Comparison of DNA extraction methods to detect Salmonella spp. from pig faeces and pork

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Abstract
The quality of DNA extract significantly influences the outcome of PCR-based detection methods. Performances of four different DNA extraction methods were evaluated for their ability to recover Salmonella DNA from artificially contaminated specimens. Swine faecal and pork samples were spiked with known concentration of Salmonella Typhimurium, DNA was then extracted by each of the methods considered and finally tested by a commercial Salmonella Real Time kit. The QIAgen and Adiafood kits were the most suitable methods to detect Salmonella in pig faeces. For pork samples the best performances were obtained using the Invitrogen kit. The use of appropriate DNA extraction methods is a critical issue for successful and valid PCR results and it is advisable that DNA extraction techniques are carefully selected with particular regard to the type of samples they should be used for.

Introduction
Salmonella is an important pathogen causing food-borne diseases and pork is recognised as one of the main sources of human infection (1,2). Salmonella Typhimurium, that is one of the main serovars involved in human illness, is frequently associated with pigs (3). Contamination of pig carcasses can occur in the slaughtering plants as a result inaccurate procedures or environmental contamination, and subsequently Salmonella may be present in pork.
Benefits of having rapid, sensitive, and specific tests for the detection of Salmonella in matrices such as faeces and foodstuffs are clear, for the possibility of timely identification of infection or contamination even at very low doses. However, some challenges associated with the usage of rapid methods based on PCR still remain and a crucial one is the removal of inhibitory compounds from target DNA (4). To date, only a limited number of studies comparing the efficacy of extraction methods to perform Real Time PCR, removing reaction inhibitors from different matrices, have been carried out. Thus, this study was aimed at identifying the most effective DNA extraction methods suitable for molecular protocols specifically for faecal and pork samples. Therefore different DNA isolation kits were tested to extract Salmonella DNA from artificially contaminated specimens and their performances were evaluated by applying a Real Time PCR kit. Preliminary results allow to identify the commercial kits yielding the best DNA products useful for further molecular analysis.

Material and methods
Spiked faecal and pork samples
Forty-four samples of pig faeces and 44 samples of pork were spiked with 3 different concentrations of S. Typhimurium (from 2 to 14 CFU/g). Each sample was prepared in triplicate. For all methods the first step was the pre-enrichment of 10 g of matrix (faeces or pork) into 90 ml Buffered Peptone Water (BPW).

DNA extraction
The following four extraction methods were used to extract DNA from faecal specimens, according to the manufacturers' instructions: - InviMag Stool DNA kit (Invitek, GmbH), combining the Invisorb® technology with the use of magnetic particles for isolation of nucleic acids at high purity level [1]; - QIAamp DNA Stool kit (Qiagen), based on an initial step with a fast spin-column, that specifically binds DNA to the silica-gel membrane, then a secondary step where the lysis using proteinase K ensures high yields of DNA in stool [2]; - Lysis reagent (iCheck-Bio-Rad), that uses a lysing solution and beads [3]; - Extraction DNA mix (AES Chemunex, AdiaFood), that uses a lysing solution combined with boiling method [4]. Similarly, DNA was extracted from pork samples using four different methods: Charge Switch gDNA mini Bacteria Kit (Invitrogen, Life technologies), that allows bacterial DNA purification by using the magnetic bead-based technology and cellular lysis with proteinase K and lysozime [1], the boiling method [4], (5).Then two methods that were tested also

for faecal samples: Lysis reagent (iCheck-Bio-Rad) [2] and Extraction DNA mix (AES Chemunex, AdiaFood) [3].
In addition, each extraction method was tested both directly on the enriched samples and on the same ones pre-treated with magnetic beads named Dynalbeads Anti-Salmonella (Dynal, Invitrogen).

Real Time PCR analysis

Eventually, a Salmonella Real-Time PCR assay (AES Chemunex, AdiaFood) was used to compare the efficacy of the extraction methods to recover Salmonella DNA. The Real Time PCR analysis was performed on triplicate nucleic extracts prepared from spiked samples (faeces and pork) at different concentrations (Table 1 and 2);. To determine the extraction efficiency also the absorbance at 260 nm (A260) was taken into account since this parameter indicates the average nucleic acid extracted from each sample.

Statistical analysis

The Linear Mixed Model for repeated measures (LMM) was used to evaluate if a significant difference exists among the methods tested taking into account also the pre-treatment with Dynabeads.
Different models with fixed and random effect and structures of variance/covariance matrix were evaluated in order to identify the most appropriate; Maximum likelihood value, histogram, Q-Q plot and distribution of residuals, and Kolmogorov Smirnov test were used to verify the goodness of the model (SAS 9.1.3), (6).

