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Rapid and Specific Detection of *Salmonella* spp. in Animal Feed Samples by PCR after Culture Enrichment

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A PCR procedure has been developed for routine analysis of viable *Salmonella* spp. in feed samples. The objective was to develop a simple PCR-compatible enrichment procedure to enable DNA amplification without any sample pretreatment such as DNA extraction or cell lysis. PCR inhibition by 14 different feed samples and natural background flora was circumvented by the use of the DNA polymerase *Tth*. This DNA polymerase was found to exhibit a high level of resistance to PCR inhibitors present in these feed samples compared to DyNAzyme II, FastStart *Taq*, Platinum *Taq*, *Pwo*, r*Tth*, *Taq*, and *Tfl*. The specificity of the *Tth* assay was confirmed by testing 101 *Salmonella* and 43 non-*Salmonella* strains isolated from feed and food samples. A sample preparation method based on culture enrichment in buffered peptone water and DNA amplification with *Tth* DNA polymerase was developed. The probability of detecting small numbers of salmonellae in feed, in the presence of natural background flora, was accurately determined and found to follow a logistic regression model. From this model, the probability of detecting 1 CFU per 25 g of feed in artificially contaminated soy samples was calculated and found to be 0.81. The PCR protocol was evaluated on 155 naturally contaminated feed samples and compared to an established culture-based method, NMKL-71. Eight percent of the samples were positive by PCR, compared with 3% with the conventional method. The reasons for the differences in sensitivity are discussed. Use of this method in the routine analysis of animal feed samples would improve safety in the food chain.

Salmonellosis is currently a zoonotic disease of considerable magnitude (15). Animal feed is a recognized source of *Salmonella enterica* for farm livestock and might also act as an indirect cause of infection of people consuming foods of animal origin. Salmonellae can survive for several years in feed (14), and low concentrations have been found to be infective in, for instance, chickens (18). Transmission of salmonellae from feed to animals such as cattle and chickens is well documented (18, 22). In several countries, salmonellae have been isolated from animal feed ingredients as well as the final product, of both vegetable and animal origin, with a prevalence of between 0 and 6% (17, 30). The levels of salmonellae in feed are low; D'Aoust and Sewell (14) quantified salmonellae in several kinds of feed ingredients and compound feeds and found the levels to be less than 1 bacterium per g of feed.

The established culture-based methods used to detect salmonellae in animal feed are laborious, time-consuming, and often not specific enough. The standard methods used today for analyzing salmonellae involve preenrichment in buffered peptone water (BPW), selective enrichment in Rappaport-Vassiliadis soy broth, plating on selective agar, and subsequent identification by biochemical tests, for example, NMKL-71 (7). The whole procedure takes at least 3 days to complete. Several alternative analysis strategies have been proposed, and PCR in particular has been found to be a highly specific molecular diagnostic tool (20, 35). Although this technique can be extremely effective on pure solutions of nucleic acids, its sensitivity may be reduced dramatically when it is applied directly to complex biological samples (28). Since feed can originate from both animal and vegetable sources, inhibition could be caused by a number of compounds commonly found in feed, such as lipids, salts, and proteins (40). DNA and cells other than the target organism have also been shown to affect both the sensitivity and specificity of PCR (39).

To achieve an efficient high-throughput PCR method suitable for routine analysis of feed samples, rapid and simple sample preparation methods are required (34). Many sample preparation methods, such as DNA extraction (13), and immunomagnetic separation (12), are laborious and expensive and do not provide the desired template quality. Therefore, there is a need for simple and robust sample preparation methods that take advantage of the use of whole cells instead of DNA before diagnostic PCR can be widely used for routine analysis of feed and food samples. The use of culture enrichment prior to PCR analysis serves many purposes, including (i) dilution of PCR-inhibitory substances present in the sample matrix, (ii) multiplication of the target organism to provide detectable concentrations, (iii) dilution of dead cells, and last but not least (iv) the possibility of isolating the target organism for complementary tests (37).

The aim of this work was to develop a rapid and simple PCR-based protocol suitable for routine analysis of viable *Salmonella* spp. in animal feed. The method should allow the detection of 1 CFU per 25 g of feed without the need for DNA extraction or treatment prior to PCR. Based on experimental data, a logistic regression model was used to describe the probability of detecting different concentrations of salmonellae in feed. Furthermore, in order to obtain a PCR-compatible sample containing enriched cells in the presence of feed and

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TABLE 1. Inhibitory effects of five different concentrations of 14 feed homogenates on the amplification capacity of eight thermostable DNA polymerases in a standardized PCR assay[a]

