Journal of Microbiological Methods 184 (2021) 106190

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A molecular assay for rapidly distinguishing the AviPro SALMONELLA VAC T vaccine strain from wild-type field isolates

Pieter-Jan Ceyssens [a,*], An Van den Bossche [a], Lac Kim Phan [a], Koenraad Van Hoorde [b], Wesley Mattheus [a]

[a] Unit of Human Bacterial Diseases, Sciensano, Belgium
[b] Unit of Foodborne Pathogens, Sciensano, Belgium

ARTICLE INFO

Keywords:
Salmonella
Vaccine strain
Molecular typing
NGS
Luminex
Validation

ABSTRACT

Rapid differentiation of the AviPro Salmonella VAC T strain from wild-type *Salmonella* ser. Typhimurium isolates is essential for the monitoring of veterinary isolates and targeted control actions. The distinction between the two strain types is routinely made by phenotypic antimicrobial resistance testing, but this sometime leads to ambiguous results with major economic implications. In this study, we used whole-genome sequencing to identify conserved and specific mutations in resistance and virulence genes which enable to distinguish field and vaccine strains. Based on this information, we developed and validated (n = 199) a Luminex-based assay targeting seven specific single-nucleotide polymorphisms. This molecular test is able to distinguish both *Salmonella* ser. Typhimurium types with 100% sensitivity and specificity within one working day.

1. Introduction

Salmonella enterica serovar Typhimurium (*Salmonella* ser. Typhimurium) is a leading global cause of foodborne illness, mostly contracted by eating contaminated food of animal origin (*EFSA J. Wiley Online Library*, n.d.). In accordance with EU Regulation 2160/2003, European targets have been set for the reduction of *Salmonella* ser. Enteritidis and *Salmonella* ser Typhimurium in flocks of breeding hens, laying hens, broilers, breeding turkeys and fattening turkeys (European Union, 2003). Each Member State has to introduce national control plans to achieve these targets.

The basis for successful control are good farming and hygienic practices together with testing and the very costly process of eliminating positive flocks (Whiley and Ross, 2015). The use of vaccines against *Salmonella* spp. is regarded as an additional measure to increase the resistance of poultry against *Salmonella* exposure and decrease the shedding (Jia et al., 2020). Currently, the EC legislation only obliges vaccination of laying hens against *Salmonella* ser. Enteritidis if the national prevalence exceeds 10% based on the monitoring program (European Union, 2011). Other vaccinations are voluntary, but can facilitate in achieving the set targets. Live vaccines can only be used if an appropriate method is available to bacteriologically distinguish *Salmonella* wild-type strains from vaccine strains.

One of the commonly used vaccine strains is the attenuated drift mutant AviPro SALMONELLA VAC T strain, found in a single-serotype Typhimurium vaccine and a combination Typhimurium/Enteritidis vaccine (AviPro VAC DUO) (Gantois et al., 2006). According to the manufacturer, the vaccine strain can be phenotypically distinguished from field strains based on susceptibility to erythromycin and resistance to both nalidixic acid and rifampicin. However, experience from our group and others (Maurischat et al., 2015) sometimes shows ambiguous results in culture-based testing, with major economic implications. In contrast to detection of the AviPro VAC E vaccine (Tang et al., 2019), no molecular detection test has been published for the VAC T vaccine strain. Given the rather long turnaround time still associated with (short-read) next-generation sequencing, we aimed to develop a same-day molecular test.

2. Methodology

2.1. Bacterial strains

All bacterial reference strains used in this study are listed in Table S1, with indication of origin. *Vibro alginolyticus* M/5035 is used as negative control strain. Bacterial cultures were grown overnight at 37 °C on Nutrient agar (Bio-Rad Laboratories, Hercules, CA, USA). For DNA

* Corresponding author.
E-mail address: pieter-jan.ceyssens@sciensano.be (P.-J. Ceyssens).

https://doi.org/10.1016/j.mimet.2021.106190
Received 14 January 2021; Received in revised form 3 March 2021; Accepted 3 March 2021
Available online 22 March 2021

Table 1
Selected SNP positions and MOL-PCR probe sequences for differentiating the AviPro Salmonella VAC T from wild-type *Salmonella* ser. Typhimurium.

