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DNA microarray for tracing *Salmonella* in the feed chain

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ABSTRACT

In the present study we investigated if the microarray platforms Premi®Test *Salmonella* (PTS) and *Salmonella* array (SA) could be applied for the identification and typing of *Salmonella* in artificially contaminated animal feed materials. The results were compared to the culture-based MSRV method and serotyping according to Kauffman–White. The SA platform showed a specificity of 100% for the identification of *Salmonella* compared to 93% with the PTS platform and a sensitivity of 99% or 100%, respectively. Among all identified *Salmonella* serotypes, 56% with the SA platform and 81% with the PTS platform were correctly identified. The difference in probe signal intensity for each probe was higher between duplicates analyzed with the SA platform than with the PTS platform. Attempts to use the microarray platforms from BPW resulted in many false negative samples and incorrect typing results. The microarray platforms tested were simple to use and might have a potential in tracing studies for *Salmonella* in the feed chain particularly when rapid information about serotypes are important.

1. Introduction

Salmonellosis is one of the most common zoonotic infections in humans in the EU. More than 160000 human cases are annually reported according to EFSA for the last 5 years (Anonymous, 2009). *Salmonella* is causing the majority of food borne outbreaks in the EU and in 2005 there were 5311 outbreaks reported with 47000 human cases where 5300 were hospitalized and 24 died (Anonymous, 2002a; Anonymous, 2006a). The main source of human salmonellosis is the consumption of contaminated food of animal or vegetable origin.

In order to decrease the burden of human *Salmonella* infections in the EU focus has been given to preventive measures in the food chain, primarily in the poultry sector but also directed to pig production (Anonymous, 2010). It is recognized that animal feed can be a source of *Salmonella* infection in food producing animals and in the EFSA opinion on microbiological risks in feed *Salmonella* was considered a major risk (Anonymous, 2008). Animal feed processing is characterized by the very large volumes of feed being processed, a high through-put, a continuous batch-wise production and lack of shelf-life of the finished product. In order to successfully trace and control *Salmonella* contamination in the feed chain, and also for epidemiological studies when animal infections were demonstrated, efficient sampling strategies as well as sensitive isolation and typing methods are necessary to apply. A recommendation in the EFSA opinion from 2008 was that microbiological criteria should be defined for *Salmonella* in feed (Anonymous, 2008), which underline the need for detailed knowledge about the performance of detection methods for feed and feed ingredients.

The internationally recognized standard reference method for isolating *Salmonella* from food and feed is the ISO culture-based method (EN ISO 6579) with the last modification (annex D) where modified semi-solid Rappaport Vassiliadis (MSRV) agar is used for the selective enrichment (Anonymous, 2002a; Anonymous, 2006b). The method requires 5–7 days for completion before serotyping results are available.

There are large numbers of different rapid methods presently being used in the surveillance of *Salmonella*, primarily in the food sector, however, only few were evaluated for animal feed (Alvarez et al., 2003; Chan et al., 2003; Maciorowski et al., 2006; Porwollik et al., 2005). Recently, we evaluated different commercial PCR-based methods for feed and feed ingredients and the results showed that those methods often fail when attempts were made to isolate the strains which are vital for the serotyping and tracing investigations (Koyuncu et al, 2010).

Microarray technologies have potential use in analysis of microbial pathogens, to be applied in research, food safety and industrial settings (Jarquin et al., 2009; Rasooly and Herold, 2008). The power of the technology is the simultaneous analysis of large numbers of DNA sequences in a sample and also the potential for automation of the analytical chain. The technology offers a wide range of food safety analysis such as multi-pathogen detection, antibiotic resistance determination and virulence factor identification being useful in tracing and epidemiological investigations as well as in different industrial applications when rapid information is important (Rasooly and Herold, 2008).

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To our knowledge, the application of microarray for *Salmonella* identification in the feed chain was not evaluated previously.

In this study we were interested to apply the commercially available DNA microarray platforms Premi®Test *Salmonella* (PTS) and the previous version the *Salmonella* array (SA) (Wattiau et al., 2008a; Wattiau et al., 2008b) as a rapid and easy to use tool for the identification and typing of *Salmonella* in feed materials. According to the instructions samples for the microarray should be analyzed from the migration boundary of the MSRV plates. The results from the microarray platforms were compared to the cultural MSRV (modified semi-solid Rappaport Vassiliadis) method (Anonymous, 2006b) and serotyping according to Kauffman–White (Bopp et al., 2003).

The principle of the commercial microarray is based on generating circular DNA molecules by using a multiplex ligation detection reaction (LDR) that are subsequently PCR amplified and then hybridized to a low-density DNA microarray spotted with probe-specific complementary oligonucleotides. The probe types included are *Salmonella* general probes, typing probes, negative control probes, DNA control probes and hybridization control probes. The *Salmonella* general probes represent markers present in all *Salmonella* with no sequence variation found between serotypes (targeted genes are srlD, InvA, and yhdA). The probes used for serotype identification are random genomic markers representing small nucleotide polymorphisms identified by multilocus sequence typing (MLST). The targeted genes are fliC, araE, ycfN, misL, gutM, nrdG, aroC, hisD, thrA, fljA, aroC, dnaN, sopD, safC, tcfA, srfJ, spvC, stdB and four random genomic sequences. Biotin labeled PCR primers are used for detection of positive hybridization. Unique microarray hybridization profiles are obtained for different *Salmonella enterica* serotypes. In each ArrayTube® three amplification reactions can be detected simultaneously. The software translates the microarray data into the name of the serotype by comparing the hybridization spot pattern from the array with the hybridization patterns in the data base.