Feed homogenate	PCR result[b] with indicated DNA polymerase at feed homogenate concentration (%, vol/vol):																																							
	DyNAzyme II					FastStart Taq					Platinum Taq					Pwo					rTth					Taq					Tfl					Tth				
	20	4	2	0.4	0.2	20	4	2	0.4	0.2	20	4	2	0.4	0.2	20	4	2	0.4	0.2	20	4	2	0.4	0.2	20	4	2	0.4	0.2	20	4	2	0.4	0.2	20	4	2	0.4	0.2
Corn gluten	−	±	+	+	+	−	+	±	−	−	−	+	+	+	+	−	±	−	±	±	−	+	+	+	+	−	+	±	±	+	−	+	+	±	+	−	+	+	+	+
Corn pellets	−	+	+	+	+	−	+	±	−	+	−	+	+	+	+	±	+	+	−	±	−	+	+	+	+	−	+	±	+	+	−	+	+	+	+	−	+	+	+	+
Distiller's waste	±	±	+	+	+	±	+	±	+	+	±	+	+	+	+	±	±	−	±	+	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	±	+	+	+	+
Fish meal	−	+	+	±	+	−	+	±	+	±	−	+	+	+	+	−	+	+	−	±	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	±	+	+	+	+
Meat meal	−	+	±	+	+	−	+	±	−	±	−	+	+	+	+	−	+	+	−	±	−	+	+	+	+	−	+	+	±	+	−	+	+	+	+	±	+	+	+	+
Oats	−	±	+	+	+	−	+	±	+	±	−	+	+	+	+	±	+	+	−	±	−	±	±	+	+	−	−	+	±	+	−	−	−	+	+	±	+	+	+	+
Palm expeller	−	±	±	+	+	−	±	−	±	±	−	−	−	+	+	−	+	+	±	±	−	±	±	+	+	−	−	−	+	±	−	−	−	−	+	−	+	+	+	+
Pellets	−	+	±	+	+	−	+	±	±	±	−	+	+	+	+	+	+	+	−	+	±	−	±	+	+	−	+	±	+	±	−	+	±	+	+	−	+	+	+	+
Rape seed	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−	−	−	+	−	+	+	−	−	±	+	+
Rape seed meal	−	−	−	±		−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	±	+	−	−	±	+	+
Soy	−	+	±	−	+	−	+	±	−	±	−	+	+	+	+	−	+	+	−	−	−	+	+	+	+	−	±	+	±	+	−	+	+	+	+	±	+	+	+	+
Soy meal	−	+	+	−	+	−	+	−	±	−	±	+	+	+	+	−	+	+	−	−	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+
Wheat	−	−	±	+	+	−	−	−	+	+	−	−	−	+	+	−	+	+	±	+	−	−	−	±	+	−	−	−	±	+	−	−	−	−	+	±	−	+	+	+
Whey	±	+	+	+	+	±	+	±	+	−	−	+	+	+	+	+	+	+	−	+	−	−	+	+	+	−	+	+	+	±	±	+	+	+	+	±	+	+	+	+

[a] Five microliters of double distilled H_2O in the reaction mixture was replaced by 5 μl of the feed homogenates, and 5×10^3 Salmonella cells were used as the target for amplification. Feeds were homogenized in BPW at a ratio of 1:10 (wt/vol), and the homogenates were diluted in distilled water prior to PCR analysis. The following feed homogenate concentrations were used in the reaction mixture: 20, 4, 2, 0.4, and 0.2% (vol/vol).

[b] +, detection of a specific band in both independent replicates; ±, detection of a specific band in one of the two replicates; −, no detection of the specific product in either of the two replicates.

medium components, a number of alternative DNA polymerases and accompanying buffer systems were tested on 14 different feed samples. Finally, the method was evaluated on natural feed samples and the relative accuracy, relative sensitivity, and relative specificity were calculated.

MATERIALS AND METHODS

Feed samples. Salmonella-free feed samples were obtained from local feed factories (7). The following feeds were used: soy (acidified and nonacidified) (Glycine max), rape seed (meal and kernels) (Brassica napus), wheat (Triticum aestivum), oats (Avena sativa), palm kernel expeller (Elaeis guineesis), maize (gluten and pellets) (Zea mays), mixed feeds, whey, draff (distiller's waste), meat meal, and fish meal. Feed homogenates were prepared with a stomacher. Twenty-five grams of each feed was homogenized in 225 ml of buffered peptone water (BPW; Lab 46, LabM, Bury, United Kingdom) in a sterile plastic bag. Each bag was thoroughly shaken before 1-ml samples were withdrawn and frozen at −20°C. The feed homogenates prepared in this way were used throughout the study.