Target mutation	Probe	Sequence [a]	MTAG [b]	Unique (%)[c]
*gyrA*_178A > T (p.Trp59Arg)	Up	*TAATACGACTCACTATAGGG*ATAGATTTAGAATGAATTAAGTGCAGATTTTTTATAGGCTTTGTTCC**T**	28	100
	Down	P-GTCATTGCCCAATACGTTCATGG*TCCCTTTAGTGAGGGTTAAT*		
*rpoB*_1579C > G[d] (p.His526Asp)	Up	*TAATACGACTCACTATAGGG*TATTAGAGTTTGAGAATAAGTAGTCCGCTGTCTGAGATTACG**C**	33	100
	Down	P-ACAAACGTCGTATCTCCGCA*TCCCTTTAGTGAGGGTTAAT*		
*cheM*_1326C > T (synomynous)	Up	*TAATACGACTCACTATAGGG*TTGTGATAGTAGTTAGATATTTGTGCGTAACGGCATTGACGATA	33	100
	Down	P-TCAGTCATGGTTTCCCCGGGCGCT*TCCCTTTAGTGAGGGTTAAT*		
*spaO*_232C > T	Up	*TAATACGACTCACTATAGG*GTAAGATTAGAAGTTAATGAAGAAGCCAGGGAACGACCAGGT**A**	39	100
(p. His78Tyr)	Down	P-CTCAGCGCCAGCAGAAACCG*TCCCTTTAGTGAGGGTTAAT*		
*sseF*_397C > T	Up	*TAATACGACTCACTATAGGG*AGTGAATGTAAGATTATGTATTTGCCGCCAGTGATAGCGTTGCT**T**	52	100
(p. Leu133Phe)	Down	P-TTGTGGTCAGTGCGCTGGCCT*TCCCTTTAGTGAGGGTTAAT*		
*stfD*_186G > A (p. Trp61fs)	Up	*TAATACGACTCACTATAGGG*TAATTGAATTGAAAGATAAGTGTCCCTATCTGGCGCAGTCCT**A**	13	98.8
	Down	P-GGTGGAAGATGAGAAGGGC*TCCCTTTAGTGAGGGTTAAT*		
*shdA*_3069C > T (synomynous)	Up	*TAATACGACTCACTATAGGG*TGTTATAGAAGTTAAATGTTAAGCGTCAGTGATAGTGGTGGC**A**	18	99.2
	Down	CCGGAATAGCTGTTGTCGCC*TCCCTTTAGTGAGGGTTAAT*		

[a] Primer (T7 and T3), anti-TAG and SNP positions are indicated by italic, underlined and bold sequences, respectively. P-, 5′-Phosphate.
[b] ID-number of the MagPlexTM-TAG microspheres.
[c] Hit percentage of SNP against non-redundant *Salmonella* ser. Typhimurium (taxid:90371) database, containing 126 sequences.
[d] the *rpoB* probe targets the wild-type allele, while all others target the VACT strain.

extraction, a single colony was added to 50 μL of DI H_2O and placed in a thermal cycler (90 °C for 10 min., cool to 4 °C). The mixture was spun (13,000 xg, 10 min.) and the supernatant was used immediately or stored at −20 °C.

2.2. Next-generation sequencing

Bacterial gDNA was extracted using the MgC Bacterial DNA Kit™ with 60 μL elution volume (Atrida, NL), following the manufacturer's instructions. Sequencing libraries were constructed using the Illumina Nextera XT DNA sample preparation kit (Illumina, San Diego, USA) and subsequently sequenced on an Illumina MiSeq instrument with a 250-bp paired-end protocol (MiSeq v3 chemistry) according to the manufacturer's instructions. Trimming of the short reads was performed with Trimmomatic (version 0.32) (Bolger et al., 2014). First, the Illuminaclip option was used to remove the Nextera adapter sequences. Then, a sliding window approach of four bases and trimming when the Phred score dropped below 30 was employed. Lastly, the leading and trailing bases of a read were removed when the Phred score dropped below 3. All reads that were smaller than 50 bp were removed. Obtained reads were mapped to the *Salmonella* ser. Typhimurium LT2 genome (NC_003197) using default settings (CLC Genomic Workbench 20.0.2, QIAGEN, Germany). Single-nucleotide variants were identified with minimal coverage of 10, prune distance of 10, and minimal required z-score of 1.96. Neighbor Joining trees were constructed with the General Time Reversible Nucleotide substitution model, with transition ratio set at 2.0, the number of substitution rate categories at 4, and a gamma distribution parameter of 1.0. All sequencing data were deposited at ENA under accession number ERP124737.

2.3. Probe design

For all targeted genes (Table 1), adjacent upstream and downstream probes were designed targeting 35–45 bp conserved regions with maximal conservation and accessibility, as scored using Visual OMP™ software (version 7.6.58.0; DNA Software, Belgium). Upstream probes are equipped with an internal anti-TAG sequence compatible to the anti-TAG of the MagPlex beads (Luminex Corp., Austin, TX, USA), and universal T7 and T3 primer sequences were added to the 5′ and 3′ ends of upstream and downstream probes, respectively. Downstream probes were 5′-phosphorylated. All primers and probes used in this project are listed in Table 1.

