Rapid method for detection of salmonella in meat

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(54) **Title**: RAPID METHOD FOR DETECTION OF *SALMONELLA* IN MEAT

(57) **Abstract**: The present invention relates to a rapid method for the detection of *Salmonella* in meat as well as to a kit for performing said method. The method provides a time-to-result of less than 8 hours.

Rapid method for detection of *Salmonella* in meat

Field of invention

The present invention relates to a rapid method for the detection of *Salmonella* in meat as well as to a kit for performing said method.

Background of invention

Meat is one of the major sources of human *Salmonella* infection and, therefore, efficient and rapid monitoring of *Salmonella* in the meat production chain is necessary. *Salmonella* infection, also known as salmonellosis, is estimated to have caused the death of 1,316 people in the United States between 1990 and 2006. According to the FDA, close to 150 000 Americans are infected each year due to contamination of egg shells. Prevention of salmonellosis is of general interest to the public and requires rapid and accurate detection of *Salmonella* in food. Besides the expected positive impact on public health, improved detection methods are of interest to the food industry. Current detection methods take at least 8 hours, and some even take several days, before results are available. Products have often already been set in motion once the results become available, and recall of products is expensive. At the same time, detection methods should be easily implementable in situ. Thus methods requiring only relatively cheap equipment are desirable.

Traditional rapid methods for detecting *Salmonella* in meat samples are based on enrichment of a given sample, typically by incubation at 37°C, followed by nucleic acid extraction and PCR-based detection methods, such as RT-PCR. The legislative demand requires that detection methods can detect one CFU (colony forming unit) in the analysed sample, typically a 25 g sample. This low detection limit often leads to long enrichment times, so that there are enough starting materials for the PCR, e.g. the RT-PCR reaction to yield a reliable result. At the same time, the nucleic acid extraction and PCR steps are the most costly steps of the procedure and may require specialised equipment.

Several methods have been developed where the sample treatment time and the investment costs have been reduced to some extent by avoiding the nucleic acid purification step.

WO 2006/085906 discloses a method where no RNA extraction is needed. However, the enrichment step requires at least overnight incubation.

WO 2012/018964 discloses a method for detecting *Salmonella* in food samples with a time-to-result of less than 27 hours. The method requires enrichment of the solid meat samples in buffered peptone water (BPW) followed by nucleic acid extraction followed by detection (e.g. by real-time PCR or qPCR).

WO 2008/025570 discloses a multiplex real-time PCR detection method. For samples with very low contamination, an enrichment step is performed, e.g. by placing 25 g of meat in BPW and incubating for 18 hours at 37°C. A nucleic acid extraction is then performed.

Josefsen et al. (2007) describe a 12 hour PCR-based method for detection of *Salmonella* in meat. The method includes enrichment at 37°C for 6, 8 or 24 h and a step of nucleic acid extraction.

All of the above methods require either or both of a longer enrichment (overnight or longer) and a nucleic acid extraction step. Thus, there is a need for a method for detection of *Salmonella* that is very fast and easy. A faster detection of *Salmonella* results in an improved allocation or "turn-over" of carcasses or meat at abattoirs.

Summary of invention

In one aspect, the invention relates to a method for detection of *Salmonella* in at least one meat sample, said method comprising the steps of:

i) enriching the at least one meat sample by incubating said at least one meat sample in pre-heated enrichment broth such as buffered peptone water optionally comprising a surfactant such as polyoxyethylene (20) sorbitan monolaurate, wherein the incubation time is less than 6 hours and the incubation temperature is between 40°C and 44°C, thereby obtaining an enriched sample;

ii) concentrating at least part of said enriched sample, thereby obtaining a concentrated sample;

iii) treating said concentrated sample with a protease, thereby obtaining a treated sample, and concentrating said treated sample, thereby obtaining a concentrated treated sample;

iv) lysing said concentrated treated sample, thereby obtaining a lysate;

v) detecting the presence of at least one *Salmonella* marker in the lysate;

wherein the time-to-result of said method is less than 8 hours.