PCR conditions. The original PCR protocol, developed by Rahn et al. (35), amplifying a 0.28-kb PCR product originating from the invA gene, was combined with an internal control (31) and modified to include the following: 0.2 μM each primer (Scandinavian Gene Synthesis AB, Köping, Sweden), 200 μM each deoxynucleoside triphosphate (Roche Molecular Biochemicals, Mannheim, Germany), 0.75 U of one DNA polymerase (Tth, Taq, Pwo, FastStart Taq [Roche], rTth [Applied Biosystems, Foster City, Calif.], DyNAzyme II [Finnzymes, Espoo, Finland], Platinum Taq [Invitrogen Life Technologies, Karlsruhe, Germany], or Tfl [Promega, Madison, Wis.]), and 1× PCR buffer (supplied with each enzyme), and if Mg^{2+} was not included in the buffer, the concentration was adjusted to 1.5 mM with $MgCl_2$. The internal control used in the evaluation of the detection probability of the method and in the evaluation of naturally contaminated samples was present at 10^4 copies per PCR tube. The sample volume used was 5 μl, and the final volume was 25 μl.

PCR was performed in a GeneAmp 9700 PCR System thermocycler (Applied Biosystems, Foster City, Calif.). The temperature program started with a denaturation step of 5 min at 94°C, followed by 36 cycles at 94°C for 30 s, 53°C (Taq DNA polymerase in the specificity study) or 60°C for 30 s, and 72°C for 40 s, and then one cycle at 72°C for 7 min. Finally, the samples were cooled to 4°C. Samples were analyzed by gel electrophoresis on 1% agarose gels stained with ethidium bromide, and bands were visualized with the GelDoc 1000 system (Bio-Rad, Hercules, Calif.) with the Molecular Analyst computer program (Bio-Rad).

PCR inhibition by feed and background flora. Fourteen kinds of feed homogenate, prepared as described above, and dilutions (undiluted, 1:5, 1:10, 1:50, and 1:100, corresponding to 20, 4, 2, 0.4, and 0.2% in the reaction mixture, respectively) of the feed homogenates in distilled water were studied (Table 1). The inhibitory effect of the stored feed homogenates was tested by PCR with the DNA polymerases mentioned above; 5 μl of double-distilled H_2O added to the PCR tube was replaced by feed sample, and 5 μl of a cell suspension containing 10^6 CFU of S. enterica serovar Senftenberg S57 (an animal feed isolate, AnalyCen Nordic AB, Kristianstad, Sweden) per ml was used as the target for amplification.

The influence of an undefined background flora originating from a soybean sample on PCR detection was investigated with the Tth PCR assay described above. The background flora was obtained as follows: 4 g of soy was enriched in 36 ml of BPW at 37°C for 24 h, and 100 μl was transferred to 10 ml of BPW and further enriched at 37°C for 6 h to obtain fresh cells. The number of CFU of background flora was determined on tryptone glucose extract (TGE; Merck, Darmstadt, Germany) agar plates. Aliquots of the cell suspensions with the addition of 10% (vol/vol) sterile glycerol (Merck) were frozen at −20°C until required. They were thawed and diluted in distilled water before analysis. The experiment was designed to study the effect of concentration of the two factors S. enterica serovar Senftenberg S57 and background flora. The levels, in terms of concentration, were 10^1 to 10^7 CFU/ml in 10-fold dilutions of S57 and 10^1 to 10^8 CFU/ml in 10-fold dilutions of background flora. Samples without S57 or background flora were analyzed as controls. The composition of the background flora in this experiment was undefined because the aim was to study the PCR amplification at a certain concentration of background flora in the PCR test tube. Five microliters of double-distilled H_2O added to the PCR tube was replaced with 5 μl of background flora, and 5 μl of a cell suspension containing the different concentrations of S. enterica serovar Senftenberg S57 was used as the target for amplification. Ten replicates of each combination of concentrations were analyzed.

The influence of the sampling time was studied by homogenizing 4 g of Salmonella-free feed (soy and rape seed) and 36 ml of BPW in separate sterile plastic tubes. The feed homogenates and 50 ml of BPW in a separate tube were inoculated with 50 CFU of a fresh culture of S. enterica serovar Senftenberg S57. The inoculated cell count was determined by viable counts on TGE agar plates. The inoculated samples were incubated at 37°C for 24 h. Samples were withdrawn at 3-h intervals after thorough shaking of the tube. The ability to detect salmonellae in undiluted and in 1:10 and 1:100 dilutions in physiological saline with the PCR assay with Tth was determined. After sample withdrawal, samples were stored at −20°C until required for PCR analysis.

