Oligonucleotide microarray for molecular characterization
and genotyping of Salmonella spp. strains

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Objectives: To characterize and subtype multidrug-resistant Salmonella isolates by determining the virulence factors, prophage sequences and antimicrobial resistance genes using a novel Salmonella-specific oligonucleotide microarray.

Methods: Preliminary screening of 24 Salmonella clinical isolates was carried out by using susceptibility testing, plasmid profiling and class 1 integron PCR. Subsequently, oligonucleotide microarray was involved in genotypic characterization and localization of monitored genetic markers. The presence of antimicrobial resistance genes was also detected and confirmed by PCR and subsequent sequencing. The potential spread of emerging blaSHV-2 was investigated by bacterial conjugation.

Results: All Salmonella strains revealed resistance to two or more (up to nine) antibiotics. Nineteen of them carried class 1 integrons including dfrA1, dfrA12, aadA1, aadA2, blaPSE-1 and blaTEM-1 gene cassettes, respectively. Twenty-three out of 24 Salmonella isolates possessed one or more plasmids. Oligonucleotide microarray characterization and typing revealed the conserved character of Salmonella pathogenicity island virulence factors among three Salmonella enterica serovars, significant variability in prophage sequences and many different antimicrobial resistance gene patterns. Differential labelling of genomic and plasmid DNA, respectively, and hybridization to the microarray made it possible to localize important resistance determinants. Microarray results were successfully confirmed and verified by using PCR. The emerging blaSHV-2 gene from Salmonella Kentucky SK10944 conferring resistance to ceftriaxone and cefotaxime was transferred via bacterial conjugation to Escherichia coli K-12 3110.

Conclusions: Salmonella isolates were quickly and thoroughly characterized by a novel oligonucleotide microarray, which could become a useful tool for detection of virulence and resistance genes and monitoring of their dissemination among salmonellae and closely related bacteria.

Keywords: multidrug resistance, virulence, conjugation

Introduction

The Salmonella genus consists of two species: Salmonella bongori and Salmonella enterica, which itself is further divided into six subspecies: enterica, salamae, arizonae, diarizonae, indica and houtenae. Based on the agglutinating properties of three different types of antigens (somatic O, flagellar H and capsular Vi), genus Salmonella can be differentiated into more than 2500 serovars, which can be further subdivided by using methods such as phage typing. Serovars within S. enterica subsp. enterica cause almost all (>95%) of salmonella infections in humans as well as in animals.1 Salmonellosis manifests itself in several forms ranging from asymptomatic colonization through gastroenteritis to severe systemic illness or typhoid fever.

The differences in pathogenicity of various Salmonella serovars result from fitness and virulence potential of the microorganism and the susceptibility of the host, which is mainly determined by age, genetics and environmental factors. It has been estimated that ~4% of the Salmonella Typhimurium

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genome including over 200 virulence genes is required for the fatal infection of a mouse. Virulence genes can be found individually within the chromosome or on plasmids, however the majority of them are clustered in regions called pathogenicity islands (PAIs or Salmonella pathogenicity islands—SPIs). To date, 12 SPIs have been described. Some of them are conserved throughout the genus, while others are specific for certain serovars. For instance, well-conserved SPI-1 and SPI-2 encode different type III secretion systems (TTSS) and effector proteins, which enable Salmonella to efficiently penetrate the intestinal epithelium and to survive and replicate within the host cells, respectively. SPI-7 is serovar specific (Salmonella Typhi, Salmonella Dublin and Salmonella Paratyphi C) and encodes Vi antigen, SopE effector protein and type IV pilus.

DNA of bacteriophage origin often comprises 10–20% of the bacterial genome. Four functional bacteriophages (Gifsy-1, Gifsy-2, Fels-1 and Fels-2) are present in the genome of Salmonella Typhimurium LT2 and similar numbers have been observed in other strains. Many of these prophages carry extra genes such as rfb, sodC, nanH as well as genes encoding for TTSS effector proteins, which can provide their hosts with a selective advantage and/or increase their virulence.

Plasmids are frequently found in the majority of serovars associated with infections of humans and animals. Eight S. enterica serovars are known to harbour a serovar-specific virulence plasmid typically 50–100 kb in size. Further, Salmonella can possess additional low molecular weight plasmids under 20 kb, which are usually of unknown function, or high molecular weight plasmids up to 200 kb, which can transfer resistance to antibiotics. The antibiotic resistance genes are often located within transposons. Moreover, resistance genes can also be found in the form of gene cassettes captured and clustered in integrons and thereafter mobilized to spread resistance among other organisms. Recent studies show the emerging increase of multidrug-resistant (MDR) isolates of Salmonella worldwide. Epidemiologically relevant is the association of the plasmids carrying multiple resistance markers with the other virulence genes of Salmonella. Such a recombination process provides Salmonella not only with a survival advantage in hostile conditions, but also with the opportunity to speed up evolution into a new genetic lineage.