Results

Comparison of extraction methods on faecal samples

DNA yields obtained for each extraction method carried out on faecal samples are summarized in table 1. Overall, the Lysis reagent [3] and the Extraction DNA mix [4] provided the greatest yield of DNA as calculated from A260 values. The InviMag Stool DNA kit [1] provided good performances in terms of DNA yielded, while the QIAamp DNA Stool kit [2] produced the least amount of DNA extracted.
The treatment with Dynalbeads did not result in relevant differences in the yield and purity of DNA extracted.

Comparison of extraction methods on pork samples

Table 2 shows the average DNA extracted from pork samples. Overall, the Lysis reagent [2] and the Extraction DNA mix [3] provided the greatest yield as calculated from A260 values. The Charge Switch gDNA mini Bacteria Kit [1] as well as the boiling method provided good performances in terms of DNA extracted.
Relevant differences were observed when the sample were pre-treated with Dynalbeads. In particular the DNA recovery from pork samples treated with Dynalbeads and extracted with methods 1 and 4 was lower than the one obtained from samples untreated with beads and extracted with the same methods.

	N	Mean	Median	Minimum	Maximum	SD
Test Herds						
DD/AY before	33	34.3	31.0	2.6	118.5	24.6
DD/AY after	33	24.4	23.4	1.5	84.8	19.3
Delta-DD/AY	33	-9.8	**-8.2**	-34.1	11.0	10.6
Control herds						
DD/AY before	29	41.8	28.0	2.9	210.8	42.8
DD/AY after	29	39.9	27.8	2.8	198.0	38.8
Delta-DD/AY	29	-1.9	**1.7**	-45.3	39.2	18.5

Table 1. Comparison of the four extraction methods used for the recovery (µg/ml) of DNA from faecal samples spiked with Salmonella Typhimurium (results are expressed as mean values determined for triplicate samples prepared at each concentration).
** samples without pre-treatment with Dynalbeads; ** samples with pre-treatment*

Initial inoculum CFU/10gr	Method	1		2		3		4	
	Name	Charge Switch gDNA mini Bacteria		Lysis reagent		Extraction DNA mix		Boiling method	
	Pretreatment	*	**	*	**	*	**	*	**
40 CFU/10gr		173	15	200	200	200	200	113	55
84 CFU/10gr		143	48	200	200	200	200	165	77
119 CFU/10gr		155	12	200	200	200	200	77	54

Table 2. Comparison of the four extraction methods used for the recovery (µg/ml) of DNA from pork samples spiked with Salmonella Typhimurium (results are expressed as mean values determined for triplicate samples prepared at each concentration).
** samples without pre-treatment with Dynalbeads; ** samples with pre-treatment*

Real Time PCR analysis of DNA extracted from spiked faecal samples

The best performance, calculated as the lowest Ct value, was obtained for samples extracted with QIAamp DNA Stool kit [2], followed by Extraction DNA mix [4]. Conversely, kit 1 and 3 failed to produce PCR products and this is probably due to the presence of inhibitors in the DNA extracted.
The best LMM for repeated measures data selected presents the significant fixed effects of the pre-treatment, the methods tested and the interaction among them ($p<0.001$). The residuals were normal ($p>0.10$) and without particular patterns.
Dynalbeads pre-treatment clearly decreases the level of detection, and specifically for methods 2 and 4, a significant increase in the Ct values was observed. In particular, for method 4 the Ct values obtained were significantly higher than the ones provided by method 2 and these differences were evident more in samples pre-treated with Dynalbeads than in ones without pre-treatment (Figure 1).