Specificity. The specificity of the PCR assays with Tth and Taq DNA polymerase was evaluated with strains isolated from food and feed, as well as strains

obtained from the AnalyCen Culture Collection, Kristianstad, Sweden; the American Type Culture Collection, Manassas, Va.; the Danish Veterinary Laboratory, Copenhagen, Denmark; H. Aarts, State Institute of Quality Control of Agricultural Products (RIKILT), Wageningen, The Netherlands (1); the Culture Collection, University of Göteborg, Göteborg, Sweden; Swedish Meats Research Institute Culture Collection, Kävlinge, Sweden; the National Veterinary Institute, Uppsala, Sweden; the National Food Administration, Uppsala, Sweden; and the Norwegian School of Veterinary Science, Oslo, Norway (see Table 3). Strains used in the study were cultured either on TGE plates or in BPW and maintained on beads (Protect bacterial preservers; Technical Services Consultants Ltd., United Kingdom) at −80°C until further use. One isolated colony of each strain was suspended in 0.5 ml of saline, and 5 μl of this mixture was added to the PCR tube.

Detection probability of the PCR assay. To study the probability of detecting salmonellae in a cell suspension of a known concentration, 45 independent PCR experiments were performed over a period of 6 months. A standard cell suspension of *S. enterica* serovar Senftenberg S57 was obtained by growth in BPW at 37°C for 4 h. Storage and concentration determination of the standard cell suspension were performed as previously described (25). However, incubation of the *Salmonella* cells for concentration measurements took place at 37°C for 24 h. The detection probability of the assay was evaluated with a dilution series with the concentration range from 10^2 to 10^7 CFU/ml in 10-fold dilution steps of the stored standard cell suspension; 5 μl of each concentration was added to the PCR tube, and the PCR assay was run, as described above, with *Tth* DNA polymerase. Each PCR trial gave a positive or negative result at the concentration tested. A graph of the detection probability of the PCR assay was obtained by plotting the observed relative frequencies of positive PCR detection versus concentration of *Salmonella* cells, as previously described (24).

Detection probability of the PCR method. Twenty-five grams of *Salmonella*-free soy was homogenized in 225 ml of BPW in a sterile plastic bag. The feed homogenates were inoculated with 0, 0.03, 0.3, 3, and 30 CFU from a dilution series of a fresh culture of *S. enterica* serovar Senftenberg S57. Ten replicates of each concentration inoculated were analyzed. The inoculated cell count was determined by measuring viable counts on TGE agar plates. The inoculated samples were incubated at 37°C for 18 h. Samples were withdrawn after 18 h after thorough shaking of the test tube. After sample withdrawal, samples were stored at −20°C until required for PCR analysis. The ability to detect salmonellae in 1:10 dilutions in physiological saline in the PCR assay with *Tth* was determined. A parallel PCR assay containing an internal control was run on samples.

Statistical analysis. The results generated by the detection probability studies for the PCR assay ($n = 45$) and the PCR method with and without internal controls ($n = 10$ in each case) were applied to a logistic regression model to assess the detection probability at different cell concentrations (24). The software Matlab (version 6.5) with the statistical toolbox Stixbox (19) was used. The slopes of the two regression curves were compared with the null hypothesis that the slopes (the value of b in the regression equation) are not equal for the two groups (before and after enrichment), whereas the intercept, a, is the same for the two groups. Logistic regression was used to test the null hypothesis. Model-based confidence intervals were calculated (5).

Evaluation of the PCR method on naturally contaminated samples. Animal feed and fecal samples, in all 155 samples (see Table 4) were analyzed for their content of *Salmonella* spp. with a standard culture-based method, NMKL-71 (7). After the enrichment step (16 to 18 h in BPW), samples were withdrawn and frozen at −20°C prior to PCR analysis. After dilution to 1:10 and 1:100 in physiological saline, PCR analysis was performed as described, with and without the internal control. The results of the NMKL and PCR methods were compared. When different results were obtained with the two methods, samples used for PCR analysis were streaked on TGE agar plates and reanalyzed as described in the section on specificity.

After confirmation, the relative accuracy, relative sensitivity, and relative specificity were calculated with the following formulas (6): relative accuracy = (PA + NA + FP)/N, relative sensitivity = (PA + TP)/(PA + FN), and relative specificity = NA/(NA + FP), where PA denotes positive agreement (positive results for both the NMKL and PCR methods), NA is negative agreement (negative results with both methods), FP denotes false-positive (negative for NMKL, positive result for PCR method not confirmed by growth), TP is true-positive (negative for NMKL, positive result for PCR method confirmed by growth), and FN denotes false-negative (negative PCR result, positive NMKL).