Virulence genes located either on the chromosome or virulence plasmids, bacteriophage sequences, transposons and integrons and finally resistance genes could be used as molecular diagnostic markers for identification of the bacterial pathogen, estimation of its pathogenic potential and its antibiotic resistance pattern. It is impossible to encompass such a broad palette of genetic markers with traditional molecular techniques based on PCR. However, recent technical advances have made it possible to detect thousands of sequences simultaneously using DNA microarray or DNA chip technology. Several studies have reported the construction of DNA microarrays for the detection of antimicrobial resistance genes and also important virulence factors either covering a whole genus/family or limited to the most important species. The first DNA chips comprised spotted PCR products, which provided high sensitivity, but failed to distinguish between closely related gene family members. The introduction of synthetic short oligonucleotides (25–40-mers) paved the way to a convenient method for the preparation of DNA chips with adequate sensitivity and additional benefit of high discrimination capability.

This study presents the development and application of an oligonucleotide DNA chip for the molecular analysis of genetic content, which allows for the identification of pathogenic potential, antibiotic resistance profile and typing of Salmonella isolates. Further, we have made a substantial effort to differentiate the localization of genetic markers carried on either chromosome or plasmids, which has significant impact on the monitoring of their transfer, dissemination and acquisition among related species. The Salmonella-specific oligonucleotide DNA chip presented also includes a control probe set carefully fine-tuned to serve for normalization, but also for relative quantity estimation of present markers.

Materials and methods

Strains, growth conditions and DNA isolation

Two sets of bacterial strains were used in this study. The first set consisted of well-characterized S. enterica serovars, MDR Escherichia coli and Staphylococcus aureus selected from the National Reference Centre — Collection of the Cultures of Pathogenic Microorganisms (Bratislava, Slovakia) and represented positive and negative controls for all oligonucleotides probes printed on the DNA chip. The rifampicin-resistant E. coli K-12 3110 recipient strain used for the conjugation assay was included in this set. Completely sequenced and microarray-characterized Salmonella Typhi CT18 was selected for further optimization of microarray analysis parameters.

The second set of 14 Salmonella Typhimurium, 7 Salmonella Kentucky and 3 Salmonella Enteritidis selected from the strain collection of the National Reference Centre for Phage Typing of Salmonellae (Bratislava, Slovakia) represented MDR clinical isolates from patients suffering from various forms of gastroenterocolitis during the years 2002–2006 (Table 1). Selected strains represent the most commonly reported serovars from cases of human infections in the Slovak Republic. The Salmonella Kentucky strains caused nosocomial outbreaks in two regions of Slovak Republic. All strains were biochemically confirmed using the ENTEROTET 16 (Lachema, Brno, Czech Republic) and were serologically characterized by slide-agglutination tests with commercial Salmonella somatic (O) and flagellar (H) antisera (Sanofi Diagnostic Pasteur, Marnes-la Coquette, France). Phage typing of Salmonella Typhimurium and Salmonella Enteritidis isolates was done according to the Anderson et al. and Ward et al. schemes, respectively.

Cells were grown on LB medium at 37 °C. Genomic DNA was isolated by either using a Genomic DNA Purification kit (Promega, USA) or a DNeasy Tissue Mini kit (Qiagen, Germany). Plasmids were purified using a Large-construct kit (Qiagen).

Susceptibility testing

Antimicrobial susceptibility was determined by the disc diffusion method according to the CLSI (formerly NCCLS) criteria on Mueller–Hinton agar plates. The following antimicrobial agents with the respective quantities of active compound (in μg/disc) were tested: ampicillin 10, cefotaxime 30, ceftriaxone 30, chloramphenicol 30, ciprofloxacin 5, gentamicin 10, streptomycin 10, sulfisoxazole 250, tetracycline 30, trimethoprim 5 and a combination of trimethoprim/sulfamethoxazole 1.25/23.75. E. coli ATCC 25922 was used as a control. The results were interpreted according to the CLSI criteria.
Salmonella genotyping by microarray

Table 1. Test set of Salmonella strains with preliminary characteristics used in this study for microarray hybridization

