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Comparison of DNA isolation methods and detection of *Salmonella* spp. from animal faeces and dust using *invA* real-time PCR

Vergleich verschiedener Methoden zur Isolierung von DNA und zum Nachweis von Salmonella spp. aus Tierkot und Staub mittels invA real-time PCR

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Summary

There is a strong interest to reduce the expenditure for the detection of *Salmonella* spp. from animal faeces and environmental samples from primary production according to ISO 6579:2002 Annex D by including a rapid and effective method to detect *Salmonella* spp. already after pre-enrichment in BPW. It has been shown that real-time PCR methods are very effective to detect *Salmonella* organisms after pre-enrichment of foods. However, materials from primary animal production compose of much higher amounts of substances which might inhibit the sensitivity of real-time PCR. Different techniques of DNA isolation after pre-enrichment of artificially inoculated bovine faecal material were used to compare their detection limit and detection probability using an *invA* 5' nuclease real-time PCR approach. A detection probability of 100% was shown at 10^5 cfu/ml using the QIAamp® DNA Stool Mini Kit (Qiagen, Germany), at 10^4 cfu/ml using the High Pure PCR Template Preparation Kit® (Roche, Germany) and at 10^3 cfu/ml using thermal cell lysis or an in-house lab protocol, respectively. In comparison DNA isolation by thermal cell lysis revealed a very good detection limit, low costs and almost no risks of contamination. Furthermore, caecal contents from pigs were analysed by ISO 6579:2002 Annex D and the *invA* real-time PCR using thermal cell lysis for DNA extraction. As a result neither false positive nor false negative findings were obtained. Inclusion of the real-time PCR after pre-enrichment of samples in BPW followed by bacterial detection of *Salmonella* only with samples positive with real-time PCR might be a valuable tool to fulfil the international standard of ISO 6579:2002 Annex D but also to diminish the expenditures. However, it must be stated that the modification of an international standard method and its use in routine diagnostic requires the validation and registration of national and/ or international competent authorities.

Keywords: *Salmonella*, polymerase-chain-reaction, DNA isolation, faecal samples, ISO 6579 Annex D

Zusammenfassung

Es gibt ein starkes Interesse, den Material- und Zeitaufwand für den Nachweis von *Salmonella* spp. aus Tierkot und Umgebungsproben entsprechend der ISO Norm 6579:2002 Anhang D durch die Einbeziehung einer schnellen und effektiven Methode zum Nachweis von Salmonellen direkt nach der Voranreicherung zu verringern. Es wurde gezeigt, dass die real-time-PCR eine sehr effektive Methode für den Nachweis von Salmonellen aus Lebensmitteln nach einer Voranreicherung ist. Im Gegensatz dazu sind in Proben aus dem Tierbereich jedoch viel größere Mengen an bakterieller Sekundärflora und an hemmenden Substanzen vorhanden, die die Sensitivität der real-time-PCR beeinträchtigen können.
Daher wurden verschiedene Methoden zur Isolation von DNA nach einer Voranreicherung von Salmonellen mit Tierkot verglichen, um das Detektionslimit und die Detektionswahrscheinlichkeit mithilfe einer *invA*-basierten real-time-PCR zu bestimmen. Eine Detektionswahrscheinlichkeit von 100 % konnte mit dem QIAamp® DNA Stool Mini Kit (Qiagen, Deutschland) erst ab 10^5 kbE/ml, mit dem High Pure PCR Template Preparation Kit® (Roche, Deutschland) ab 10^4 kbE/ml und

sowohl bei der Kochlyse als auch bei einem neu entwickelten Hausprotokoll ab 10^3 kbE/ml erreicht werden. Im Vergleich aller verwendeten DNA-Extraktionsmethoden besitzt die Kochlyse eine sehr gute Nachweisgrenze, ein geringes Kontaminationsrisiko und verursacht sehr geringe Kosten. Darüber hinaus wurden Proben aus dem Zäkum-Inhalt von Mastschweinen nach ISO 6579:2002 Annex D und der *invA*-basierten real-time-PCR mit Kochlyse zur DNA-Isolation untersucht. Der Vergleich zeigte, dass weder falsch positive noch falsch negative Ergebnisse auftraten. Die Einbeziehung der *invA*-real-time-PCR nach einer Voranreicherung der Proben und die nachfolgende Fortsetzung des Nachweises von Salmonellen nur mit den Proben, die mittels real-time-PCR positiv waren, könnte ein Verfahren darstellen, um sowohl die Anforderungen der ISO 6579:2002 Anhang D zu erfüllen aber gleichermaßen den Aufwand für diese Methode zu verringen. Es muss jedoch darauf hingewiesen werden, dass die Modifikation einer international gültigen Standardmethode und deren Anwendung in der Routinediagnostik eine entsprechende Validierung und Zulassung durch die zuständigen nationalen und internationalen Behörden erfordert.

Schlüsselwörter: *Salmonella*, Polymerasekettenreaktion, DNS-Isolierung, Tierkot, ISO 6579 Anhang D

Introduction

Salmonelloses belong to the most prominent foodborne zoonoses throughout the world (EFSA, 2010). Sources of highest significance for human infection with non-host adapted *Salmonella* organisms represent contaminated foods. Raw meat, especially pork, undercooked products of poultry meat, eggs and products containing raw eggs as well as unpasteurised milk are foods posing the greatest hazard to public health. Therefore, effective control of *Salmonella* in primary animal production is an important prerequisite to prevent these organisms from entering the food chain. To analyse sources of entry, routes of spreading or to evaluate effects of hygienic as well as other prophylactic and control measures to combat *Salmonella* at farm level, rapid and effective detection of these organisms is necessary. In order to analyse the frequency of *Salmonella* at farm level different materials which can serve as source or vector (e. g. faeces, dust, feed, rodents, insects) of *Salmonella* organisms need to be examined (Rowse and Fleet, 1982; Nakamura et al., 1994; Gast et al., 1998; Doyle and Erickson, 2006; Harbaugh et al., 2006).