2.4. MOL-PCR

The multiplex oligonucleotide ligation-PCR (MOL-PCR) reactions were assembled in cooled 96 well plates in a 10 μL reaction volume containing 2 nM of each probe, 2 units Taq DNA Ligase (New England Biolabs, Ipswich, USA), 1× Taq DNA ligase buffer, 2 μL DNA template and nuclease-free water. Ligation was performed by initial denaturation (95 °C, 10 min.), followed by 25 cycles of ligation (58 °C, 30 s) and denaturation (96 °C, 25 s). 3 μL of the ligation product was amplified in a 10 μL PCR containing 0.25 units HotStartTaq DNA polymerase (Qiagen, Hilden, Germany), 1× DNA polymerase buffer, 125 nM T7- and 500 nM 5′-biotin-T3 primer, and 200 μM dNTPs. Reaction conditions were 15 min. of denaturation at 95 °C, followed by 35 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s) and a final extension step at 72 °C for 5 min.

2.5. Data acquisition and analysis

Hybridization of the PCR product to colored microspheres was performed in a total volume of 20 μL per reaction. MagPlex™-TAG microspheres (750 beads/target) with the covalently bound complementary anti-TAGs were added to hybridisation buffer (final concentration of 0.1 M Tris-HCl pH 8.0, 0.2 M NaCl, 0.08% Triton-X). To this mixture, 5 μL PCR product was added, followed by a denaturation step (90 s. at 96 °C) and 30 min. Hybridisation at 37 °C. Subsequently, 100 μL of a reporter mix containing 4 μg/mL streptavidin-R-phycoerythrin (SAPE, Life Technologies, Carlsbad, USA) in hybridisation buffer was added, and the samples were incubated for 15 min. at 37 °C in the dark. Subsequent read-out was performed at 37 °C using 100 μL of these samples, on a MAGPIX® device with a minimal bead count of 50 microspheres per target. For each marker, the signal-to-noise (S/N) ratios were calculated by dividing the median fluorescence intensity (MFI) by the corresponding MFI of the NC. During assay design, an S/N ≥ 3.0 indicates a positive identification. For the analysis of SNP markers, the following formula was used:

$$\text{Allele call}_{\text{Sample A}} = \frac{\text{S/N(sample A)}}{\text{S/N(sample A)} + (\text{average (S/N(variant alleles))})}$$

3. Results and discussion

To design a new molecular assay, we first sequenced the original AviPro SALMONELLA VAC T strain, 1 vaccine strain from the monitoring program (S19BD09271), and 21 wild-type Typhimurium strains with varying MLVA profiles (Table S1). Pairwise comparison of the processed sequence data identified 3307 high-quality variable positions between the isolates in our dataset. While there were no single

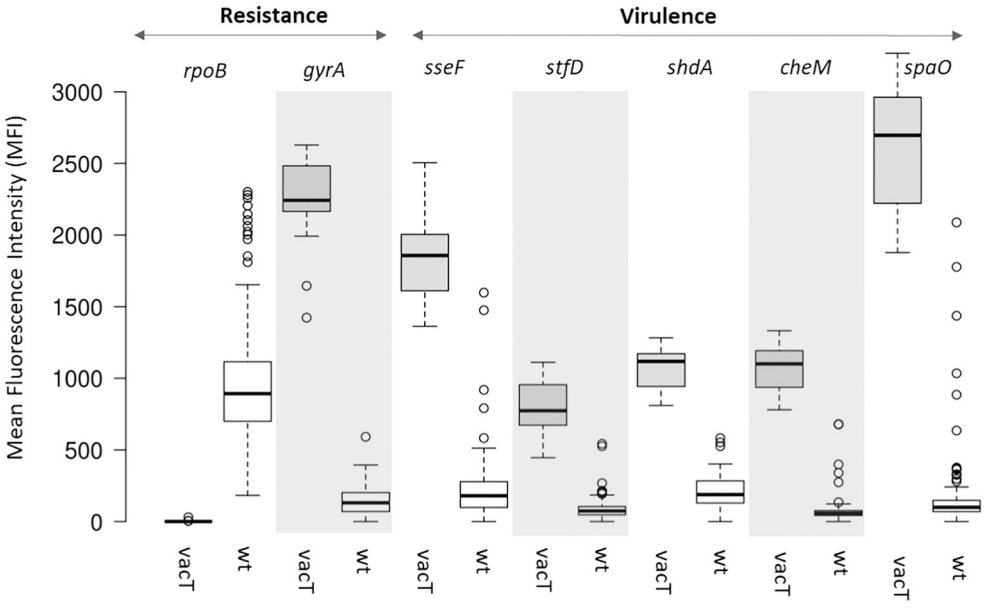

Fig. 1. Luminex test to distinguish vaccine from field strains of *Salmonella* ser. Typhimurium, based on the specific detection of seven SNPs. The boxplot shows aggregated results from 13 VacT and 198 wild-type (wt) strains, with whiskers extending to data points that are less than 1.5 x IQR away from 1st/3rd quartile.

nucleotide polymorphisms (SNPs) identified between VAC T and sample S19BD09271, field strains had on average 682 ± 60.6 SNPs difference with the vaccine strain (Fig. S1).