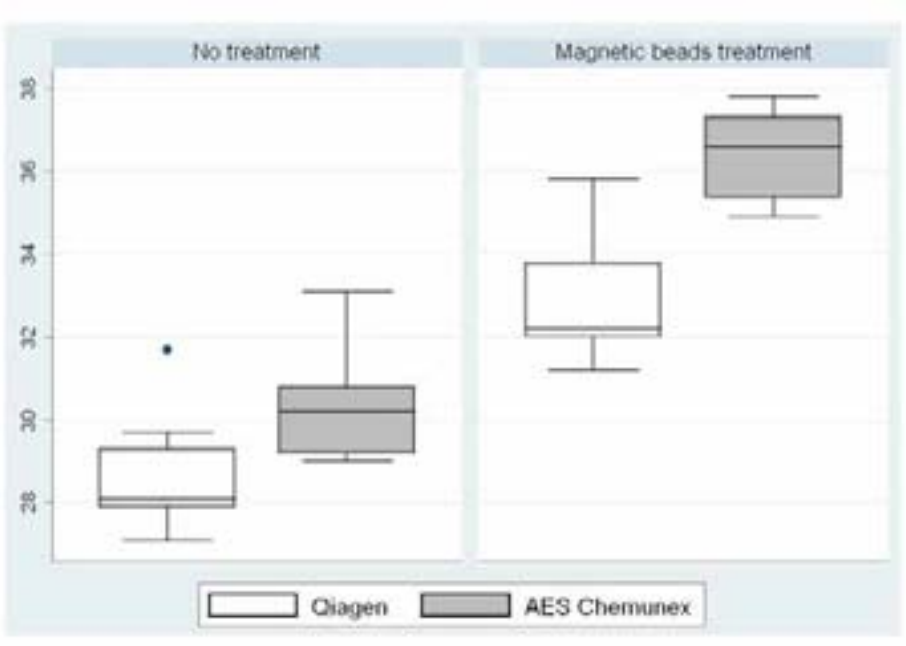

Figure 1: Box-and-whisker plots of distribution Ct values from different extraction methods on faecal samples pre-treated or not with Dynalbeads

Real Time PCR analysis of DNA extracted from spiked pork samples

The best method, identified as the one providing the lowest Ct values by Real Time PCR analysis, was the Charge Switch gDNA miniBacteria Kit [1], followed by the Extraction DNA mix [3] and the boiling method [4]. Conversely, method 2 failed to produce PCR products, probably due to the presence of inhibitors.

The best LMM presented the significant fixed effects of pre-treatment, methods of extraction and their interaction ($p<0.001$). The residuals are normal ($p>0.10$;) and without particular patterns.
For method 1 a significant change in terms of Ct values was not noted after the treatment with Dynalbead, on the contrary for methods 3 and 4 a certain difference was evidenced and more pronounced as far as method 4 is concerned. In particular the treatment with Dynalbeads resulted in an increase on the Ct values. (Figure 2).

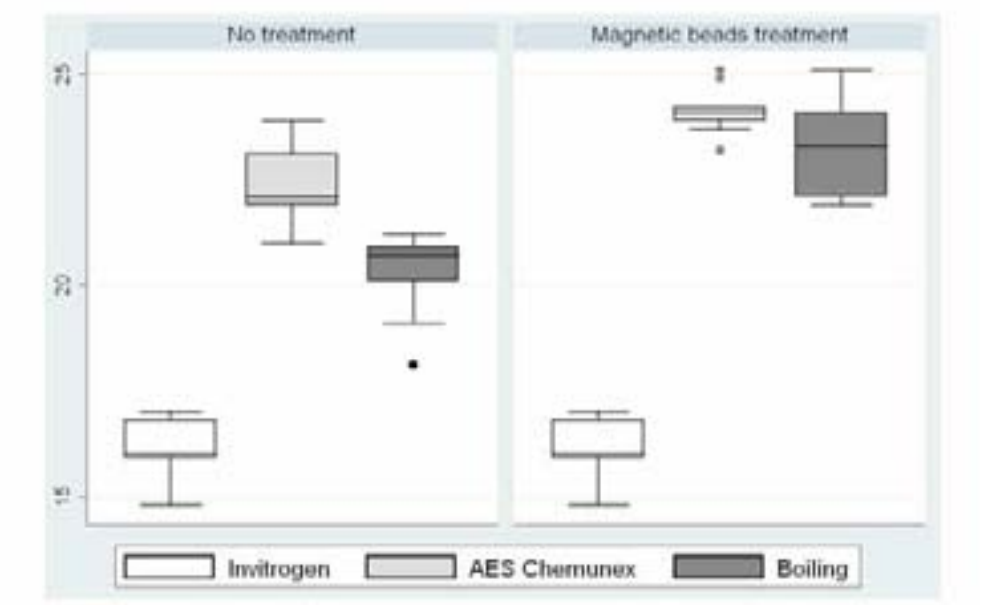

Figure 2: Box-and-whisker plots of distribution Ct values from different extraction methods on pork samples pre-treated or not with Dynalbeads

Discussions and conclusions

The efficacy of Real Time PCR analysis, as well as of other molecular methods can be enhanced by using suitable extraction methods for the matrix tested. On the other hand, many commercial DNA extraction kit are patented and this precludes the possibility of a comprehensive comparison of their technical features.
Although further and more extensive studies are needed, our results show that QIAamp DNA Stool kit [2], and Extraction DNA mix [4] are the most suitable methods to detect Salmonella in pig faeces. As far as pork samples are concerned, the best performances were obtained using the Charge Switch gDNA miniBacteria Kit [1]; however, the Extraction DNA mix [4] and the boiling method, possibly with some improvements, represent an inexpensive, handling and time-saving method to obtain Salmonella DNA from different pig matrices. Eventually, the pre-treatment of samples with Dynalbeads did not result in an improvement of the DNA recovery.

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