In another aspect, the invention relates to a kit for performing the method of the invention, said kit comprising:

- a protease or a protease mixture such as bacterial alkaline protease and/or serine endopeptidase from *Bacillus licheniformis*;
- enrichment broth powder;
- reagents for detecting the presence of at least one *Salmonella* marker such as a real-time composition for detecting a *Salmonella*-specific locus such as the *ttrRSBCA* locus, the *Inv*A locus, the *Sii* locus or the *Bcf*D locus;
- instructions for performing said method; and
- optionally, at least one Stomacher bag with a filter with a mesh size of 200-300 µm and tubes for performing the method.

Description of Drawings

Figure 1 shows the timeline for performing one embodiment of the present method when two technicians handle 50 samples, including sample preparation and labelling.

Definitions

Amplification product: refers to a nucleic acid fragment produced during a primer-directed amplification reaction such as real-time PCR.

Colony Forming Units (CFU): refers to the number of cells in a sample that produce a colony on an agar plate. CFU may also refer to the number of cells producing colonies that are expected to be in a sample by comparison to a similar assayed sample. This is an equivalent number of CFU, which is referred to as simply CFU or cells. Likewise, cells or CFU may also refer to the equivalent number of CFUs present in a sample prior to treatment or lysis.

Enrichment: the term herein refers to cultivation of a sample containing or suspected of containing a pathogenic bacterium in order to obtain enriched concentration of the bacteria in the sample and allow detection.

Lysis: perturbation or alteration of a cell membrane facilitating access to or release of the cellular RNA or DNA. Neither complete disruption nor breakage of the cell membrane is an essential requirement to the concept of lysis.

Meat sample: as used herein the term refers preferably to a 25 g sample of meat sampled from e.g. a carcass or from a processed piece of meat. Different sizes of meat may be used as meat samples as further described herein below.

Pathogen: a pathogen or a contaminant refers herein to a bacterial agent that can cause a disease.

Protease: the term protease refers to an enzyme that performs proteolysis. Different classes of proteases can perform the same reaction by completely different catalytic mechanisms. Proteases can be derived from animals, plants, bacteria, archaea and viruses.

Real-time polymerase chain reaction (real-time PCR): also known as quantitative PCR (qPCR) is a method allowing simultaneous amplification and detection of a targeted DNA molecule. A first method for product detection involves the use of non-specific fluorescent dyes that intercalate with any double-stranded DNA. Another method is based on sequence-specific DNA probes consisting of oligonucleotides labelled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary sequence.

Salmonella: this pathogenic enterobacterium is Gram-negative. There are two species of Salmonella; *Salmonella bongori* and *Salmonella enterica* of which there are around six subspecies and innumerable serovars.

Detailed description of the invention

The present inventors have developed a method for fast detection of *Salmonella* in meat samples. The method is based on specific conditions for enrichment of

Salmonella from meat sample: the meat samples are incubated with pre-heated buffered peptone water and incubated for less than 6 hours at a temperature between 40°C and 44°C. The sample is then concentrated and treated with a protease or a protease mixture. This is followed by a step of cell lysis, where the sample can be further concentrated. Finally, *Salmonella* is detected. The present method does not require any lengthy step of DNA extraction and detection is performed directly on the lysate. The time-to-result is less than 8 hours. Importantly, it appears to be the combination of an incubation step at a higher temperature than typically used for *Salmonella* enrichment with a step of treatment with a protease or a protease mixture combined with a fast DNA release by lysis of the treated cells at high temperature, which allows shortening of the time-to-result. The method can also be performed on pooled samples, as illustrated in the examples.