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phage type</th>
<th>Phenotype</th>
<th>Plasmid profile (kb)</th>
<th>Class I integrons (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella Typhimurium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STM10/02</td>
<td>DT193</td>
<td>AMP, CHL, CIP, GEN, STR, SUL, TET, TMP</td>
<td>90, 12, 6, 5</td>
<td>1.9 (dfrA12-ORFX-aadA2)</td>
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<td>AMP, STR, SUL, TET, TMP</td>
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<td>1.6</td>
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<tr>
<td>STM3/03</td>
<td>NT</td>
<td>GEN, STR, SUL, TET</td>
<td>10, 8, 6, 5, 2.1</td>
<td>1.0 (aadA1)</td>
</tr>
<tr>
<td>STM8/03</td>
<td>U302</td>
<td>AMP, STR, SUL, SXT, TET, TMP</td>
<td>90</td>
<td>1.6 (dfrA11-aadA1)</td>
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<tr>
<td>STM13/03</td>
<td>DT41</td>
<td>STR, SUL</td>
<td>90, 10, 7, 5, 5, 4</td>
<td>—</td>
</tr>
<tr>
<td>STM21/03</td>
<td>DT104</td>
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<td>1.0</td>
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<tr>
<td>STM29/03</td>
<td>DT104</td>
<td>AMP, STR, SUL, TET</td>
<td>90</td>
<td>1.2 (blaTEM-1)</td>
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<tr>
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<td>DT104</td>
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<td>1.0</td>
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<tr>
<td>STM35/04</td>
<td>DT104</td>
<td>AMP, CHL, STR, SUL, TET</td>
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<td>1.0</td>
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<td>DT104</td>
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<td>90, 70, 35</td>
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<td>STM13/04</td>
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<td>1.2</td>
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<tr>
<td>STM25/04</td>
<td>DT119</td>
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<td>5.6, 3.5</td>
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<td>NT</td>
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<td>90, 50, 6, 7, 4.3</td>
<td>1.6, 1.2, 0.65</td>
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<tr>
<td>STM8/05</td>
<td>NT</td>
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<td>90</td>
<td>6.7, 4.3</td>
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<td>Salmonella Enteritidis</td>
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<tr>
<td>SENT74/02</td>
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<td>AMP, SUL, TET</td>
<td>60, 9, 8, 4.1, 2.7, 1.8, 1.6</td>
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<tr>
<td>SENT81/02</td>
<td>NT</td>
<td>AMP, SUL, TET</td>
<td>80, 60, 4, 9, 2.9</td>
<td>—</td>
</tr>
<tr>
<td>SENT4/03</td>
<td>NT</td>
<td>AMP, STR, SUL, TET, TMP</td>
<td>60</td>
<td>—</td>
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<tr>
<td>Salmonella Kentucky</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK4854</td>
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<td>AMP, CHL, CIP, GEN, STR, SUL, SXT, TET</td>
<td>3.6, 2.4</td>
<td>1.6</td>
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<tr>
<td>SK11411</td>
<td>N/A</td>
<td>AMP, CHL, CIP, CRO, CTX, GEN, STR, SUL, TET</td>
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<td>1.6</td>
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<td>SK3297</td>
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<td>90, 3.6, 2.4</td>
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<td>SK173/05</td>
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<td>AMP, CIP, GEN, STR, SUL, TET</td>
<td>—</td>
<td>1.6</td>
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<tr>
<td>SK10944</td>
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<td>AMP, CHL, CIP, CRO, CTX, GEN, STR, SUL, TET</td>
<td>3.6, 2.4</td>
<td>1.2, 1.0</td>
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<tr>
<td>SK4707</td>
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<td>AMP, STR, SUL, TET</td>
<td>3.6, 2.4</td>
<td>1.6</td>
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<tr>
<td>SK14515</td>
<td>N/A</td>
<td>AMP, CIP, STR, SUL</td>
<td>40, 3.6, 2.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

NT, non-typeable; N/A, not available; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; GEN, gentamicin; STR, streptomycin; SUL, sulfisoxazole; SXT, sulfamethoxazole/trimethoprim; TMP, trimethoprim; TET, tetracycline.

Plasmid profiling

Plasmid profiling was essentially conducted as described previously. All strains were grown on blood agar plates at 37°C overnight. Inoculum of corn size was used for plasmid isolation. Samples were analysed by electrophoresis in 1× TBE buffer in 0.7% agarose gel. Plasmid sizes were determined according to the reference E. coli V517 strain, Salmonella Typhimurium LT2 strain and supercoiled DNA ladder (Gibco BRL, Paisley, UK) by using Gel-Pro Analyzer v4.5 (MediaCybernetics, USA).

Class I integron detection and sequence analysis

The PCR detection of class 1 integron content was performed essentially as described by Majtan et al. The PCR products were excised after electrophoresis on a 1% agarose gel and subsequently purified using the QIAEX II kit (Qiagen). Sequencing was performed in both directions by the BigDye method on an ABI 3100-Avant Genetic Analyzer (Applied Biosystems, USA). Obtained sequences were combined using Vector NTI Advance 10 (Invitrogen, USA). The similarity search was carried out using the BLAST algorithm.

Oligonucleotide probe and PCR primer design

The target genes were selected mainly from virulence genes, bacteriophage elements and antimicrobial resistance genes. After rigorous selection of target genes and obtaining the primary template sequences from the GenBank database, BLAST analysis was performed against the NCBI database to retrieve as many similar and related sequences for each target gene as possible and also to determine the segments of each gene with highest cross homology to unrelated sequences. The relevant BLAST results were aligned with a primary template resulting in 59 consensus sequences, which were subsequently used for the design of 59 oligonucleotide probes specific either for the individual gene or for a group of closely related genes with the same biological function. The set of possible 33–35-mer probes for each consensus sequence was designed by using Primer3 with regards to the following parameters: melting temperature Tm, GC content, oligonucleotide self-complementarity and the presence of polymeronucleotide and dinucleotide repeats. The probes for each of the genes, which were closest to an average Tm of 74°C and which had a GC content between 35–65%, were again analysed by BLAST to determine uniqueness of the probe to the target gene of interest and by M-fold to check for the presence of stable secondary structures. The probes which did not pass both analyses were discarded and the selection process was repeated until a suitable probe was designed for each target gene. A similar procedure was applied for the design of spike-in control probes, which were selected from a Salmonella-unrelated biological system from our previous microarray transcriptional profiling study. The final 66 probes were synthesized and HPLC-purified by VBC-Genomics (Austria). Probe sequences and their properties are listed in Table S1 [available as
Supplementary data at JAC Online (http://jac.oxfordjournals.org/) and also stored in the GEO database under accession number GPL5093.