Therefore, there is a strong demand to detect *Salmonella* organisms from different materials with high sensitivity and specificity. The traditional culture method is used as a "reference method" to detect *Salmonella* organisms and since 2002, ISO 6579 Annex D, represents the legislative norm for the detection of *Salmonella* from animal faeces and environmental samples from primary production of farm and other animals (Anonymous, 2007). This culture method includes the non-selective pre-enrichment in buffered peptone water followed by selective enrichment using modified semisolid Rappaport-Vassiliadis medium and plating on two solid selective media. Colonies of interest are confirmed biochemically and serologically. However, the procedure according to ISO 6579:2002 Annex D takes 4–5 days to be completed. For this reason, there is a strong interest to reduce the expenditure for the bacterial examination by including a rapid and effective method to detect *Salmonella* spp. already after pre-enrichment. Recently, 5'-nuclease real-time PCR methods have been developed as technology to detect bacterial contaminants in foods (Chen et al., 2000; Malorny et al., 2004; 2009; Piknova et al., 2005). Non-selective enrichment combined with real-time PCR has been applied for the detection of *Salmonella* in foods in order to improve both, sensitivity and time management (Malorny et al., 2009). For the isolation of the genomic DNA from the pre-enrichment broth, commercial kits (Hein et al., 2006; Gonzalez-Escalona et al., 2009) or thermal cell lysis (Malorny et al., 2003a, 2007a; Sareyyüpoglu et al., 2008) are used. However, compared to pre-enrichment broth of food samples, pre-enrichment broth of faecal or environmental samples compose of much higher quantities of secondary flora and inhibitory substances (Wilson, 1997; Malorny and Hoorfar, 2005) which might decrease the sensitivity of the real-time PCR.

One aim of this study was to compare different DNA isolation methods which may reduce such inhibitory substances. A modified real-time PCR protocol detecting the *invA* gene (Hadjinicolaou et al., 2009) was used to analyse the detection limit of these DNA isolation methods using artificially contaminated faeces. In order to imitate naturally contaminated samples, faeces and dust were artificially inoculated with very low numbers of *Salmonella* organisms, stored at room temperature for several days and examined by both, microbiological examination and real-time PCR. To evaluate the *invA* based real-time PCR samples of caecal content from pigs collected in an abattoir were analysed and compared with the standard culture method according to ISO 6579:2002 Annex D.

Material and Methods

Bacterial strains and isolation of genomic DNA

All bacterial strains used in this study are listed in Table 1. A total of 18 *Salmonella* strains and 14 non-*Salmonella* strains were used for inclusivity and exclusivity tests. The non-*Salmonella* strains were chosen because of the close relation to *Salmonella* or as they are found in the same environment and grow under the same conditions. To extract genomic DNA for use in the

molecular detection assay, bacteria were cultivated in 5 ml nutrient broth (SIFIN, Germany) for 18 h at 37°C. 1 ml of this culture was transferred in a 1.5 ml tube for centrifugation for 10 min at 13 000 rpm. The supernatant was discarded and the cell pellet was used for DNA extraction using DNeasy® Blood and Tissue Kit (Qiagen, Germany). *Salmonella* strains used for artificial inoculation were incubated in nutrient broth for 18 h at 37°C to a cell density of approximately 10^8 cfu/ml. Using these cultures, 10-fold dilution series required for the different experiments were prepared in PBS.

Pre-cultured and artificially inoculated faeces

Faeces from bovines were collected and tested for the absence of natural contamination with *Salmonella* organisms. To calculate the detection limit of *Salmonella* in faeces 10-fold dilution series of three different very well described (Berndt et al., 2007; Methner et al., 2010) nalidixic-acid (N) resistant *Salmonella* (*S.*) strains (Enteritidis 147N, Typhimurium 9098N and Infantis 1326N) were prepared and mixed with an 18 h pre-cultured suspension containing 1 g faeces and 9 ml buffered peptone water (BPW). The final concentration of *Salmonella* in these suspensions ranged from 10^8 to 10^0 cfu/ml. These artificially inoculated samples were additionally incubated for 30 min at 37°C and afterwards the genomic DNA was extracted. Additionally, the number of *Salmonella* organisms was detected by plate counting on deoxycholate-citrate agar supplemented with 50 µg/ml nalidixic-acid (Methner et al., 2010).

