serotyping. This means that some serovars were characterized by more than a single microarray pattern, which is a good indication of the system's sensitivity. Another critical sensitivity issue of the Premi®Test system was addressed by testing strains belonging to the closely related serovars Enteritidis, Gallinarum and Dublin (Porwollik et al., 2005). The results show that all these strains matched 4 distinct genetic identifiers, 2 of these being associated with serovar Enteritidis. In a more general way, clustering of *Salmonella* strains into discrete genetic profiles matched remarkably the serovar assignment, knowing that both typing methods rely on totally different markers. This observation is reminiscent to the one observed by authors working on totally different genetic markers (Sukhnanand et al., 2005) and argue in favor of a parallel evolution of the global genome diversity on one hand and of the variability of surface antigens on the other. The multiple microarray patterns observed within a single serovar were sometimes very similar, differing at a single spot only (sv. Brandenburg, Infantis, Lexington, Liverpool, Manhattan, Poona). Other serovars were much more heterogeneous (sv. Livingstone, Newport, Oranienburg, Schwarzengrund). The detailed strain typing data are provided as Supplementary material (Tables S3 and S4).

In conclusion, the microarray system assessed here was found robust and globally sensitive. As few as 10^5 CFU or genome equivalents were used routinely for strain typing. It gave satisfactory results on most crude *Salmonella* extracts in the absence of DNA purification. The analysis time (8 h) was relatively short compared to the barely predictable time scale of classical typing. The single-tube processing of the samples was found particularly convenient compared to other multiplexed analysis systems. The reading of the microarray results required a very simple device hosted in a standard molecular biology laboratory and required no peculiar technical expertise. The system could differentiate 2 or 3 genotype variants in 19 out of the 62 serovars tested, indicating superior discriminating capacity compared to serotyping. At the other hand, its current design did not allow to differentiate sv. Münster from sv. Sandiego. Complementary serotyping tests are thus required to finalize the identification of strains belonging to these peculiar serovars. Given this limitation, it can be anticipated that other instances of *Salmonella* serovars with overlapping Premi®Test identifiers may be found if much more serovars are analyzed. The open design of the LDR method on one hand and of the ZIP-code microarray on the other, renders the system easily improvable to reach the sensitivity level required for the typing of hundreds of different serovars. Future releases of the Premi®Test *Salmonella* should therefore include additional genetic markers to increase the discriminating power of the test. In addition, extensive strain testing will be required to strongly validate the system before it can be envisioned as a trustable alternative to classical serotyping. The current system sensitivity, however, is sufficient for the preliminary identification and typing of *S. enterica* subsp. *enterica* by first-line laboratories as well as laboratories in charge of sanitary controls in breeding farms and food industries. Assessment of the Premi®Test *Salmonella* on DNA directly extracted from food samples following enrichment procedures should be also investigated in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijfoodmicro.2008.01.006.

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