Primers for PCR validation of microarray results were designed only for selected resistance genes for several reasons. First, we expected the highest variability in the microarray results for this group of genes, which should be verified. Second, the obtained PCR products could be sequenced and thus provided the ultimate proof about hybridization target identity. Third, primers against resistance genes could be used independently in other studies. The list of primer pairs synthesized by Invitrogen (Austria) with all relevant properties is shown in Table S2 [available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)].

Spike-in mix preparation
A new spike-in control system was developed for labelling and hybridization quality control, normalization and estimation of signal quantity of hybridized targets. Briefly, the spike-in control system consists of 7 oligonucleotide probes and 200 bp PCR products corresponding to the probes adopted from our previous microarray experiment. Control oligonucleotide probes were spotted along with the rest of Salmonella-related probes. Appropriate amounts of PCR products corresponding to final 10 ng DsRed, 5 ng dotA CB, 2.5 ng BFK20ORF02, 1.0 ng BFK20ORF12 and 0.5 ng BFK20ORF41 probe per one labelling reaction were mixed together and spiked into each labelling reaction to provide positive and dynamic range controls. PCR products corresponding to BFK20ORF24 and BFK20ORF47 were not included in the labelling mix, thus providing negative controls.

Oligonucleotide chip construction
Lyophilized oligonucleotide probes were dissolved in 1× Micro Spotting Solution Plus (Telechem Int., USA) to a final concentration of 50 μmol and transferred into sterile 384-well microplate. The plate was subsequently attached into a Pixsys 5500 microarrayer (Telechem Int.). Probes were deposited onto the SuperAmine or SuperEpoxy slides (Telechem Int.) in four replicates per grid and two grids per slide, which allowed for two separate experiments at one time. Each grid thus consists of two identical subgrids, whose layout is shown in Figure 1. DNA microarray printing was performed at constant temperature (20°C) and humidity (42%). DNA microarrays were dried overnight at room temperature. The SuperAmine slides were briefly rehydrated over boiling water and subsequently baked for 2 h at 80°C. The spotting result was verified using a CheckIt Chips Kit (Telechem Int.).

DNA fluorescent labelling
The total DNA from all bacterial strains was fluorescently labelled via random priming. Briefly, the reaction consisting of 9 μg of random primer (Invitrogen, USA), 5 μg of total DNA and control spike-in mix was heated for 10 min at 70°C and immediately chilled on ice. Total DNA was labelled using 50 U of Klenow fragment exonuclease (-NEB, UK) in the presence of 2 mM dATP, dTTP, dGTP, 1 mM dCTP and 1 mM Cy5-dCTP (Amersham Biosciences, UK). The reaction was incubated at 37°C overnight. Unreacted dye was removed using a QIAquick PCR Purification Kit (Qiagen). Labelling of plasmid DNA followed essentially the same protocol except Cy5-dCTP was substituted with Cy3-dCTP. All dual-channel microarray experiments were performed as dye swapped experiments.

Hybridization and washing
Microarray slides were incubated in preheated (42°C) pre-hybridization buffer (5× SSC, 0.1% SDS and 1% BSA) for 1.5 h with an initial 20 min of vigorous shaking. After incubation, slides were rinsed 4× with double distilled water (ddH2O) and subsequently immersed into boiling ddH2O for 2 min. DNA probes were immediately fixed by dipping the slides into pre-chilled 100% isopropanol for 5 s. Slides were dried with compressed air and placed into a thermostat at 42°C until hybridization.

Hybridization was carried out in a Hybridization Chamber (Corning, USA). Vacuum concentrated fluorescently labelled DNA (either only Cy5-labelled genomic DNA or a mixture of Cy5-labelled genomic and Cy3-labelled plasmid DNA and vice versa) was completely dissolved in 15 μL of the 1× HybIt 2 buffer (Telechem Int.). Samples were subsequently denatured at 95°C for 5 min, immediately chilled on ice and spun in a microcentrifuge. Afterwards, they were incubated at 60°C until applied onto the DNA chip. The hybridization was performed overnight for 16 h at 42°C. Then the slides were washed using an initial wash in 2× SSC and 0.1% SDS preheated to 50°C for 5 min, followed by a 5 min wash in 1× SSC and finished by 1 min wash in 0.5× SSC both at room temperature. The slides were immediately dried with compressed air.