Methods of DNA isolation

Three different methods were used to isolate genomic DNA from pre-cultured and artificially inoculated faeces from bovines. (i) Thermal cell lysis: 1 ml of pre-cultured and artificially inoculated sample was centrifuged at 14 000 g and the pellet was washed once with 1 ml PCR-gradient water. After another centrifugation at 14 000 g the pellet was resuspended in 200 µl water and boiled for 15 min. Cell fragments were separated by centrifugation at 14 000 g and 1 µl of the supernatant was directly used for the real-time PCR. (ii) In-house lab-protocol: 1 ml of pre-cultured and artificially inoculated sample was centrifuged and the pellet was homogenised in 600 µl stool lysis buffer (SLP: 500 mM Tris-HCl pH 9.0, 50 mM EDTA, 10 mM NaCl) and heated for 10 min at 70°C (Deuter et al., 1995). Afterwards, 600 µl SLP containing 150 mg of absorption matrix (potato flour, www.neuform-international.de or Qiagen InhibitEx Tablets®) was added to the homogenate. The suspension was mixed vigorously for 10 min and centrifuged at 2000 and 14 000 g each for 5 min to precipitate cell debris and absorption matrix. After digesting the clear supernatant with proteinase K (2.5 mg/ml), the DNA was precipitated by ethanol (99.8%) and sodium acetate (3 M, pH 5.2) followed by centrifugation at 14 000 g for 20 min. The DNA pellet was washed once with ethanol (70%), dried at 50°C and subsequently resuspended in 200 µl PCR water. One µl of the DNA suspension was used in the real-time PCR. (iii) Commercial DNA isolation kits: one ml of pre-cultured and artificially inoculated sample was centrifuged at 14,000 g and the genomic DNA was isolated using the (a) Roche High Pure PCR Template Preparation Kit® or the (b) Qiagen QIAamp® Mini Stool DNA Preparation Kit.

Internal amplification control (IAC) target synthesis, amplification and quantification

IAC target sequence was synthesised by PCR amplification using long overlapping primers (Hadjinicolaou et al., 2009). Briefly, the PCR reaction was performed in a 25 µl reaction volume containing 24 µl Qiagen Taq PCR Mastermix (Qiagen, Germany) and 50 pmol each of primers TFIAC and TRIAC (Tab. 2). Amplification was performed with an activation step of 94°C for 30 s, followed by 20 cycles, each consisting of 94°C for 20 s, 68°C for 30 s and 72°C for 20 s, followed by a final extension step of 72°C for 5 min in an Eppendorf Mastercycler (Eppendorf AG, Germany). 3 µl of the product from the first PCR was used in the secondary PCR in a 50 µl reaction volume containing 25 µl Qiagen Taq PCR Mastermix and 50 pmol of each primer 302 and 437 (Tab. 2). Amplification was performed with

TABLE 1: *Salmonella and non-Salmonella strains used for inclusivity and exclusivity tests in the real-time multiplex PCR*

Bacterial strain	O-group	O-antigen	H-antigen		real-time PCR
			phase 1	phase 2	
Salmonella Enteritidis	D_1	1, 9, 12	(f), g, m, (p)	-	+
Salmonella Dublin	D_1	1, 9, 12(Vi)	g, p	-	+
Salmonella Typhimurium	B	1, 4, (5), 12	i	1, 2	+
Salmonella Choleraesuis	C_1	6, 7	c	1, 5	+
Salmonella Gallinarum	D_1	1, 9, 12	-	-	+
Salmonella Panama	D_1	1, 9, 12	l, v	1, 5	+
Salmonella Brandenburg	B	1, 4, 12	l, v	e, n, z_{15}	+
Salmonella Oranienburg	C_1	6, 7, 14	m, t	(z57)	+
Salmonella Paratyphi B	B	1, 4, (5), 12	b	1, 2	+
Salmonella Mbandaka	C_1	6, 7, 14	z_{10}	e, n, z_{15}	+
Salmonella Bovismorbificans	C_2–C_3	6, 8	r, (i)	1, 5	+
Salmonella Hadar	C_2–C_3	6, 8	z_{10}	e, n, x	+
Salmonella Infantis	C_1	6, 7, 14	r	1, 5	+
Salmonella Senftenberg	E_4	1, 3, 19	g, (s), t	-	+
Salmonella Münster	E1	3, (10), (15), (34)	e, h	1, 5	+
Salmonella Manhattan	C_2–C_3	6, 8	d	1, 5	+
Salmonella Newington	E_1	3, 15	e, h	1, 6	+
Salmonella Derby	B	1, 4, (5), 12	f, g	(1, 2)	+
Enterobacter cloaceae					-
Citrobacter diversus					-
Proteus mirabilis					-
Proteus vulgaris					-
Escherichia fergusonii					-
Vibrio spp.					-
Pseudomonas spp.					-
Enterobacter spp.					-
Enterobacter cloacae					-
Escherichia coli 11775					-
Escherichia coli 35421					-
Klebsiella pneumoniae					-
Citrobacter freundii 8090					-
Escherichia coli 1266					-

TABLE 2: *Oligonucleotide primers and probes used in the real-time multiplex PCR assay for the detection of Salmonella*

Designation	Sequence	Position	Reference
Primer 302	TTGGCGATAGCCTGGCGGTG	302–321	Hadjinicolaou et al. (2009)
Primer 437	TGTTTACCGGGCATACCATCCAGAG	413–437	Hadjinicolaou et al. (2009)
Probe invA	FAM-CCGCGACTTCCGCGACACGTTC-BBQ	382–403	this study
Probe IAC	YAK-GCTACTCAGCAGAGGCTCCCTCG-BBQ	N/A	this study
TFIAC	TTGGCGATAGCCTGGCGGTGGCTGTATCGACGATGATCTGCTACTAGCTCGAGGGAGCCTCTGCTGAG-TAGCGACACTGATCGCCCTCGACTAGCTCGGTACAT	N/A	Hadjinicolaou et al. (2009)
TRIAC	TGTTTACCGGGCATACCATCCAGAGATGTACCGAGCTAGTCGAGGGCGATCAGTGTCGCTACTCAGCA-GAGGCTCCCTCGAGCTAGTAGCAGATCATCGTCGATACAGC	N/A	Hadjinicolaou et al. (2009)

