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The development of a screening protocol for *Salmonella* spp. and enteropathogenic *Yersinia* spp. in samples from wild boar (*Sus scrofa*) also generating MLVA–data for *Y. enterocolitica* and *Y. pseudotuberculosis*

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ABSTRACT

Salmonellosis and yersiniosis are notifiable human diseases that are commonly associated with contaminated food. Domestic pigs as well as wild boars and other wild-life have been identified as reservoirs of these bacteria. Methods for cultivation and molecular epidemiological investigations of *Salmonella* spp. are well established, however, cultivation of enteropathogenic *Yersinia* spp. is time- consuming and the commonly used method for molecular epidemiological investigations, pulsed-field gel electrophoresis, lack in discriminatory power. The aim of this study was to develop and evaluate a screening protocol well suited for wildlife samples and other highly contaminated samples. The method is based on PCR-screening followed by Multiple Loci Variant number tandem repeat Analysis (MLVA) on enrichment broth to obtain molecular epidemiological data for enteropathogenic *Yersinia* spp. without the need for pure isolates. The performance of the protocol was evaluated using wild boar samples (n = 354) including tonsils, faeces and lymph nodes from 90 Swedish wild boars. The new protocol performed as well as or better than the established ISO-standards for detection and cultivation of *Y. enterocolitica* and *Salmonella* spp., however for cultivation of *Y. pseudotuberculosis*, further development is needed. The selection for motility seems beneficial for the enrichment of *Salmonella* spp. and *Y. enterocolitica*. Further, the selective enrichment prior to PCR-analysis eliminates inhibitory factors present in the original sample. In total, ten isolates of *Y. enterocolitica* of various bio-serotypes were obtained, and the MLVA-profile of these isolates were consistent with the profiles from the corresponding enrichment broth. Further, 22 isolates of *Salmonella* spp. comprising six different serovars were obtained with *S*. Fulica, *S*. Hadar and a monophasic *S*. Typhimurium being the most common. In conclusion, the presented screening protocol offers a rapid and efficient way to obtain prevalence data from a large sample set as well as MLVA-data within a short time frame. These results can hence improve the knowledge on the epidemiology and distribution of these pathogens and their importance to public health.

1. Introduction

Salmonellosis and yersiniosis are notifiable human diseases that are commonly associated with contaminated food, and with an annual notification rate of 21.2 and 2.2 European cases/100 000 population, respectively (European Food Safety et al., 2016).

The genus *Salmonella* includes more than 2500 serovars that exhibit varying pathogenicity for different animal species, humans included (Issenhuth-Jeanjean et al., 2014), with *Salmonella* Enteritidis being the most commonly reported serovar. Reliable diagnostic methods for detection and molecular epidemiology of *Salmonella* spp. are well established and the data generated are contributing to new insights into the epidemiology of *Salmonella* spp. in both humans and farm animals (Leekitcharoenphon et al., 2016).

The situation regarding the epidemiology of *Y. enterocolitica* and *Y. pseudotuberculosis* is different. The most common *Yersinia* species

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associated with yersiniosis is *Y. enterocolitica* of the bio-serotype 4/O:3. The current ISO-standards for detection of *Salmonella* spp. EN ISO 6579/A1:2007 (Anonymous, 2002) and *Y. enterocolitica* ISO 10273:2017 (Anonymous, 2017) are based on cultivation and developed for food matrices. However, the cultivation of *Yersinia* spp. is time-consuming and lack sensitivity especially in the analyses of highly contaminated samples (Fredriksson-Ahomaa and Korkeala, 2003). Thus, many studies depend solely on PCR-detection for prevalence estimates (Arrausi-Subiza et al., 2016; Cheyne et al., 2010), excluding culture-dependent techniques such as bio-serotyping and molecular epidemiological investigations. The Gold Standard method previously used for molecular epidemiology is Pulsed Field Gel Electrophoresis (PFGE) that is time consuming, results are difficult to interpret, and with poor discrimination between isolates (Gilpin et al., 2014). More recently, protocols for Multi Locus Sequence Typing (MLST) and Multiple Loci Variant number tandem repeat Analysis (MLVA) have been developed (Hall et al., 2015; Halkilahti et al., 2013; Sihvonen et al., 2011b). MLST have been used for studies on bacterial evolution (Laukkanen-Ninios et al., 2011) while MLVA have been used to study the distribution of and the relations between different clones of *Y. enterocolitica* in slaughter pigs, showing the usefulness of the method in epidemiological studies (Virtanen et al., 2014).

Domestic pigs as well as wild boars are known carriers of *Salmonella* spp. and *Yersinia* spp. (Sanno et al., 2014; Nesbakken and Kapperud, 1985). The wild boar population and the consumption of wild boar meat is increasing, thus the need for screenings of various zoonotic pathogens has also increased. The aim of the present study was to develop and evaluate a time-efficient protocol that is suitable for screening a large number of samples from wildlife and other sources. The protocol should be robust, easily adopted in standard laboratory settings, and cost-effective while also generating molecular epidemiological data for enteropathogenic *Yersinia* spp. that can be used for source attribution in outbreaks and for epidemiological research.

2. Materials and methods

In total, 354 tissue samples from 90 wild boars were obtained through a network of hunters organized by the Swedish hunters' association. Details on sampling and sample material has been previously described (Sannö et al., 2018).