TABLE 2. PCR results in the presence of different concentrations of *S. enterica* serovar Senftenberg and background flora[a]

Background flora (CFU/ml)	PCR detection probability at *Salmonella* concn (CFU/ml):					
	3×10^2	3×10^3	3×10^4	3×10^5	3×10^6	3×10^7
10^8	0.6	0.7	1.0	1.0	1.0	1.0
10^7	0.8	1.0	1.0	1.0	1.0	1.0
10^6	0.4	0.8	1.0	1.0	1.0	1.0
10^5	0.5	0.7	1.0	1.0	1.0	1.0
10^4	0.7	0.8	1.0	1.0	1.0	1.0
10^3	0.4	0.8	1.0	1.0	1.0	1.0
10^2	0.5	0.9	1.0	1.0	1.0	1.0
10^1	0.3	0.8	1.0	1.0	1.0	1.0

[a] Detection probability for 10 replicates, calculated as the number of positive samples divided by the total number of replicates; 5′ μl of salmonellae and 5 μl of background flora were added to the PCR tube. The total reaction volume was 25 μl, and the *Tth* assay was used.

RESULTS

PCR inhibition by feed samples and background flora. To evaluate their inhibitory effects on PCR, five different concentrations of homogenates of 14 different feed samples (20, 4, 2, 0.4, and 0.2% [vol/vol] in the reaction mixture) were added to reaction mixtures containing 5.0×10^3 CFU of *S. enterica* serovar Senftenberg cells and different DNA polymerases (Table 1). The homogenized feed samples in BPW were all found to be inhibitory to PCR, but the level of inhibition was different when different DNA polymerases were employed. In the *Tth* assay, amplification of target cells in all feed homogenates tested was achieved in the presence of 0.4% (vol/vol) homogenate, in contrast to the assays with the other DNA polymerases. Rape seed and rape seed meal were found to be highly inhibitory for all thermostable DNA polymerases tested except *Tth*. Therefore, *Tth* and its accompanying buffer were used in further experiments.

Different concentrations of a background flora obtained from a soybean sample and *S. enterica* serovar Senftenberg S57 were added to the *Tth* assay to determine the detection sensitivity in the presence of an undefined background flora (Table 2). The detection sensitivity of the *Tth* assay was not affected by the presence of 10^1 to 10^8 CFU of background flora/ml when 3×10^4 to 3×10^7 CFU of salmonellae per ml were present in the PCR mixture.

To determine the appropriate time for sample withdrawal when using the *Tth* assay for routine analysis of feed and to verify that detection was unaffected by feed matrices and background flora from feed, a growth experiment was performed with soy and rape seed homogenized in BPW and inoculated with *S. enterica* serovar Senftenberg S57. PCR amplification was inhibited to a large extent when the undiluted samples were subjected to PCR analysis, as shown for the nonenriched feed samples (Table 1). However, diluting the samples could circumvent the inhibition by *Tth* DNA polymerase. In the soy sample detection was possible after 9 to 24 h of incubation and by diluting the sample 1:10 (corresponding to a concentration of 2% in the PCR tube). For the rape seed sample, detection was possible after 12 to 24 h of incubation and 9 to 24 h of incubation when the samples were diluted 1:10 and 1:100, respectively. Based on these data and the choice of the same preenrichment time as with the NMKL-71 method (18 h), a

protocol for the detection of salmonellae in feed was developed: (i) homogenization of 25 g of feed in 225 ml of BPW; (ii) enrichment for 18 h at 37°C; (iii) withdrawal of 100-μl sample; (iv) dilution 1:10 in 0.9% NaCl solution; and (v) addition of 5 μl to *Tth* PCR assay.

Specificity. To verify that the previously reported specificity (11, 31, 35) of the *invA* primer set designed by Rahn et al. (35) was not affected by changing the DNA polymerase from the originally used *Taq* DNA polymerase to *Tth* DNA polymerase, the specificity of two PCR assays with the two DNA polymerases was evaluated on 101 *Salmonella* strains and 43 non-*Salmonella* strains (Table 3). To demonstrate the usefulness of the assay for routine analysis of feed, isolates of the most commonly found serotypes (8) were included in the study. No difference in specificity was observed when comparing the assay with *Tth* DNA polymerase with that employing *Taq* DNA polymerase. The modified *Salmonella* assay was found to be specific, since all *Salmonella* strains resulted in a 0.28-kb PCR product, whereas none of the other strains gave specific amplicons. Strains from six non-*Salmonella* species generated nonspecific amplicons. However, in all instances the nonspecific products could be distinguished from the specific amplicon based on size.