The objective of this study was to apply the microarray from the selective enrichment (MSRV) for identification and typing of *Salmonella* in artificially contaminated feed samples. We also wanted to investigate if the microarray could be applied directly from the pre-enrichment broth (BPW) after the non-selective enrichment in order to further reduce the time for analysis.

2. Materials and methods

2.1. Microarray platforms, feed materials and Salmonella strains

Two microarray platforms Premi®Test *Salmonella* (PTS) and *Salmonella* array (SA) (Check-Points, Wageningen, The Netherlands) were used. Different feed materials representing samples from different parts of the feed chain were used in the study, namely wheat grain, soybean meal, rape seed meal, palm kernel meal, pellets of finished pig feed and also scrapings from a feed mill elevator. The *Salmonella enterica* ssp. *enterica* serotype Typhimurium (*S. Typhimurium*), *S. Cubana*, *S. Bareilly*, *S. Agona*, *S. Reading* and *S. Yoruba*, respectively, isolated from feed and serotyped according to Kauffman–White, were added to 25 g of each feed material, respectively. Non-spiked feed samples were used as controls. The preparation of the bacterial cells for the spiking experiments and the procedure to measure the concentration of *Salmonella* in the inoculum was previously described in (Koyuncu and Haggblom, 2009).

2.2. Spiking of feed materials and culturing

Two spiking levels of the feed materials were used in order to assure detection of *Salmonella* since the levels are close to the detection limits (Koyuncu et al., 2010). The spiking levels of *Salmonella* were 7 or 70 CFU/25 g except for rape seed meal which was spiked with 0.7 or 7 CFU/25 g and palm kernel meal with 70 or 700 CFU/25 g and the volumes used for spiking were approximately 350 µl. The samples were left in room temperature for 4 h before 225 ml of buffered peptone water (BPW) (Oxoid CM 0509, Basingstoke, England) was added, followed by incubation at 37 °C ± 1 °C for 18 h. Three drops (equivalent to approximately 0.1 ml) of the BPW were inoculated at separate positions on the surface of Modified Semi-solid Rappaport Vassiliadis agar plates (MSRV) (Oxoid CM 0910) with 1.0% Novobiocin and then incubated at 41.5 ± 0.5 °C for 24 ± 3 h. In addition, with the SA platform, an extra 1 ml sub-sample from the BPW was taken for direct DNA extraction.

2.3. Sample preparation and analysis

After incubation the MSRV plates were examined for typical *Salmonella* growth and a sample was plated on Xylose Lysine Deoxycholat agar (XLD) (Lab M lab 32, Axel Johnson Lab System Inc. Solna, Sweden) (with 1.5% Novobiocin) and Brilliant Green agar (BGA) (Oxoid CM 0329). If no migration was observed the plates were incubated for an additional 24 h at 41.5 °C ± 0.5 °C and the procedure was repeated. Typical *Salmonella* colonies on XLD and BGA were plated on blue-agar and were then serotyped according to Kauffman–White. For the SA and the PTS platforms a 5 µl aliquot from the migration boundary of the MSRV-plate suspected to contain *Salmonella* or from the inoculation point was resuspended in 100 µl lysis buffer following the manufacturer's instructions version 3.1 (SA) or version 4.4 (PTS). With the SA or the PTS platform 146 or 80 samples, respectively, were analyzed including non-spiked samples.

For direct DNA extraction the sub-samples from the BPW were centrifuged at 12 000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 200 µl of the supplied lysis reagent. The suspension was vortexed and incubated at 99 °C for 15 min using a Thermo Mixer (400 rpm) for lysis. After a final centrifugation for 5 min at 12 000 rpm the supernatant was used for the SA platform according to the manufacturer's instruction from the DNA recognition step A version 3.1. In total 97 sub-samples from the BPW were analyzed with the SA platform.

For studies of reproducibility random samples with the different *Salmonella* serotypes, feed materials and spiking levels were chosen. Wheat grain, soybean meal or pellets of finished pig feed spiked at the highest level with *S. Yoruba*, *S. Bareilly* or *S. Reading* were analyzed. Similarly Palm kernel meal, rape seed meal or scrapings from a feed mill elevator were analyzed for S. Typhimurium, S. Agona or S. Cubana (total of 18 samples). The SA platform was tested using single samples in two separate experiments and the results were compared. The PTS platform was tested in two separate experiments, where duplicate and single samples were analyzed and the results were compared. The SA platform was also tested from BPW for all feed materials, except for scrapings spiked with S. Agona or S. Bareilly at the highest level (total of 10 samples).

2.4. Microarray readings

The microarray results were recorded on a single-channel ATR03 reader (Clondiag, Jena, Germany) connected to a computer, where the data was translated into a *Salmonella* serotype by the software 2.7.0 for the SA platform and software 30.06.2009 for the PTS platform.

2.5. Data analysis and statistical calculations

The relative accuracy (AC), sensitivity (SE) and specificity (SP) were calculated according to the validation protocol of NordVal (Anonymous, 2002b) as described in Koyuncu and Haggblom (2009). Test results were recorded as true positive (TP) when both the microarray and culture methods indicated presence of *Salmonella* and as false positive (FP) when the microarray indicated presence of *Salmonella* and also the culture method yielded negative result. Test results were recorded as true negative (TN) when the microarray and the culture method both