We analyzed variable positions in the AviPro SALMONELLA VAC T strain with a focus on antimicrobial resistance markers and virulence genes. We found that erythromycin susceptibility is associated with a 10-bp deletion, leading to a premature termination codon in the gene for the RND transporter permease subunit AcrB (Leu876fs), which has been correlated to impaired efflux of the drug (Yamasaki et al., 2016). A second deletion (*acr*B:912_916del) mutation was identified in the same gene. Rifampicin resistance in VAC T is caused by a His526Asp mutation in *rpoB* (Jin and Gross, 1988), while four unique mutations were found in the Gyrase A protein (Ala866Ser, Asp87Gly, Gly75Ala and Trp59Arg), of which Asp87Gly is most likely the cause of the quinolone resistance phenotype (Hopkins et al., 2005).

As AviPro SALMONELLA VAC T is a live, attenuated vaccine, we expected mutations in virulence gene sequences as well. Using SRST2 read mapping against the VFDB database (Liu et al., 2019), we identified 273 potential virulence genes in the VAC T genome, with 49/273 carrying at least one SNP/indel as compared to the reference allele. By comparing these with the other sequenced *Salmonella* ser. Typhimurium strains, we retained 9 virulence-related SNP positions which are unique to VAC T in our dataset (Table S2). All resistance- and virulence-related candidate positions were checked for conservation using a BLASTn database search against the non-redundant database limited to *Salmonella* ser. Typhimurium (taxid: 90371), and scored for probe accessibility using Visual OMP (version 7.6.58.0; DNA Software). Finally, seven SNPs positions were retained for assay development (Table 1). Two of the targeted genes (*rpoB* and *gyrA*) are related to drug resistance, five others (*cheM, spaO, sseF, stfD* and *shdA*) encode virulence factors.

To allow a same-day response, we opted to expand an in-house multiplex protocol for genoserotyping of *S. enterica* (Gand et al., *2020a*). This assay is based on Multiplex Oligonucleotide Ligation – PCR (MOL-PCR), and uses commercially available MagPlex™-TAG microspheres for detection. Probe design, assay workflow and data interpretation are performed as described elsewhere (Ventola et al., 2019; Gand et al., 2020b), and described in detail in the supplementary methods. For a sample to flag positive as a vaccine strain, all seven VAC T-specific SNPs need to be unequivocally identified using a $MFI_{test\_runX}/MFI_{VAC\text{-}T\_runX} = 1.0 \pm 0.3$ (Mean Fluorescent Intensity) threshold.

A total of 202 *Salmonella* ser. Typhimurium strains were used to evaluate the sensitivity (using inclusivity tests), the specificity (using exclusivity tests) and the accuracy of the newly developed MOL-PCR test (Table S1). Human isolates were selected with a maximum variety of MLVA profiles, while veterinary strains were sampled at random from national monitoring programs for pigs and poultry. The raw data are shown in Table S1 and summarized in aggregated form in Fig. 1. In eight different runs, the Luminex-based assay correctly identified the AviPro SALMONELLA VAC T strains (100% inclusivity), and excluded all the wild-type Typhimurium strains (100% exclusivity).

Interestingly and in contrast to what was expected based on BLASTn searches, 17/198 (8.5%) validation strains contained one of the VAC T-specific positions (Table S1). Moreover, we encountered a single field TM isolate (S19FP07247) sampled from duck meat, which carried 3/7 VAC T-type SNPs. Whole-genome analysis of this strain clearly showed relatedness with VAC T, although both strains still differ in 271 positions (Fig. S1). In general, this observation points at the underrepresentation of veterinary TM strains in public databases.

In conclusion, we used SNP typing to develop a rapid molecular test to distinguish wild-type *Salmonella* ser. Typhimurium field isolates from the AviPro SALMONELLA VAC T vaccine strain. This test has 100% accuracy, and will replace the phenotypic vaccine identification method in the National Reference Center in Belgium.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106190.

Declaration of Competing Interest

None.

Acknowledgements

The National Reference Center is partially supported by the Belgian Ministry of Social Affairs through a fund within the Health Insurance System.

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