Thus in a first aspect the invention relates to a method for detection of *Salmonella* in at least one meat sample, said method comprising the steps of:

i) enriching the at least one meat sample by incubating said at least one meat sample in pre-heated enrichment broth such as buffered peptone water optionally comprising a surfactant such as polyoxyethylene (20) sorbitan monolaurate, wherein the incubation time is less than 6 hours and the incubation temperature is between 40°C and 44°C, thereby obtaining an enriched sample;

ii) concentrating at least part of said enriched sample, thereby obtaining a concentrated sample;

iii) treating said concentrated sample with a protease, thereby obtaining a treated sample, and concentrating said treated sample, thereby obtaining a concentrated treated sample;

iv) lysing said concentrated treated sample, thereby obtaining a lysate;

v) detecting the presence of at least one *Salmonella* marker in the lysate;

wherein the time-to-result of said method is less than 8 hours.

Samples

The present method is useful for detecting the presence of *Salmonella* in at least one meat sample. The meat sample may be a sample of poultry meat (white meat), of red meat, of game meat or other meat. Examples of poultry meat include meat originating from chicken, duck, goose, turkey and others. Examples of red meat include meat

originating from beef, pork, veal, sheep, pig, piglet and others. Examples of game meat include deer meat and others. Examples of other meat include rabbit meat, horse meat and others.

In some embodiments, the at least one meat sample is obtained from animal carcasses being processed in the abattoir.

Meat sample as used herein may also be understood as a swab sample obtained from e.g. a carcass. Such swab samples may be sampled according to ISO 17604 in accordance with EU directive 2073/2005/EC employing the non-destructive swab method with gauze swabs. The sites on e.g. a carcass such as a pig carcass that can be swabbed may include the ham, back, belly and jowl. A single swab may correspond to a single meat sample, or swabs may be pooled as described herein for meat samples.

In some embodiments, the at least one meat sample is one meat sample, such as at least two meat samples, such as at least three meat samples, such as at least four meat samples, such as at least five meat samples, such as at least six meat samples, such as at least seven meat samples, such as at least eight meat samples, such as at least nine meat samples, such as at least ten meat samples, such as at least fifteen meat samples, such as at least twenty meat samples.

In some embodiments, the at least one meat sample is a pooled sample derived from at least two meat samples. The at least two meat samples can be two samples originating from the same carcass or from different carcasses. In some embodiments, the at least two meat samples is at least two meat samples from two different carcasses, such as at least three meat samples from three different carcasses, such as at least four meat samples from four different carcasses, such as at least five meat samples from five different carcasses, such as at least six meat samples from six different carcasses, such as at least seven meat samples from seven different carcasses, such as at least eight meat samples from eight different carcasses, such as at least nine meat samples from nine different carcasses, such as at least ten meat samples from ten different carcasses. In other embodiments, the at least two meat samples is at least two meat samples from the same carcass, such as at least three meat samples from the same carcass, such as at least four meat samples from the

same carcass, such as at least five meat samples from the same carcass, such as at least six meat samples from the same carcass, such as at least seven meat samples from the same carcass, such as at least eight meat samples from the same carcass, such as at least nine meat samples from the same carcass, such as at least ten meat samples from the same carcass. In other embodiments, the at least two meat samples are at least two meat samples from two different carcasses, such as at least three meat samples from at least two different carcasses, such as at least four meat samples from at least two different carcasses, such as at least five meat samples from at least two different carcasses, such as at least six meat samples from at least two different carcasses, such as at least seven meat samples from at least two different carcasses, such as at least eight meat samples from two different carcasses, such as at least nine meat samples from at least two different carcasses, such as at least ten meat samples from at least two different carcasses. Preferably meat samples in a pooled sample originate from one or more animals of similar species.

The appropriate sample size is preferably such that the sample surface is between 10 and 100 cm^2, such as between 15 and 75 cm^2, such as between 20 and 50 cm^2, such as about 25 cm^2. Meat samples of about 5 X 5 cm, preferably as thin as possible e.g. 2-3 mm, are suitable for performing the present method. In some embodiments, the meat sample weighs between 2 and 100 g, such as between 2.5 and 90 g, such as between 5 and 80 g, such as between 10 and 70 g, such as between 15 and 50 g, such as between 20 and 40 g, such as between 20 and 30 g, such as between 21 and 29 g, such as between 22 and 28 g, such as between 23 and 27 g, such as between 24 and 26 g, such as 25 g. In some embodiments, the meat sample weighs 25 g. In other embodiments, the meat sample weighs 10 g. In other embodiments, the meat sample weighs 15 g.