an activation step of 94°C for 30 s, followed by 40 cycles, each composing of 94°C for 20 s, 60°C for 30 s and 72°C for 30 s, followed by a final elongation step of 72°C for 5 min in an Eppendorf Mastercycler. The PCR product was run in a 2% agarose gel with a low range DNA ladder (Fermentas GmbH, Germany) and then cleaned with Qiagen gel extraction kit (Qiagen, Germany). The 129 bp fragment was cloned in pGEM T-Easy (Promega GmbH, Germany), resulted in the vector pGEM_IAC. The vector pGEM_IAC was transferred into Ca^{2+}-competent cells of *Escherichia coli* JM109 and transformants were selected on LB agar plates containing ampicillin (50 mg/l), X-Gal (20 g/l) and IPTG (200 g/l). The *E. coli* containing the plasmid pGEM_IAC was cultivated overnight and the plasmid was isolated using QIAprep® Spin Miniprep Kit (Qiagen, Germany). The concentration was measured on an Eppendorf BioPhotometer (Eppendorf AG, Germany). The number of molecules was calculated from the measured concentration and the molecular weight of the plasmid pGEM_IAC. The plasmid was then diluted to a concentration of approximately 100 copies/µl. This concentration was used for the duplex real-time PCR.

InvA based duplex real-time PCR

All sequences of probes, primers and the IAC are listed in Table 2. According to Hadjinicolaou et al. (2009) the duplex real-time PCR in this study was modified using LightCycler480 TaqMan probes instead of molecular beacons (MBINVA, MBIAC). The target sequences of the probes detecting the gene *invA* or the IAC were not modified. The compounds for one real-time PCR-reaction are listed in Table 3. Amplification was performed with an UNG incubation step at 50°C for 5 min, an activation step of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s. PCR water was used as negative control and genomic DNA of *S.* Enteritidis in a concentration of approximately 10^6 copies/µl was used as positive control in all real-time PCR experiments. Samples were estimated as *Salmonella*-positive if a positive result was observed in the real-time PCR and the negative controls (water and *Salmonella*-free extraction samples) turned out negative. In this case the IAC could be positive or negative. Samples were assessed as *Salmonella*-free if a negative result was observed in the real-time PCR and the positive controls (e. g. genomic DNA of *Salmonella*) were positive. In this case the IAC had to be positive to exclude an inhibitory effect of the DNA extract. All samples used in the real-time PCR were analysed twice and the mean C_t- value was directly calculated by the LightCycler480 software (Roche GmbH, version 1.5)

TABLE 3: *Compounds for a single real-time multiplex PCR reaction for the detection of Salmonella*

Reagent	Volume µl	Final concentration
Roche LightCycler 480 Probes Master	10	1x
Primer 302	1	0.5 pmol/µl
Primer 437	1	0.5 pmol/µl
Probe invA	0.4	0.2 pmol/µl
Probe IAC	0.4	0.2 pmol/µl
pGEM_IAC	1	app. 100 copies
Uracil-N-glykosylase (UNG)	0.25	0.25 U
BSA	0.2	1x
H_2O	4.75	-
isolated DNA	1	
total volume	20	

Standard curve and detection limit

Duplex real-time PCR reactions as described above were performed on 10-fold serial dilutions (10^7-10^0 copies/µl) of the genomic DNA of strain *Salmonella* Enteritidis 147N. The DNA concentration was measured using an Eppendorf BioPhotometer and the number of DNA molecules was calculated from the measured concentration and the molecular weight. The reactions were performed on a LightCycler 480 system (Roche GmbH, Germany).

Determination of the detection probability

The detection probability of the PCR assay was obtained by plotting the relative number of positive PCRs observed against the number of *Salmonella* of artificially inoculated faeces from cattle. A sigmoidal line fitting was performed using the Sigmaplot program (SPSS inc., version 8.02). The determination of the detection probability was done using the extracted DNA of the different isolation methods. The number of *Salmonella* in the artificially inoculated faeces was determined by plate counting on deoxycholate-citrate agar supplemented with 50 µg/ml nalidixic-acid (Methner et al., 2010). The detection probability was determined in the presence of 100 copies of IAC DNA. Data points were generated by 9 repetitive PCRs from 3 independent experiments.

Detection of *Salmonella* in faeces and dust

To simulate as close as possible conditions of naturally occurring environmental samples faeces from bovines and dust from a farm building were collected and tested for the presence of natural contamination with *Salmonella* organisms. These sample pools, free of *Salmonella*,