Detection probability. A logistic regression model was fitted to the observed PCR data generated before and after enrichment (Fig. 1). The response of PCR can be described by a binary variable, taking the values 1 for detection and 0 for no detection. A model commonly used to describe methods where the response variable is binary is the logistic regression model (5, 21, 24), where the dependence of the detection probability on the *Salmonella* concentration, x, is described by $P(x) = e^{(a + bx)}/[1 + e^{(a + bx)}]$. From PCR analysis, the regression parameters a and b can be estimated with the maximum likelihood method (5). Inserting these parameters into the equation gives a function that illustrates the detection probability at various *Salmonella* concentrations. By plotting the concentration of salmonellae against the observed relative frequencies of positive PCR detection, it can be verified that the model applied fits the observed data. The model can then be used to describe the detection probability of the PCR method employed and to calculate the detection probability at various target concentrations or to determine the probability of detecting a certain amount of target, i.e., 1 CFU/25 g of sample if the model is applied to a PCR method.

The results showed that detection of small numbers of salmonellae was possible after 18 h of enrichment and that PCR detection could be described by a logistic regression model, estimated from a total of 40 independent observations (Fig. 1, curve A). The regression parameters were estimated to be: $a = 1.4560$ and $b = 1.9186 \pm 0.5986$, and the detection probability was 0.81 when 1 CFU was inoculated in 25 g of feed. The same regression parameters and detection probability were obtained when the internal control was included (Fig. 1, curve B). Finally, the detection probability for *S. enterica* serovar Senftenberg S57 cells in water was established at different cell concentrations with the *Tth* assay from data generated by 270 PCR results (Fig. 1, curve C). The applied statistical model estimated the regression parameters to be: $a = -11.3783$ and $b = 2.1517 \pm 0.2927$, which, inserted into the equation above, give

a detection level of 5×10^6 CFU/25 g of feed after enrichment, with a detection probability of 0.95.

Evaluation of the PCR protocol on naturally contaminated feed samples. To confirm the sensitivity and specificity of the PCR method, 155 naturally contaminated feed and fecal samples were analyzed and the results were compared with those from a standard culture-based method (Table 4). Twelve positive samples were found in total; all were detected with the PCR assay, but only four were detected with the NMKL method. To verify the positive PCR results, eight samples with positive PCR results and negative NMKL results were cultured on nonselective agar plates, and colonies were identified by PCR. As a result, two of the PCR-positive and NMKL-negative acidified soy samples were confirmed as positive, i.e., they contained living *Salmonella* cells (data not shown). The relative accuracy, sensitivity, and specificity were found to be 0.99, 1.50, and 0.96, respectively.

DISCUSSION

Several obstacles are encountered when analyzing salmonellae in animal feed samples, namely, low concentration and uneven distribution of the target organism in the feed. Furthermore, the presence of PCR inhibitors and background flora make this matrix unsuitable for PCR analysis. Biological samples such as feed as well as the enrichment broth or its components have been found to affect PCR (29). BPW has also been found to be inhibitory to real-time PCR with the DNA polymerase AmpliTaq Gold (26).

The various animal feeds tested in this study were found to be highly inhibitory to PCR. In an attempt to reduce PCR inhibition, different DNA polymerases were studied and it was found that by using *Tth* DNA polymerase, the PCR-inhibiting effect of the 14 various feed samples could be mitigated to a high degree. This finding is in accordance with those of previous investigations in which DNA polymerases from *Thermus thermophilus* (*Tth* and r*Tth*) were found to exhibit a high level of resistance to PCR-inhibitory components in different biological samples (2–4, 10, 27, 33) as well as to increase the sensitivity of the PCR assay (13, 23, 36). The choice of DNA polymerase depends, however, on the application. In this study, the PCR method should be able to function with a wide variety of samples, and therefore *Tth* DNA polymerase could be considered the best choice. On the other hand, if the method is intended to be used with a specific sample, another DNA polymerase may be optimal, e.g., *Pwo* for the pellet samples in this study or r*Tth* for blood samples (3). The biochemical composition of the PCR mixture, including the sample to be analyzed, should therefore be considered when optimizing the PCR protocol.

The small numbers of salmonellae in feed present an additional problem in analysis. The concentration of salmonellae in feed is generally considered to be below the established detection level of the PCR assay (Fig. 1, curve C), which was found to be 5×10^6 CFU/25 g of feed, corresponding to 2×10^4 CFU/ml of feed homogenate for *S. enterica* serovar Senftenberg, with an estimated detection probability of 0.95. To increase the number of cells and dilute feed inhibitors, an enrichment step at 37°C for 18 h in BPW, a commonly used nonselective medium for enrichment (7), was studied. When

TABLE 3. PCR results obtained with 144 bacterial strains in the *Taq* assay and the *Tth* assay[a]