While the conditions described herein are particularly well suited for performing the present method on a meat sample of 25 g, it will be understood that the skilled person can adapt the present method, in particular as regards volumes of the different compositions added to the sample, centrifugation and incubation times, to perform the method on a meat sample having a weight different from 25 g. For example, the method may be performed on a pooled sample of 10 samples of 25 g meat obtained from 10 different carcasses.

Enrichment

The method comprises a first step of enrichment, which allows potentially present bacteria such as *Salmonella* to proliferate so that detection is possible. Although *Salmonella* can grow over a relatively wide range of temperatures, it is customary in the art to incubate samples to be analysed for the presence of *Salmonella* at 37°C, which is considered to be its optimal growth temperature.

Thus in one embodiment, step i) is performed by incubating said sample with a volume of pre-heated enrichment broth, wherein:

- said volume is between 20 and 100 mL, such as between 30 and 90 mL, such as between 40 and 80 mL, such as between 50 and 70 mL, such as 60 mL;
- said pre-heated enrichment broth further comprises between 0.1% (volume/volume) and 1.0% of a surfactant such as polyoxyethylene (20) sorbitan monolaurate, such as 0.1%, such as 0.2%, such as 0.3%, such as 0.4%, such as 0.5%, such as 0.6%, such as 0.7%, such as 0.8%, such as 0.9%, such as 1%, preferably 0.5% of a surfactant such as polyoxyethylene (20) sorbitan monolaurate; and
- said pre-heated enrichment broth is at a temperature between 40°C and 50°C, such as 40°C, such as 41°C, such as 41.5°C, such as 42°C, such as 43°C, such as 44°C, such as 45°C, such as 46°C, such as 47°C, such as 48°C, such as 49°C, such as 50°C, preferably at 45°C.

Preferably the meat samples are not pre-heated before incubated with a volume of pre-heated enrichment broth.

Enrichment of *Salmonella* in the present method can be performed after placing the meat sample in a filter stomacher bag. The filter stomacher bag is preferably sterile and has a pore size allowing the bacterial cells to travel through the filter of the stomacher bag. A volume of non-selective, pre-heated enrichment broth, such as buffered peptone water optionally comprising a surfactant such as polyoxyethylene (20) sorbitan monolaurate (Tween 20), is then added to the filter stomacher bag.

The enrichment step may be performed by incubation of the filter bag comprising pre-heated enrichment broth optionally comprising a surfactant at a temperature between 40°C and 44°C, thereby obtaining an enriched sample.

In some embodiments, step i) is performed by incubating said sample with a volume of pre-heated enrichment broth, wherein said volume is between 20 and 100 mL, such as between 30 and 90 mL, such as between 40 and 80 mL, such as between 50 and 70 mL, such as 60 mL.

In some embodiments, the sample is incubated with a volume of pre-heated enrichment broth further comprising between 0.1% (volume/volume) and 1.0% of a surfactant such as polyoxyethylene (20) sorbitan monolaurate, such as 0.1%, such as 0.2%, such as 0.3%, such as 0.4%, such as 0.5%, such as 0.6%, such as 0.7%, such as 0.8%, such as 0.9%, such as 1%, preferably 0.5% of a surfactant such as polyoxyethylene (20) sorbitan monolaurate.