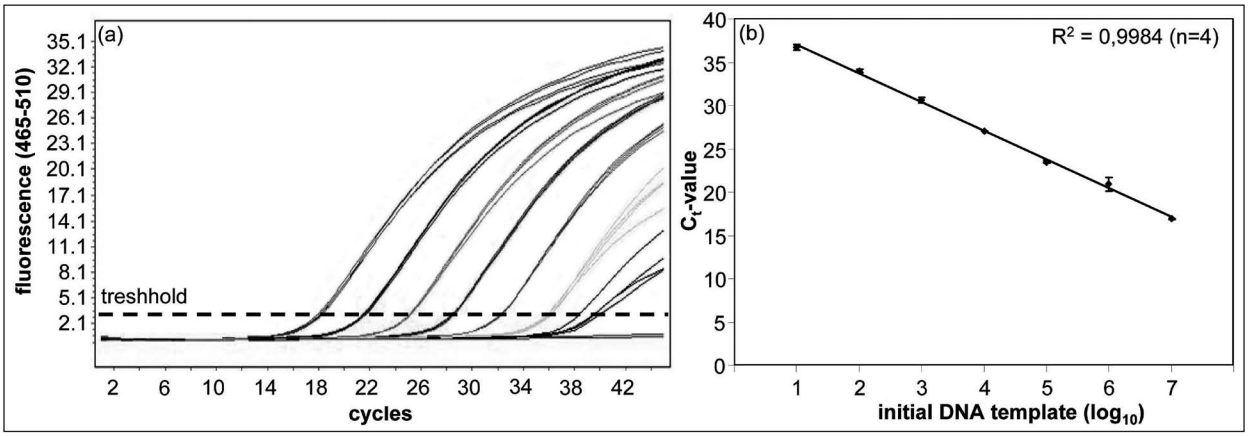

FIGURE 1: *Fluorescence plot (a) and standard curve (b) for Taqman-probe based duplex real-time PCR detection of target invA. The plot of the standard curve illustrates the relationship of known number of DNA copies per PCR reaction to the threshold cycle of detection (Ct).*

were used for artificial inoculation with *S.* Enterititidis 147N, *S.* Typhimurium 9098N and *S.* Infantis 1326N to final concentrations of 10^2, 10^1 or 10^0 cfu/g faeces or dust. Sterile PCR water was used as negative control. All samples were stored for 0, 3 or 5 days at room temperature. After storage samples were diluted 1:10 in BPW and incubated for 18 h at 37°C. Afterwards the number of *Salmonella* organisms was detected by plate counting on deoxycholate-citrate agar supplemented with 50 µg/ml nalidixic-acid (Methner et al., 2010). For the duplex real-time PCR the genomic DNA was prepared by thermal cell lysis using 1 ml of the pre-enrichment broth.

Evaluation of the real-time PCR using naturally contaminated samples

61 samples of caecal content from pigs taken during slaughtering at an abattoir were used to validate the real-time PCR. Samples were diluted 1:10 in BPW and further examined for the occurrence of *Salmonella* according ISO 6579:2002 Annex D. For the duplex real-time PCR the genomic DNA of samples was prepared by thermal cell lysis using 1 ml of the pre-enrichment broth.

Results

Standard curve and limit of detection

Standard curve was plotted to ensure the ability of the *invA*-probe to detect its specific *Salmonella* target and to calculate the detection limit of the assay (Fig. 1). The copy number of the target standard used ranged from 10^0 to 10^7 copies per reaction. Using the duplex real-time PCR the minimum detection of the *invA* target was 10 copies per reaction with a C_t-value of 36.66 ± 0.32 (n = 4). The small standard errors calculated and the R^2-correlation value of 0.998 with 100% efficiency suggest that the PCR amplification is highly reproducible. Based on the standard curve and the limit of detection in this assay, negative results were defined as those exhibiting C_t-values higher than 45.

Selectivity

Table 1 shows the results of the inclusivity and exclusivity tests. All 18 *Salmonella* strains tested were positive in the duplex real-time PCR and the non-*Salmonella* strains were not detected. Positive results had C_t-values ranging from 15 to 25 and for the IAC probe, the threshold cycle ranged from 36 to 38.

Methods of DNA isolation and detection limit of *Salmonella* from artificially inoculated faeces

Due to the identical results in the duplex real-time PCR for all three Samonella strains the mean C_t-values were calculated (Tab. 4). The detection limits of the thermal cell lysis and the in-house lab-protocol (potato flour or Qiagen InhibitEx Tablets®) was 10^2 cfu/ml, whereas the detection limits of the commercial DNA isolation kits were 10^2 cfu/ml (Roche, High Pure PCR Template Preparation Kit®) and 10^3 cfu/ml (Qiagen, QIAamp® DNA Stool Mini Kit), respectively. Furthermore, it was shown that in all isolation methods the IAC was amplified (Tab. 4). Therefore, it can be stated that inhibitory

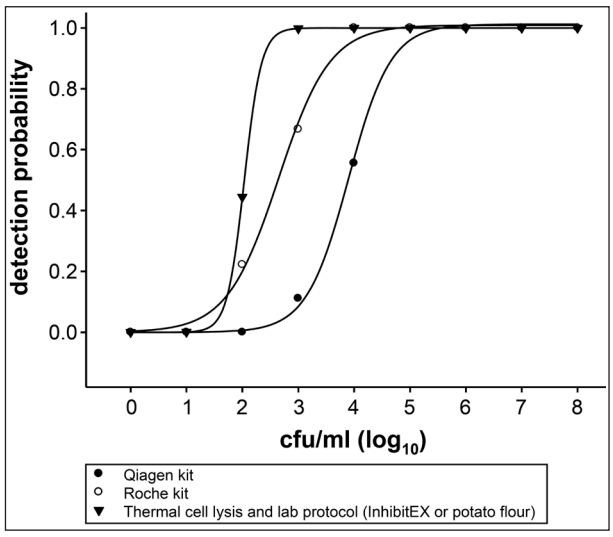

FIGURE 2: *Comparison of the detection probability between the different DNA isolation methods. The detection probability was determined using one microlitre of extracted DNA as template in the presence of 100 copies of IAC DNA. The detection probability between the three Salmonella strains was similar so that data were pooled. Data points were generated by nine repetitive PCRs from three independent experiments.*