All samples were analysed using a protocol developed within this study ("The new protocol"; Appendix A) and additional analyses were performed to evaluate the protocol. In brief, the samples were incubated in BPW for 20 ± 2 h at +28 °C. Thereafter, 10 µL of the top-layer of the broth was streaked on selective agar plates that were incubated for 20 ± 2 h. Colony material from the plates was suspended in BHI and used to prepare a template for PCR using Instagene Matrix® (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. All PCR-positive BHI-suspensions were analysed by MLVA and subsequently cultivated to obtain isolates for further evaluation of the method. The detection efficiency of the new protocol was evaluated by comparison with established protocols for *Salmonella* spp. and *Y. enterocolitica*. The selection step for motility was evaluated by analysing the pre-enrichment broth (BPW) by PCR and the results were compare to those obtained by PCR-analysis of the BHI-suspensions. The presence of PCR-inhibitory factors, and the reproducibility and precision of acquired MLVA data were also evaluated.

2.1. Evaluation of the new protocol

2.1.1. Evaluation of the detection rate by the new protocol in comparison with established protocols

The two tonsil specimens from each of 50 animals were used for this evaluation. The right tonsil was minced in small pieces using a sterilised scalpel, split into two subsamples, and 1.5 g was used to analyse the presence of *Salmonella* spp., utilizing the new protocol. In parallel, 1.5–2 g of the sample was analysed by the standard protocol EN ISO 6579/A1:2007 (Anonymous, 2002) in the accredited laboratory at the National Veterinary Institute (SVA), and the results were compared.

In the detection of *Y. enterocolitica* and *Y. pseudotuberculosis*, the left tonsil was similarly cut in small pieces, split in two subsamples, and 1.5 g tonsil tissue was analysed by the new protocol, in parallel to analyses of 1.5–2 g tissue sample by the ISO 10273:2017 protocol developed to detect *Y. enterocolitica* in food (Anonymous, 2017).

All isolates obtained by cultivation were stored at -80°C with 15% glycerol added, until further analysis.

2.1.2. Evaluation of any possible adverse effect on method performance caused by the selection step for motility

In the new protocol, a step was included for the selection of motile bacteria. To investigate the occurrence of non- or low motile bacteria presumably present at the bottom of the BPW tube following the primary incubation on day 2, a subset of samples (n = 220) was analysed. The BPW was vortexed, 2 mL collected, and frozen in −80 °C with 15% glycerol added. Later, this sample was thawed, vortexed and 100 µL was used to prepare a template as described in Appendix A, Section 6, and analysed by PCR to detect the presence of *Salmonella* spp., *Y. enterocolitica* and *Y. pseudotuberculosis* (Appendix A, Section 7). The results were compared to those obtained in the analysis by the new protocol.

2.1.3. Evaluation of the presence of PCR-inhibitory factors

To investigate the possible presence of PCR-inhibitors, all PCR-negative samples (n = 268) were re-analysed with an Internal Positive Control (IPC: EXO IPC/VIC Mix; Life technologies, Grand Island, New York, USA) according to the manufacturer's instructions, but with a higher dilution *i.e.* 1:50 of the IPC DNA. Similarly, in the analysis of the BPW (Section 2.1.2), the IPC was added to all reactions. If PCR inhibition was indicated, the template was diluted 1:10 and re-analysed. The IPC was added to the Master mix for *Y. enterocolitica* and *Salmonella* spp., while in the analysis of *Y. pseudotuberculosis*, the IPC was analysed in parallel but in separate wells since the probes for both the IPC and *Y. pseudotuberculosis* utilized the same fluorophore.

2.1.4. Reproducibility of the MLVA on pure culture of Y. enterocolitica and Y. pseudotuberculosis

2.1.4.1. MLVA-analysis on isolates of Y. enterocolitica and Y. pseudotuberculosis. The protocols by Sihvonen et al. (2011b) and Halkilahti et al. (2013) were used for MLVA analysis of *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively. Briefly, pure isolates of *Y. enterocolitica* and *Y. pseudotuberculosis* were cultured on Brain Heart Infusion (BHI; Oxoid, Hampshire, UK) agar plates at 30 °C for 24 h, followed by inoculation into BHI broth and incubation for additionally 24 h to ensure a high concentration of bacteria. Genomic DNA was purified (GeneJETGenomic DNA Purification Kit Fermentas, St. Leon-Rot, Germany), according to the manufacturer's instructions and eluted twice to ensure a maximum yield. In the analysis of *Y. enterocolitica*, primers (Gierczynski et al., 2007) labelled with ABI PRISM® fluorescent dyes were used in two separate multiplex PCRs (Qiagen, Hilden, Germany) with the VNTR loci of V2A (PET), V4 (NED), V6 (6-FAM) included in the first, and V5 (NED), V7 (VIC), and V9 (PET) in the second PCR in a total volume of 25 µL. In the analysis of *Y. pseudotuberculosis*, the forward primers were labelled with a fluorescent 6-FAM dye and the reactions were carried out in single reactions according to (Halkilahti et al., 2013) in order to amplify the loci YPbF1, YPbF3, YPbF5, YPbF7, YPbF8, YPbF9 and YPbF10. All amplifications were done in a BioRad DNA Engine Dyad Peltier thermal cycler (Hercules, California, USA).

The six PCR-products from the two multiplex-PCR reactions in the *Y. enterocolitica* protocol were mixed, while the seven PCR-products from the *Y. pseudotuberculosis* protocol were analysed in single wells. The products were diluted 1:500 (*Y. enterocolitica*) and 1:85 (*Y.*