In one embodiment, the surfactant is polyoxyethylene (20) sorbitan monolaurate. Other suitable surfactants are polyoxyethylene (80) sorbitan monolaurate (Tween 80) and polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100). Thus in some embodiments, the sample is incubated with a volume of pre-heated enrichment broth further comprising between 0.1% (volume/volume) and 1.0% of polyoxyethylene (20) sorbitan monolaurate, such as 0.1%, such as 0.2%, such as 0.3%, such as 0.4%, such as 0.5%, such as 0.6%, such as 0.7%, such as 0.8%, such as 0.9%, such as 1%, preferably 0.5% of polyoxyethylene (20) sorbitan monolaurate. In other embodiments, the sample is incubated with a volume of pre-heated enrichment broth further comprising between 0.1% (volume/volume) and 1.0% of polyoxyethylene (80) sorbitan monolaurate, such as 0.1%, such as 0.2%, such as 0.3%, such as 0.4%, such as 0.5%, such as 0.6%, such as 0.7%, such as 0.8%, such as 0.9%, such as 1%, preferably 0.5% of polyoxyethylene (80) sorbitan monolaurate. In other embodiments, the sample is incubated with a volume of pre-heated enrichment broth further comprising between 0.1% (volume/volume) and 1.0% of polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether , such as 0.1%, such as 0.2%, such as 0.3%, such as 0.4%, such as 0.5%, such as 0.6%, such as 0.7%, such as 0.8%, such as 0.9%, such as 1%, preferably 0.5% of polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether.

In some embodiments, the pre-heated enrichment broth is at a temperature between 40°C and 50°C, such as 40°C, such as 41°C, such as 41.5°C, such as 42°C, such as

43℃, such as 44℃, such as 45℃, such as 46℃, such as 47℃, such as 48℃, such as 49℃, such as 50℃, preferably at 45℃.

In one embodiment, the enrichment broth is buffered peptone water. Other suitable enrichment broths are Brain Heart Infusion (BHI), Tryptic Soy Broth (TSB), Nutrient broth (NB) and Luria-Bertani (LB). The enrichment broth may further comprise agents specifically favouring growth of *Salmonella*, such as growth enhancers known in the art. For example, the enrichment broth may comprise chemical agents known in the art, such as bile (salts) or surfactants/detergents. Such growth enhancers may promote growth of *Salmonella* while inhibiting growth of other potentially competing microorganisms, also known as background flora, in the sample.

The enrichment step is performed by incubation at a temperature supporting growth of *Salmonella*. In some embodiments, the incubation is thus performed between 40℃ and 44℃, such as at a temperature of 40℃, such as 40.5℃, such as 41℃, such as 41.5℃, such as 42℃, such as 42.5℃, such as 43℃, such as 43.5℃, such as 44℃, preferably 41.5℃.

In some embodiments, it is desirable that the enrichment phase of the present method is as short as possible, but is long enough to allow sufficient enrichment of *Salmonella* in order to enable detection. In some embodiments, the incubation time is less than 6 hours, such as less than 5.5 hours, such as less than 5 hours, such as less than 4.5 hours, such as less than 4 hours, such as less than 3.5 hours, such as less than 3 hours, such as less than 2.5 hours, such as 2 hours. In some embodiments, the incubation time is between 2 and 6 hours, such as between 2.5 hours and 5 hours, such as between 2 hours 45 minutes and 4 hours 30 minutes, such as between 2 hours 50 minutes and 4 hours, such as between 2 hours 55 minutes and 3.5 hours, such as 3 hours.

In some embodiments, the volume of enrichment broth is such that the subsequent steps of the method do not require numerous concentration steps. In some embodiments, the volume of enrichment broth is between 20 and 100 mL, such as between 30 and 90 mL, such as between 40 and 80 mL, such as between 50 and 70 mL, such as 60 mL.

Incubation of the samples in the enrichment phase may be performed with or without shaking; in a preferred embodiment, the incubation is performed without shaking.