Normalization and data analysis
Fluorescence images were recorded by scanning the slides using a GeneTAC UC4 microarray scanner (Genomic Solutions, USA) at 10 μm resolution. Fluorescence signal intensities were measured using SpotFinder20 by adaptive shape quantification followed by local background subtraction. The medians of the quadruplicate spots were recorded and further analysed by using Microsoft Excel. Intensity values from spike-in controls were used to calculate the correction factor, which was subsequently applied to adjust intensities from spots of interest. All spots with adjusted intensity over signal from negative controls plus three standard deviations were considered as positive. Dual-channel experiments followed exactly the same procedure; however, intensities were additionally corrected according to ratio factor from spike-in controls and dye swap experiments. Technical replicates were tested for statistical significance by using standard deviation. Biological replicates were compared with each other for statistical correlation by using Student’s t-test in case of two independent isolations for single channel experiments or ANOVA from three independent isolations for dual-channel experiments.

PCR validation
PCRs were performed only for antimicrobial resistance genes except genetic markers conferring resistance against macrolides [erm(A), erm(B)], tetracyclines [tet(A), tet(G)] and ammonium compounds [qac(D)]. PCRs were conducted on the UNO-II thermocycler (Biometra, Germany) in a total volume of 50 μL containing 0.5 mM each dNTP and an appropriate primer pair at a concentration of 0.5 μmol each, 1.0 U of Taq DNA polymerase (NEB) and 10 ng of template DNA. The cycling conditions were as follows: an initial denaturation at 96°C for 5 min, followed by 36 cycles at 94°C for 1 min, 56°C for 45 s and 72°C for 1 min and completed by a final extension for 5 min at 72°C. The quality of PCR amplicons was examined by electrophoresis on a 1.2% agarose gel along with GeneRuler 100 bp DNA Ladder (Fermentas, Germany) as a marker.
To determine the transmissibility of the resistance determinants, donor *Salmonella* Kentucky SK10944 was conjugated with recipient *E. coli* K-12 3110 as described by Allen and Poppe with modifications. Briefly, both donor strain and recipient strain were grown at 37°C to an OD<sub>550</sub> of ~0.5. The recipient strain was grown for 15 min at 45°C prior to mating. The donor and recipient cells (1:1) were conjugated for 60 min at 37°C and then pelleted by short centrifugation. Cell pellet was resuspended in 250 μL of LB and subsequently plated on the Tergitol-7 (HiMedia, India) agar plates with all possible antibiotic combinations: 100 mg/L rifampicin (recipient marker) and 10 mg/L streptomycin, 30 mg/L chloramphenicol and 15 mg/L cefotaxime or ceftriaxone (donor markers). Purified plasmid DNA from the obtained transconjugants was finally analysed using DNA chip and PCRs, respectively.

**Results and discussion**

**Preliminary characterization of test strains**

The 24 *Salmonella* clinical isolates were subjected to preliminary characterization by using antibiotic susceptibility testing,
plasmid profiling and analysis of class 1 integrons. The antimicrobial resistance phenotypes, plasmid profiles and class 1 integron analysis are summarized in Table 1 supplemented with phage type information for serovars Typhimurium and Enteritidis.

Two DT104 strains exhibited characteristic pentarresistance to antibiotics (ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline—ACSSuT), two DT104 strains were resistant to four antibiotics (ampicillin, streptomycin, sulfisoxazole, tetracycline—ASSuT) and in one isolate with pentarresistance, the additional resistance to trimethoprim/sulfamethoxazole was found. Isolates of DT193, U302 phage types, as well as non-typeable isolates were resistant to four to eight antibiotics. Only one isolate of DT193 phage type was resistant to ciprofloxacin.

The incidence of MDR Salmonella Typhimurium strains has increased in the past decades worldwide. Most of this increased incidence is due to the concurrent upsurge of DT104, but other phage types, such as U302, DT12, DT21 and DT193 may also play a role in this development. Multidrug resistance was found in three non-typeable Salmonella Enteritidis strains. Selected Salmonella Kentucky strains were characterized as MDR to four to nine antibiotics, including ciprofloxacin and the extended-spectrum β-lactams ceftriaxone and cefotaxime.

Nineteen (86.4%) of the MDR Salmonella isolates were found to carry class 1 integrons. Out of five Salmonella Typhimurium DT104 strains, four yielded a 1.0 kb and one yielded a 1.2 kb PCR amplicon. The 1.2 kb PCR amplicon was sequenced and found to carry a blaTEM-1 gene cassette. One DT193 strain yielded a PCR amplicon 1.9 kb in length, which was found to carry a dfrA12-ORFX-aadA2 gene cassette arrangement. Three Salmonella Enteritidis isolates showed multidrug resistance phenotypes, but did not contain any integrons. Seven MDR Salmonella Kentucky strains carried integrons with PCR amplicons 1.0, 1.2 and 1.6 kb in size (Table 1).

Sequencing of PCR amplicons from Salmonella Kentucky SK3297 revealed the presence of blatem-1 and aadA2 gene cassettes embedded within class 1 integrons. Such integrons have been described previously in studies on animal, clinical and food isolates.

Table 1 shows the distribution of plasmids among isolates included in the study. All Salmonella Typhimurium isolates, except one, carried a 90 kb serovar-specific virulence plasmid alone or with additional plasmids. The 60 kb serovar-specific virulence plasmid was detected in all Salmonella Enteritidis strains. The plasmid profile with two plasmids of 3.6 and 2.4 kb in length was observed in six Salmonella Kentucky strains. One isolate harbouring a 90 kb plasmid and one isolate harbouring a 40 kb plasmid. One isolate did not possess any plasmid.