Bacteria	No. of strains	PCR result[b]	
		Taq	*Tth*
S. enterica serovars			
Adabraka	1	+	+
Agona	4	+	+
Anatum	3	+	+
Arizonae	1	+	+
Blockley	1	+	+
Bovismorbificans	1	+	+
Cubana	3	+	+
Derby	3	+	+
Dublin	1	+	+
Ealing	1	+	+
Enteritidis	5	+	+
Havanna	2	+	+
Heidelberg	1	+	+
Indiana	1	+	+
Infantis	2	+	+
Lexington	3	+	+
Lille	1	+	+
Livingstone	2	+	+
Mbandaka	9	+	+
Mikawasima	1	+	+
Morehead	1	+	+
Rissen	3	+	+
Senftenberg	7	+	+
Tennessee	1	+	+
Thompson	2	+	+
Typhimurium	29	+	+
Vancouver	1	+	+
Wien	1	+	+
Yoruba	5	+	+
Zanzibar	1	+	+
Virginia	1	+	+
Salmonella sp.	1	+	+
Salmonella spp.	2	+	+
Citrobacter freundii	6	−	—
Citrobacter koseri	1	−	−
Enterobacter agglomerans	1	−	−
Enterobacter amnigenus	1	−	−
Enterobacter areogenes	1	−	−
Enterobacter cloacae	1	−	−
Enterobacter intermedium	1	−	−
Enterobacter sakazakii	2	−	−
Enterobacter taylorae	2	−	−
Escherichia adecarboxylata	1	−	−
Escherichia coli	8	—	—
Escherichia hermanii	1	−	—
Escherichia vulneris	1	−	−
Hafnia alvei	1	−	−
Klebsiella oxytoca	1	−	−
Klebsiella pneumoniae	4	−	−
Kluyvera ascorbata	1	−	—
Morganella morganii	1	−	−
Providencia rettgeri	1	−	—
Pseudomonas fluorescens	1	−	−
Pseudomonas fragi	1	−	−
Serratia ficaria	1	−	−
Serratia fonticola	1	−	−
Serratia liquefaciens	1	−	−
Serratia rubidea	1	−	—
Yersinia enterocolitica	1	−	−

[a] A PCR assay optimized for *Taq* DNA polymerase and a PCR assay optimized for *Tth* DNA polymerase with previously described (34) primers. The concentration of the bacterial suspension was approximately 10^6 CFU/ml. The following phage types were included for serovar Enteritidis: PT1, PT4, and PT21 (one strain each) and NPT (two strains). The following phage types were included for serovar Typhimurium: DT104 (three strains), DT195 (one strain), PT3 (one strain), PT10 (one strain), PT40 (three strains), PT41 (five strains), PT130 (two strains), PT193 (five strains), PT506 (one strain), and NPT (seven strains).

[b] +, detection of band with high intensity, −, no detection of the specific product; —, detection of unspecific bands with sizes different from that of the specific product.
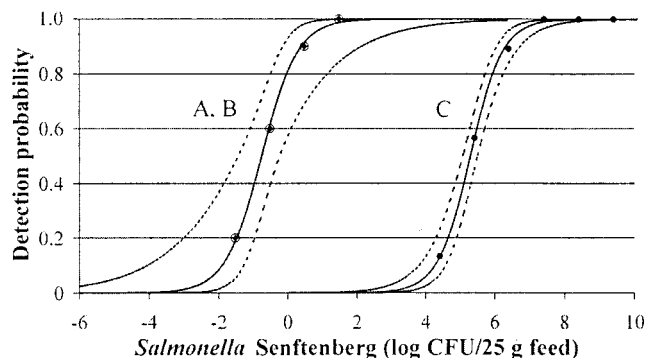

FIG. 1. Detection probability for *S. enterica* serovar Senftenberg (log CFU/25 g of feed) with the *Tth* assay and the PCR procedure. PCR detection is described by a logistic regression model, estimated from a number of replicate experiments, and is represented by the curves. The dotted lines represent the upper and lower model-based confidence intervals. By plotting the concentration of the target organism against the observed relative frequencies calculated as the number of positive samples divided by the total number of replicates for (●) pure cells in a water system analyzed without enrichment (*n* = 45 for each concentration, curve C), and artificially contaminated soy samples analyzed with the PCR procedure (♦) with (*n* = 10 for each concentration, curve A), and (○) without (*n* = 10 for each concentration, curve B) an internal control, it was verified that the model applied fits the observed data. The model was then used to describe the detection probability of the PCR studied, to calculate the detection probability at various target concentrations, and to determine the probability of detecting a certain amount of target.

this enrichment step was included in the method, the probability of detecting 1 CFU in 25 g of feed was 0.81, as estimated by a logistic regression model (Fig. 1, curves A and B). No significant difference could be observed when comparing the values of the slopes of the detection probability curves for *Salmonella* cells in water without enrichment and *Salmonella* cells in soy analyzed with the PCR procedure. If the PCR method had been inhibited by the presence of feed and background flora in the sample, the slope of the curve would have decreased. This means that the inhibition was overcome and the level of *Salmonella* cells had increased to a detectable level by employing the PCR procedure.