Concentration

The enriched sample obtained in step i) of the present method is then concentrated, thereby obtaining a concentrated sample. Prior to concentration, the enriched sample may be transferred to a container having an appropriate volume, such as a tube with a 100 mL capacity, a tube with a 50 mL capacity, a tube with a 25 mL capacity or a tube with a 10 mL capacity. The container is preferably suitable for being transferred to a centrifuge. Such containers are known in the art. In some embodiments the container has the capacity for the entire enriched sample. In other embodiments, a fraction of the enriched sample is transferred to the container so that the container is filled to its maximal capacity. In some embodiments, between 10 and 100 mL of the enriched sample is transferred to the container, such as between 20 and 90 mL of the enriched sample, such as between 30 and 80 mL of the enriched sample, such as between 40 and 70 mL of the enriched sample, such as between 45 and 60 mL of the enriched sample, such as 50 mL of the enriched sample. In some embodiments, the container is a 1.5 mL or 2 mL tube. In other embodiments, the container is a plate such as a 96-well plate, for example a deep-well plate, and the volume transferred to each well is between 0.5 and 2 mL, such as between 0.6 and 1.9 mL, such as between 0.7 and 1.8 mL, such as between 0.8 and 1.7 mL, such as between 0.9 and 1.6 mL, such as between 1.0 and 1.5 mL, such as between 1.1 and 1.4 mL, such as between 1.2 and 1.3 mL. In a particular embodiment, 1.8 mL is transferred to one or more well of a 96 deep-well plate.

Methods for concentrating samples are known in the art, and include concentration by centrifugation of the sample, followed by discarding of at least part of the supernatant.

The force and duration of centrifugation are preferably such that they allow separation of cellular material within the enriched sample. In some embodiments, the centrifugation is carried out at a centrifugal force between 2000 and 5000 g, such as between 2500 and 4000 g, such as 3000 g. The duration of the centrifugation may vary depending on the force of centrifugation. In some embodiments, the duration of the centrifugation is between 1 and 10 minutes, such as between 2 and 9 minutes, such as between 3 and 8 minutes, such as between 4 and 7 minutes, such as between 4 and 6

minutes, such as 5 minutes. In a preferred embodiment, the centrifugation is performed at 3000 g for 5 minutes.

In some embodiments, at least part of the supernatant is discarded. Preferably, as much of the supernatant as possible is discarded.

Protease treatment

The third step of the present method involves a protease treatment of the concentrated sample, thereby obtaining a treated sample.

In the present method, the protease of step iii) is a protease or a protease mixture such as bacterial alkaline protease and/or serine endopeptidase from *Bacillus licheniformis*, provided in a treatment mixture, the treatment mixture further comprising a buffer such as PBS, wherein the volume of the protease relative to the volume of buffer is preferably between 5 and 20%, such as between 5 and 15%, such as between 6 and 14%, such as between 7 and 13%, such as between 8 and 12%, such as between 9 and 11%, such as 10%.

In some embodiments, the concentrated sample is resuspended in a suitable buffer. The buffer may comprise a protease. Suitable buffers are known in the art and include PBS (phosphate buffered saline). It will be understood that the buffer and the protease can be added to the concentrated sample one after the other or simultaneously. In some embodiments, the buffer and the protease are thus mixed together in a treatment mixture before being added to the concentrated sample.

Proteases suitable for treating the concentrated sample according to the present method include, but are not limited to: bacterial alkaline proteases, serine proteases and serine endopeptidases. Thus the proteases suitable for performing the present method include enzymes generally acting on peptide bonds, such as enzymes of the EC class 3.4, more particularly of the EC class 3.4.21, such as of the EC class 3.4.21.62. In some embodiments, the protease is derived from *Bacillus licheniformis*. The protease treatment may be performed with a mixture of one or more proteases.

The volume of treatment mixture, i.e. the volume of protease and buffer, is preferably such that it is suitable for resuspending the concentrated sample. In some

embodiments, the volume of protease and buffer is between 2 and 10 mL, such as between 2.5 and 9 mL, such as between 3 and 8 mL, such as between 3.5 and 7 mL, such as between 4 and 6.5 mL, such as between 4.5 and 6 mL, such as between 5 and 5.5 mL. The volume of protease relative to the volume of buffer is preferably between 5 and 20%, such as between 5 and 15%, such as between 6 and 14%, such as between 7 and 13%, such as between 8 and 12%, such as between 9 and 11%, such as 10%.

In some embodiments, the treatment mixture comprises 5 mL buffer and 500 µL protease. In other embodiments, the treatment mixture comprises 1 mL buffer and 100 µL protease.