**Microarray analysis of test strains**

Microarray genotypic profiles of 24 clinical isolates and one transconjugant for the presence/absence of mainly pathogenicity-related genes, phage-associated genes and antimicrobial resistance genes are summarized in Figure 2. Several probes gave discordant results when compared with either PCR analysis or resistance phenotypes, i.e. the aminoglycoside resistance probes aadA1/2/7 and aph(3’)-Ic/A7 provided a false negative signal in three Salmonella Typhimurium strains when compared with susceptibility testing and PCR analysis. The inability to detect particular sequences may occur for different reasons. The most likely reason is related to the divergence in sequence region corresponding to the probe. Such a situation is equally likely as a failure of PCR detection could be due to divergence within the region complementary to the PCR primers.

Conversely, microarray detected catA1 in Salmonella Typhimurium STM29/03, catB3/8 in Salmonella Typhimurium STM25/04 and floR in Salmonella Typhimurium STM32/03, which did not correspond to susceptibility testing. The PCR analysis, however, agreed with the microarray results. This result demonstrates the advantage of molecular methods in the detection of silent genes that are either not phenotypically expressed in vitro or are defective in promoter sequences or other regulatory regions. Screening for the presence of silent genes using molecular typing methods such as DNA microarray should be very helpful in the rapid identification and further tracking of silent genes within the resistance gene pool, which could be easily activated by simple transposition.

**Pathogenicity-related markers**

The 16 pathogenicity-related markers were monitored using DNA microarray. These include mainly genes coding for effector proteins associated with SPIs and fimbriae.

Our results show the minimal variability of virulence factors encoded by SPIs, which suggests that their integrity is important for virulence. However, we observed that two Salmonella Typhimurium isolates were defective in spaO and three Salmonella Kentucky strains were missing stpP. PCR analysis verified this observation (data not shown). Both virulence factors are located within SPI-1. If the pathogenicity of these strains is altered by missing virulence factors, the symptoms of infection could be more moderate.

Using DNA microarray, we also detected genes encoding putative virulence factors bigA, Salmonella enterotoxin stn and type 1 fimbriae chaperone fimC in all strains tested. It was demonstrated that all S. enterica serovars carry stn, but only a limited portion of them also express the enterotoxin phenotypically. Type 1 fimbriae encoded by the fim gene cluster are the best characterized out of 13 identified Salmonella fimbrial gene clusters so far. Unlike chromosomally located fim fimbriae, the pef fimbrial operon is localized on the 90 kb Salmonella virulence plasmid. On the contrary, our results suggest that the association of pef fimbrial cluster should not be so strictly bound to the Salmonella virulence plasmid. The Salmonella Typhimurium STM29/03 strain possesses a 90 kb virulence plasmid, as was confirmed by both plasmid profile analysis and strong fluorescence signal from the spvA probe. However, microarray analysis indicated the absence of pefA signal for this strain. Negative microarray result was supported by PCR analysis (data not shown). The independent distribution of pef fimbrial genes from the Salmonella virulence plasmid is further supported by microarray analysis of all Salmonella Kentucky strains tested. We found that the analysed Salmonella Kentucky strains did not possess virulence plasmids (no signal from spvA probe), but five out of the seven tested strains provided strong signal from the pefA probe. Plasmid profiling of these strains revealed the presence of two small plasmids 3.6 and 2.4 kb in length, respectively, whereas Salmonella Kentucky SK3297 contained an additional 90 kb plasmid. Together, all these results suggest that the pef fimbrial locus is most likely not strictly associated with Salmonella virulence plasmid. However, the
Figure 2. DNA microarray profiles of the 24 *Salmonella* test strains and one *E. coli* transconjugant compared with PCR and susceptibility testing results, respectively. Positive results by both DNA microarray and PCR analysis compared with susceptibility testing are indicated by black filled blocks, whereas negative results are indicated by open white blocks. A white block with black diagonal lanes indicates target sequence detected by antimicrobial susceptibility testing and also PCR analysis, but was not confirmed by microarray analysis. A white block with black horizontal lanes indicates target sequence detected by DNA microarray and confirmed by PCR analysis, but does not correspond to susceptibility testing. The PCR validation was performed only for antimicrobial resistance genes except genetic markers conferring resistance against macrolides (*erm*(A), *erm*(B)), tetracyclines (*tet*(A), *tet*(G)) and ammonium compounds (*qac*(DE)).
question of whether the pef operon can be carried on a chromosome or on a low molecular weight plasmid should be answered by further analysis.

Salmonella Enteritidis-specific fimbrial operon sef was detected in all three Salmonella Enteritidis strains. Moreover, all three Salmonella Enteritidis strains possess all of the pathogenicity-related genetic determinants monitored by DNA microarray.

Prophage-associated genes

Prophages or phage-like elements may contribute to the pathogenicity of respective host. Moreover, since the distribution of bacteriophages in different strains varies, detection of specific bacteriophage sequences could serve as a method for the subtyping of isolates, especially those that are non-typeable by other methods or even substitute phage typing.