substances did not impair the quality of the real-time PCR. However, it was observed that the IAC was not amplified when using high numbers of *Salmonella*, but through the identical primer set, primer were bound to the high concentrated genomic DNA extracted by the different isolation methods. The detection probability calculated for isolation methods and numbers of *Salmonella* is shown in Figure 2. A detection probability of 44% for a cell density of 10^2 cfu/ml was noticed for the thermal cell lysis and the lab protocol (potato flour or Qiagen InhibitEx Tablets). For these protocols a detection probability of 100% was observed at a concentration of 10^3 cfu/ml. The commercial kits from Roche (High Pure PCR Template Preparation Kit®) and Qiagen (QIAamp® DNA Stool Mini Kit) revealed a detection probability of 100% at concentrations of 10^4 cfu/ml and 10^5 cfu/ml, respectively. The ranking of isolation time was thermal cell lysis (1 h), Roche kit (High Pure PCR Template Preparation Kit®) (2 h), in-house lab-protocol (3 h) and Qiagen kit (QIAamp® DNA Stool Mini Kit) (4 h), whereas the ranking of DNA-purity was Qiagen (QIAamp® DNA Stool Mini Kit), Roche (High Pure PCR Template Preparation Kit®), in-house lab-protocol and thermal cell lysis (data not shown).

Detection of *Salmonella* in faeces and dust

Due to the minimal difference of the number of *Salmonella* organisms and the C_t-values between the three artificially inoculated *Salmonella* strains within one treatment only the mean values were displayed in Figure 3. One treatment is a combination of the final concentration (10^2, 10^1, 10^0 cfu/ml) of the strains and the storage time (0, 3, 5 days), e. g. 10^0 cfu/g stored for 5 days. It was shown that in all samples *Salmonella* strains could be detected with both methods, by plating and by using the duplex real-time PCR. Even after 5 days storage of *Salmonella* strains in faeces at the lowest start concentration, the number of *Salmonella* reached approximately 10^6 cfu/ml and a C_t-value of 30 could be detected. After 5 days of storage the number of *Salmonella* organisms in dust was lower than in faeces with approximately 5 x 10^4 cfu/ml with a C_t-value of 35. Furthermore, a clear correlation was observed between the number of *Salmonella* organisms detected by plate counting and the C_t-value detected by the duplex real-time PCR (Fig. 3)

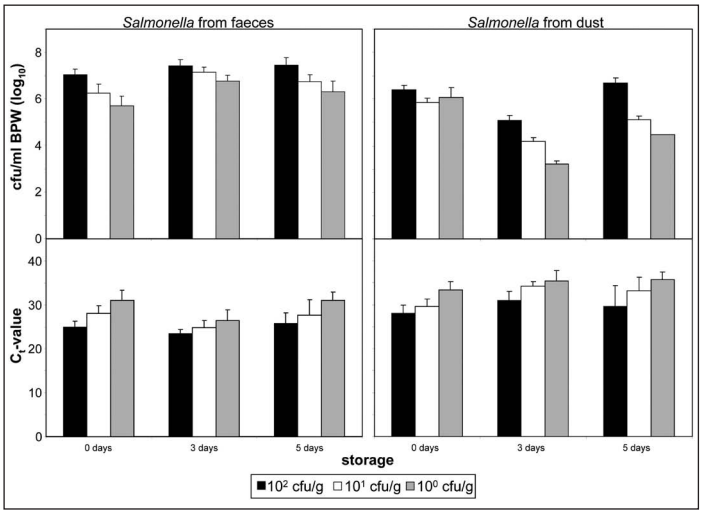

FIGURE 3: *Number of Salmonella organisms and the corresponding C_t-values after 18 h incubation of dust and faeces artificially inoculated (10^2, 10^1, 10^0 cfu/g) with S. Enteritidis 147N, S. Typhimurium 9098N or S. Infantis 1326N and storage (0, 3 or 5 days at room temperature) before incubation in BPW. Due to the minimal differences in the number of Salmonella organisms and the corresponding C_t-values between the three Salmonella serovars in one treatment (e. g. 10^0 cfu/g, 5 days), the mean value was calculated and presented in one data column. All tests were repeated twice (n = 6).*

Evaluation of the real-time PCR with naturally contaminated samples

A total of 61 samples of caecal content from pigs were analysed by the culture method according to interna-

TABLE 4: *Comparison between the mean C_t- and s_r-values of different isolation methods in the presence of 100 IAC copy numbers. 18 h pre-enrichment broth (1g faeces + 9ml BPW) was artificially inoculated with S. Enteritidis 147N, S. Typhimurium 9098N or S. Infantis 1326N to a final concentration ranging from 10^8 to 10^0 cfu/ml. One ml of spiked samples was used to isolate the DNA followed by the real-time PCR. Due to the minimal differences of the C_t-values between the three Salmonella serovars, the mean of the C_t-values was calculated. All isolation methods were repeated with all three serovars three times (n = 9)*