Furthermore, enrichment prior to PCR has been reported to

TABLE 4. Evaluation of the PCR method relative to the conventional culture method (NMKL) for the detection of *Salmonella* spp. in 155 naturally contaminated and noncontaminated feed and fecal samples

Sample type	No. of samples	Results (PCR/NMKL)[a]			
		+/+	−/−	−/+	+/−
Feces and intestines	22	0	22	0	0
Fish meal	4	0	3	0	1
Maize gluten	1	0	0	0	1
Meat meal	1	0	1	0	0
Mixed feed	24	0	24	0	0
Rape meal	8	0	8	0	0
Soy	59	3	56	0	0
Soy, acidified	36	1	29	0	6
Total	155	4	143	0	8

[a] +/+, positive PCR result, positive NMKL result; −/−, negative PCR result, negative NMKL result.

increase the number of target microorganisms and to minimize the risk of detecting DNA from dead cells (37). Bacteria that are naturally present in feed could have reduced viability due to, for example, low water activity, high salt concentrations, unfavorable pH, freezing, or heating. As a result, for most microorganisms nonselective enrichment increases the probability of recovery and detection of damaged cells (16). BPW is considered a good choice for the growth of sublethally damaged salmonellae in, for instance, heat-treated or dry products such as feed (38). However, BPW allows the growth of other bacteria, which may cause inhibition of PCR due to high cell counts, and the growth of competing microorganisms could mask the presence of salmonellae.

It has been shown that the practical operating range of a PCR assay is affected by the presence of DNA and cells in the reaction mixture (39). Furthermore, previous studies have shown that high concentrations of either background flora or target cells interfere with DNA amplification (29). Since feed samples are often highly contaminated with other microorganisms and the levels of background flora remain high after the nonselective enrichment step applied in the detection of salmonellae (9), background flora can be considered a limiting factor for efficient PCR analysis. The detection probability for *Salmonella* concentrations of $>2 \times 10^4$ CFU/ml in this study was found to be the same with and without the addition of background flora at levels as high as 10^8 CFU/ml with the DNA polymerase *Tth*. It has formerly been indicated that the use of r*Tth* improves the detection limit in the presence of large amounts of microorganisms in comparison with the use of *Taq* DNA polymerase (13).

When 155 feed samples were analyzed, more positive samples were identified with the PCR procedure than with the standard culture-based assay. Uneven distribution of salmonellae in the feed sample or in the preenrichment broth may account for some of these conflicting results. Furthermore, strains might not be able to grow in the selective broth or on the agar plates employed (12). It is known that to be able to distinguish between salmonellae and other bacteria growing on selective agar plates, the number of *Salmonella* cells must be at least 10^4 CFU/ml after the enrichment step (9). The presence of a *Salmonella* strain with atypical morphology, for instance, H_2S-negative strains, is also possible (38). Atypical strains are likely to cause problems in methods relying on manual judgment. The prevalence of salmonellae in feed found in this study is not representative of feed in Sweden, since samples were obtained to verify the accuracy of the PCR method, not to study prevalence.

Using a PCR-based method for routine diagnostic purposes on a large number of samples presents several specific demands such as speed, ease of use, and lack of false-positive or false-negative results. In this study, the total time to perform the analysis was decreased from at least 3 days for the standard culture-based method (7) to less than 24 h for the PCR procedure. The specificity of the PCR assay can be studied by testing several different target and nontarget strains, and hence the rate of false-positive samples can be controlled. The presence of PCR-inhibitory compounds can be monitored by the addition of an internal control DNA fragment in each reaction (32). However, the sensitivity of the PCR assay has been shown to be lowered by the addition of an internal control, and the use of low copy numbers of the internal control has been suggested to favor the amplification of the DNA fragment from the target organism (31). On the other hand, if the number of internal controls is too low, the detection probability of the internal control itself falls below 1, introducing the possibility of false-negative results. In this study, the detection probability for the PCR method was not altered by the addition of an internal control at a rather high concentration.

In conclusion, when optimizing a PCR-based detection method for a specific application, the intended samples should be considered from the beginning because the sample itself affects the biochemical composition of the PCR mixture and thus the outcome of the analysis. The use of more robust DNA polymerases is a convenient way of circumventing the inhibitory effect of many kinds of biological samples; in this study, inhibition by different kinds of feed samples was relieved by the use of *Tth* DNA polymerase. The specificity, an important factor in diagnostic PCR, was also shown to be unaffected by changing the DNA polymerase. Furthermore, the use of mathematical modeling provides an objective way of interpreting the results of PCR-based analysis, and in this study it was used to determine the probability of detecting small numbers of salmonellae inoculated in feed which were analyzed with the developed PCR protocol.

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