In our study, we monitored the presence or absence of seven bacteriophage-associated genetic determinants. Distribution of genes coding for two phage-encoded type III secreted effector proteins GipA and GtgB was quite variable within the set of tested strains (Figure 2). The bacteriophage ST104 ORF45 gene was detected exclusively in the Salmonella Typhimurium strains with the exception of two phage non-typeable strains Salmonella Typhimurium STM6/05 and STM8/05. The bacteriophage ST104 encoded sieB was found in 11 out of 14 Salmonella Typhimurium strains and also in Salmonella Kentucky SK173/05. The rest of the tested prophage genetic markers eae, g13 and g8 are encoded by bacteriophage P22 and several P22-like bacteriophages. The g8 coding for scaffolding protein was present in all Salmonella Kentucky as well as Salmonella Typhimurium strains except Salmonella Typhimurium STM13/03. On the other hand, holin-encoding gene g13 was not detected in any strain tested. The eae gene was found only in two Salmonella Typhimurium strains, STM15/02 and STM8/03. All together, our results show great variability in selected prophage markers within all tested S. enterica serovars, which is in agreement with other studies. Our results pointed out that DNA microarray analysis of prophage elements could be a promising tool for subtyping and clustering of strains within certain ‘phage type’ groups. After addition or extension of the presented DNA oligochip with other phage-derived probes, such a DNA chip could serve as a molecular alternative to phage typing.

Antimicrobial resistance genes

More than half of the probes spotted on the DNA chip are devoted to the determination of an antimicrobial resistance profile. The DNA microarray contains 31 probes identifying eight different classes of antibiotics and antimicrobial compounds as well as 2 additional probes for the detection of class 1 and class 2 integrons, respectively.

Resistance against aminoglycosides was detected by four probes. We detected almost universal presence of the aadA gene among all tested Salmonella serovars. The aadB gene was detected in two Salmonella Typhimurium strains STM13/03 and STM13/04. According to the results of Vo et al., the aadA2 gene present on the SGI-1 could be replaced by aadB, thus creating a new SGI-1 variant. The aph gene was detected only in Salmonella Typhimurium STM25/04. However, PCR validation and subsequent sequencing of relevant PCR amplicons showed the presence of an aph gene in two Salmonella Typhimurium strains STM25/04 as well as STM13/04.

From 10 different types of β-lactamases monitored by this DNA chip, the most widespread β-lactamase among Salmonella Typhimurium and Salmonella Enteritidis serovars is non-ESBL blaPSE-1, contrary to blaTEM-1 mainly detected in Salmonella Kentucky isolates. In Salmonella Typhimurium STM10/02, we detected blaCXA-30 besides blaPSE-1. The most important finding was the detection of ESBL blaSIV-2 in two Salmonella Kentucky SK11411 and SK10944 strains, respectively, conferring resistance against ceftriaxone and cefotaxime. The ESBL blaSIV-2 enzymes are now becoming increasingly common in S. enterica serovars from Europe and the UK.

Resistance against chloramphenicol was determined by the presence of catA1 in eight Salmonella strains, whereas catB3/8 was present in only one strain. In all Salmonella Typhimurium DT104, but one, the floR gene was detected.

Dihydrofolate reductases dfrA1 and dfrA5/14 conferring resistance to sulphonamide inhibitors were found to be the most prevalent among the tested strains. dfrA7/17 was detected only in Salmonella Typhimurium STM5/05 and dfrA12/21 in Salmonella Typhimurium STM10/02, respectively. Closely related to sulphonamide resistance is resistance against trimethoprim. We detected the sul1 gene in all tested strains except two Salmonella Typhimurium and one Salmonella Kentucky (Figure 2). The sul2 gene was also detected in a majority of analysed strains. Our results clearly support the high prevalence of trimethoprim and sulphonamide resistance among Salmonella and pathogenic bacteria in general as has been documented recently.

Another very common type of antimicrobial resistance is resistance to tetracycline. The tet(G) gene usually associated with SGI-1 was present in all tested tetracycline-resistant Salmonella strains except Salmonella Kentucky SK173/05, which contains the tet(A) gene. Apart from that, the tet(A) gene was detected in five other strains and always along with the tet(G).

The DNA microarray contained probes int1 and int2 for detection of class 1 and class 2 integron-specific integrase genes, respectively. The signal from int1 probes follows the presence of PCR amplicons for detection of class 1 integrons. We did not detect the presence of class 2 integron by using DNA microarray.

The resistance markers ere(A) and erm(B) conferring resistance to erythromycin and other macrolides were not detected in any of the Salmonella isolates tested. Our findings are consistent with previously published studies as these resistance genes are widespread predominantly in Gram-positive bacteria.