No. of *Salmonella* (cfu/ml)	Thermal cell lysis		High Pure PCR Template Preparation Kit® (Roche)		QIAamp® DNA Stool Mini Kit (Qiagen)		Lab protocol (potato flour)		Lab protocol (Qiagen InhibitEx Tablets®)	
	Dye									
	FAM[a] (*Salmonella* probe)	Yakima Yellow (IAC probe)	FAM (*Salmonella* probe)	Yakima Yellow (IAC probe)	FAM (*Salmonella* probe)	Yakima Yellow (IAC probe)	FAM (*Salmonella* probe)	Yakima Yellow (IAC probe)	FAM (*Salmonella* probe)	Yakima Yellow (IAC probe)
	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$	$C_t \pm s_r$
10^8	20.13 ± 2.00	n. d.	20.98 ± 1.23	n. d.	26.19 ± 2.43	37.29 ± 1.33	20.62 ± 2.24	n. d.	21.91 ± 1.79	n. d.
10^7	23.26 ± 1.63	n. d.	24.26 ± 1.54	n. d.	29.32 ± 2.09	36.06 ± 0.78	23.90 ± 1.20	n. d.	25.22 ± 1.67	37.02 ± 1.33
10^6	26.29 ± 1.29	40.23 ± 1.73	27.84 ± 1.49	37.86 ± 1.66	32.84 ± 2.06	36.15 ± 1.16	27.00 ± 1.87	36.30 ± 0.79	28.70 ± 1.94	35.89 ± 1.63
10^5	29.92 ± 1.54	38.41 ± 2.71	31.24 ± 1.49	36.50 ± 1.26	35.71 ± 1.72	36.50 ± 1.15	30.37 ± 1.92	37.80 ± 2.07	32.40 ± 2.19	36.57 ± 1.83
10^4	33.05 ± 1.52	37.40 ± 1.95	34.25 ± 1.72	37.16 ± 1.60	37.50 ± 1.30	37.11 ± 0.95	33.80 ± 2.06	36.85 ± 1.56	34.99 ± 2.83	35.45 ± 1.55
10^3	36.86 ± 1.71	36.94 ± 0.92	37.19 ± 2.98	36.45 ± 1.49	37.77 ± 0.00	36.74 ± 1.44	36.81 ± 1.06	35.99 ± 1.38	37.95 ± 2.25	35.65 ± 1.56
10^2	38.90 ± 2.03	37.46 ± 1.21	38.44 ± 1.45	36.31 ± 1.97	n. d.	37.01 ± 1.86	38.21 ± 1.91	37.13 ± 1.54	38.43 ± 2.71	36.02 ± 1.79
10^1	n. d.[a]	37.09 ± 1.20	n. d.	36.34 ± 1.18	n. d.	36.25 ± 0.85	n. d.	37.03 ± 1.39	n. d.	35.31 ± 1.45
10^0	n. d.	37.07 ± 1.02	n. d.	36.31 ± 1.64	n. d.	36.53 ± 0.91	n. d.	37.42 ± 1.19	n. d.	35.79 ± 1.58
0	n. d.	36.98 ± 1.05	n. d.	36.39 ± 1.45	n. d.	36.77 ± 1.93	n. d.	37.13 ± 1.44	n. d.	36.33 ± 1.76
water	n. d.	35.67 ± 0.88	n. d.	36.68 ± 2.30	n. d.	36.46 ± 0.58	n. d.	37.17 ± 1.53	n. d.	36.32 ± 1.75

[a] FAM – 6-FAM-phosphoramidit.

[b] n. d. – not detectable.

TABLE 5: *Detection of Salmonella from caecal content of pigs by bacteriology (ISO 6579:2002 Annex D) and duplex real-time PCR*

Type of sample	Number of samples	ISO 6579:2002 Annex D		Duplex real-time PCR		Sensitivity (%)	Specificity (%)	Accuracy (%)
		No. of positive samples	No. of negative samples	No. of false negative samples	No. of false positive samples			
Caecal content	61	33	28	0	0	100	100	100

tional standard ISO 6579:2002 Annex D and the real-time PCR method. Of these 61 samples, 33 were positive and 28 were negative by both methods (Tab. 5). The sensitivity, specificity and accuracy were 100% and no false-positive or false-negative samples were obtained by PCR.

Discussion

Prerequisite for the effective control of *Salmonella* infection at primary animal production is the detection of *Salmonella* from different materials which can serve as source or vector of these organisms with high sensitivity and specificity. ISO 6579:2002 Annex D represents not only the international but also the "reference standard" for detection of *Salmonella* spp. from animal faeces and environmental samples at primary production (Anonymus, 2007). As this method takes about 4–5 days to be completed, there is a strong interest to reduce both time and expenditure. Particularly real-time PCR methods have been described to be rapid, robust, effective and most suitable to detect *Salmonella* spp. after pre-enrichment of food samples (Malorny et al., 2007b; Lee et al., 2009). A number of different real-time PCRs to detect *Salmonella* spp. in foods have been described using target genes like *fimC* (Piknova et al., 2005), *himA* (Chen et al., 2000), *ttrC/A* (Malorny et al., 2004) or *spaQ* (Kurowski et al., 2002), but real-time PCR using *invA* as target gene was often described as effective and highly selective. All species and subspecies of *Salmonella* could be detected using *invA* as target but other bacteria related to *Salmonella* or organisms present in the same environment as well as grow under the same conditions were not identified as *Salmonella* (Rahn et al., 1992; Chen et al., 1997; Hoorfar et al., 2000; Perelle et al., 2004; Hadjinicolaou et al., 2009; Suo et al., 2010). However, Ginocchio et al. (1997) reported that some strains of *S.* Litchfield and *S.* Senftenberg partly lost their *invA* gene and were not detectable when *invA* primer were used in PCR. However, Malorny et al. (2003b) tested a number of *Salmonella* serovars and also strains from serovars *S.* Litchfield and *S.* Senftenberg using *invA* PCR and concluded that absence of the *invA* gene in *Salmonella* seems to be very rare.