Localization of monitored genetic markers

Purified genomic DNA and plasmid DNA from the same Salmonella isolate were differentially labelled, mixed together and hybridized to the Salmonella oligonucleotide microarray. After successful hybridization of differentially labelled chromosomal DNA and plasmids from completely sequenced Salmonella Typhi CT18, a localization study was performed on one representative MDR Salmonella strain possessing one or more plasmids from each of the tested serovars. Corresponding microarray scans are shown in Figure 1(b). For Salmonella Typhimurium STM10/02 (Figure 1b), dual-channel microarray analysis revealed the presence of a serovar-specific virulence
plasmid (spvA) and plasmid-encoded fimbriae (pefA) on a plasmid. In addition, five resistance genes (aadA, dfrA12/21, sul1, sul2 and qacΔE) and class 1 integrase (int1) were also harboured on plasmids. Regarding the class 1 integron PCR analysis result, we suggest that aminoglycoside (aadA2) and trimethoprim (dfrA12) resistance gene cassettes are harboured in the same class 1 integron, which is carried on a plasmid. The virulence plasmid and Pef fimbriae were also detected in *Salmonella* Enteritidis SENT4/03 (Figure 1b). A different palette of resistance genes (blaTEM, dfrA5/14 and sul2) was located on serovar-specific virulence plasmid considering the presence of only one 60 kb plasmid in this isolate according to plasmid profile analysis. The results obtained from plasmid profiling, class 1 integron PCR analysis and single channel microarray analysis are also consistent with dual-channel microarray results for *Salmonella* Kentucky SK10944 (Figure 1b).

**Conjugative transfer of resistance genes**

Dual-channel microarray analysis of *Salmonella* Kentucky SK10944 revealed the presence of emerging ESBL blaSHV-2 and catA1 on a plasmid. Transfer was assayed for by selection for the resistance phenotype against ceftriaxone and chloramphenicol. Bacterial conjugation revealed high transferability of *Salmonella* Kentucky plasmids (efficiency of transfer was 74.9 ± 5.8%). Transconjugant *E. coli* TC12.2 expressed a resistance phenotype against both ceftriaxone and chloramphenicol.

Microarray hybridization of the transconjugant detected signal from *blaSHV* and catA1 probes, the same as for the donor strain (Figure 1b). The presence of both genetic determinants in *E. coli* TC12.2 was confirmed by susceptibility testing and PCR analysis.

**PCR validation of microarray results**

PCR analysis was performed for the majority of monitored resistance genes in order to validate microarray results. The majority of *Salmonella* strains and also transconjugant PCR results were consistent with the microarray data. Different results from both analyses are indicated by hatched boxes in Figure 2. All of them were PCR positive and microarray negative only for three resistance genes aadA, aph and catA. We suggest that the observed discrepancy between microarray and PCR analysis is most likely caused by the presence of nucleotide mismatch(es) within the query sequence complementary to the respective oligonucleotide probe, as was verified by sequencing of relevant PCR amplicons and subsequent alignment with the respective probe (data not shown). It is equally likely that failure of PCR analysis to verify a positive DNA microarray result could be due to divergence within the primer annealing region on the respective sequence.12 However, we did not detect any in this case.

Several *Salmonella* Kentucky and *Salmonella* Typhimurium STM10/02 revealed a resistant phenotype or reduced susceptibility to ciprofloxacin. Our previous study revealed identical sequence patterns in ciprofloxacin-resistant *Salmonella* Kentucky strains with point mutations in gyrA and parC genes.8 Recently, a plasmid-borne determinant conferring low-level resistance to fluoroquinolones from the qnr family was detected in *Salmonella* Enteritidis.34 However, we did not detect any *Salmonella* strain possessing the qnr gene.

In conclusion, the presented oligonucleotide microarray proved itself to be a simple and inexpensive molecular tool capable of identifying a variety of *Salmonella*-specific virulence factors and resistance genetic markers. Such microarray data provides genotypic characterization of each *Salmonella* isolate suitable for risk assessment studies with an emphasis on dissemination of antimicrobial resistance. Characterization of *Salmonella* strains using oligonucleotide chip has many advantages over traditional methods. Serotyping is time consuming and requires a large number of specific antisera. Phage typing has limited discriminatory power and requires specialized phage collections that are available to only a few references laboratories.28 Many molecular methods suffer from poor reproducibility and their standardization for interlaboratory comparison is difficult. The current gold standard for subtyping of *Salmonella* serovars often exhibits limited discriminatory power and interpretation of PFGE patterns can be ambiguous and susceptible to subjective errors.28,35 The presented oligonucleotide array covers several important genetic determinants from pathogenicity-, prophage- and antimicrobial resistance-related marker groups. Furthermore, it is an easily expandable platform for the accommodation of new markers, such as plasmid replication and partitioning elements.36 Differential labelling and analysis of genomic and plasmid DNA introduces substantial advances in the localization of resistance genes with great implications for monitoring of their transfer, dissemination and acquisition via horizontal gene transfer. A significant improvement for standardization of low-density detection oligonucleotide array is the introduction of external spike-in controls to DNA microarray analysis. The use of several spikes allows not only data normalization, but also the evaluation of several parameters of the platform quality including the sensitivity and specificity of the microarray experiments, the accuracy and reproducibility of the measurements and the assessment of technical variability.37,38

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**Transparency declarations**

None to declare.

**Supplementary data**

Tables S1 and S2, and a colour version of Figure 1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).
References