In this study we examined the duplex real-time PCR according to Hadjinicolaou et al. (2009) modified by us for their potential to be included as a rapid method during the bacteriological detection of *Salmonella* according to ISO 6579:2002 Annex D. In contrast to foods, faeces, dust and other material from primary production compose of much higher amounts of secondary flora and inhibitory substances which might substantially decrease the sensitivity of especially PCR methods (Schrank et al., 2001). Therefore, a suitable isolation method must be developed to yield sufficient genomic *Salmonella* DNA for the real-time PCR. For this approach the methods of thermal cell lysis, an in-house isolation protocol as well as two commercial DNA isolation kits from Roche (High Pure PCR Template Preparation Kit®) and Qiagen (QIAamp® DNA Stool Mini Kit) were compared with regard to their detection limit for *Salmonella* in artificially inoculated faeces. In some studies faeces were exposed intendedly with different quantities of *Salmonella* prior to the 18 h pre-enrichment step followed by DNA isolation and real-time PCR analyses (Kurowski et al., 2002). In this case *Salmonella* will grow until a concentration of approximately 10^4 to 10^8 cfu/ml pre-enrichment broth and this concentration will definitely be detected (Klerks et al., 2006; Rapp, 2010). In this study we inoculated a 18 h incubated 1:10 mixture of faeces from bovines and BPW with a defined concentration of *Salmonella* followed by the DNA isolation step and the real-time PCR analysis. Therefore, we were able to exactly determine which concentration of *Salmonella* is detected by the different DNA isolation methods combined with our *invA* based in-house real-time PCR.

Using the commercial kits the detection limit of *Salmonella* was 10^2 to 10^3 cfu/ml BPW/faeces broth for Roche (High Pure PCR Template Preparation Kit®) and Qiagen (QIAamp® DNA Stool Mini Kit), respectively. However, using these kits a very low detection probability was observed (22% Roche [High Pure PCR Template Preparation Kit®], 11% Qiagen [QIAamp® DNA Stool Mini Kit]). Similar results were found in detecting *Escherichia coli* (Gioffre et al., 2004) or *Campylobacter* (Inglis and Kalischuk, 2003). As the purity of DNA extracted with these kits is very high, they have been used successfully for bovine faeces (Inglis and Kalischuk, 2003). However, both kits are complex, expensive and time-consuming, so that their use in routine analyses might be limited. Considerably cheaper is the in-house lab protocol using the potato flour which binds inhibitory substances. Similar in composition as the potato flour is the patented InhibitEx Tablet® produced by Qiagen. Therefore, it was not surprising that results between the in-house lab protocol for DNA isolation using the potato flour or InhibitEx Tablet® did not differ. In both cases the detection limits of *Salmonella* were 10^2 cfu/ml of BPW/faeces broth with a detection probability of 44%. This in-house protocol is economical (using potato flour) and the purity of the DNA was only somewhat lower than the DNA isolated with the commercial kits. The detection limit of *Salmonella* using thermal cell lysis was also 10^2 cfu/ml BPW/faeces broth with a detection probability of 44%. Although the purity of DNA was rather low, the IAC was amplified at all times. Therefore, substances possibly present after thermal cell lysis did not interfere with the real-time PCR reaction. It rather seems that the loss of DNA during the different isolation procedures plays a more decisive role for the detection limit of *Salmonella* (Klerks et al., 2006). The excellent detection limit of the thermal cell lysis could be due to the low losses of DNA and the robustness of the real-time PCR reaction which might be also the result of the use of bovine serum albumin which is most effective to eliminate PCR inhibitory

substances from faeces (Malorny and Hoorfar, 2005). A further advantage of the thermal cell lysis is the lower risk of contamination, because all steps are handled in one reaction cup. The other methods use different cups, substances or also silica columns, therefore, handling in numerous steps with several materials pose a risk of contamination (Malorny and Hoorfar, 2005). Our results, therefore, indicate that thermal cell lysis of faecal samples pre-enriched in BPW is an appropriate method to gain sufficient amounts of *Salmonella* DNA in adequate purity to perform the duplex real-time PCR with high sensitivity. Using this method we were able to detect approximately 1 cfu *Salmonella*/g artificially inoculated faeces or dust, respectively. Therefore, thermal cell lysis followed by a real-time PCR to detect *Salmonella* organisms is not only a valuable technique for foods as shown earlier (Chen et al., 1997; Malorny et al., 2004, Perelle et al., 2004; Piknova et al., 2005) but also for samples from primary animal production.

To evaluate the real-time PCR caecal contents from pork collected during slaughtering were examined. These samples were analysed by both the standard method ISO 6579:2002 Annex D as "reference standard" and the real-time PCR. Comparing these methods neither false positive nor false negative results were obtained.

In conclusion, the results in this study favour DNA extraction by thermal cell lysis as this method reveals a very good detection limit, is cheap and almost free of contamination risks. The efficacy of this method could be confirmed in this study using dust and faecal material from bovines or pigs, faecal material from other animal species still needs to be tested. In combination with the presented modified duplex real-time PCR a rapid and robust method with high accuracy might be available which could be used to reduce expenses and time for the bacterial examination according to ISO 6579:2002 Annex D. Inclusion of the *invA* real-time PCR after pre-enrichment of samples in BPW and the continuation of *Salmonella* detection and identification afterwards only with samples which were positive with real-time PCR after pre-enrichment might be a valuable tool to fulfil the international standard of ISO 6579:2002 Annex D but also to diminish the expenditures. However, it must be stated that the modification of an international standard method and its use in routine diagnostic requires the validation and registration of national and/or international competent authorities.

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