In some embodiments, the volume of protease is such that the treatment mixture comprising the protease comprises between 30000 and 200000 U, such as between 40000 and 150000 U, such as between 50000 and 125000 U, such as between 55000 and 90000 U, such as between 60000 and 80000 U, such as between 60000 and 75000 U, such as between 61000 and 70000 U, such as between 62000 and 65000 U. In one embodiment, the volume of protease comprises 62100 U. In another embodiment, the volume of protease comprises 31050 U. In yet another embodiment, the volume of protease comprises 124200 U.

In some embodiments, the protease has an activity of between 200000 and 800000 U/g, such as between 300000 and 700000 U/g, such as between 400000 and 600000 U/g, such as 500000U/g, such as 580000 U/g. Thus in some embodiments, the treatment mixture comprises 100 µL of a protease having an activity between 200000 and 800000 U/g. In one embodiment, the treatment mixture comprises 100 µL of a protease having an activity of 580000 U/g. In some embodiments, the protease has an activity between 300000 and 2000000 U/mL, such as between 350000 and 1500000 U/mL, such as between 400000 and 1250000 U/mL, such as between 500000 and 1000000 U/mL, such as between 550000 and 900000 U/mL, such as between 600000 and 800000 U/mL, such as between 600000 and 750000 U/mL, such as between 620000 and 650000 U/mL, such as 621000 U/mL. Thus in one embodiment, the treatment mixture comprises 100 µL of a protease having an activity of 621000 U/mL.

In some embodiments, the volume of protease is such that the treatment mixture comprising the protease comprises between 30000 and 200000 DU, such as between

40000 and 150000 DU, such as between 50000 and 125000 DU, such as between 55000 and 90000 DU, such as between 60000 and 80000 DU, such as between 60000 and 75000 DU, such as between 61000 and 70000 DU, such as between 62000 and 65000 DU. In one embodiment, the volume of protease is 62100 DU. In another embodiment, the volume of protease is 31050 DU. In yet another embodiment, the volume of protease is 124200 DU.

In some embodiments, the protease has an activity of between 200000 and 800000 DU/g, such as between 300000 and 700000 DU/g, such as between 400000 and 600000 DU/g, such as 500000 DU/g, such as 580000 DU/g. Thus in some embodiments, the treatment mixture comprises 100 µL of a protease having an activity between 200000 and 800000 DU/g. In one embodiment, the treatment mixture comprises 100 µL of a protease having an activity of 580000 DU/g. In some embodiments, the protease has an activity between 300000 and 2000000 DU/mL, such as between 350000 and 1500000 DU/mL, such as between 400000 and 1250000 DU/mL, such as between 500000 and 1000000 DU/mL, such as between 550000 and 900000 DU/mL, such as between 600000 and 800000 DU/mL, such as between 600000 and 750000 DU/mL, such as between 620000 and 650000 DU/mL, such as 621000 DU/mL. Thus in one embodiment, the treatment mixture comprises 100 µL of a protease having an activity of 621000 DU/mL.

After addition of the treatment mixture to the concentrated sample, the concentrated sample may be completely resuspended by methods known in the art such as pipetting or mixing, e.g. by vortexing. In some embodiments, the concentrated sample is resuspended in the treatment mixture by vortexing for less than 1 minute, such as less than 50 seconds, such as less than 40 seconds, such as less than 30 seconds, such as 20 seconds.

The resuspended concentrated sample comprising the treatment mixture is then incubated at a temperature where the protease is active. In some embodiments, step iii) is performed by incubating the concentrated sample resuspended in the treatment mixture at a temperature between 30 and 60°C, such as between 31 and 41°C, such as between 32 and 40°C, such as between 33 and 39°C, such as between 34 and 39°C, such as between 35 and 38°C, such as between 36 and 38°C, such as at 37°C.

In some embodiments, the incubation time is between 1 and 20 minutes, such as between 2 and 19 minutes, such as between 3 and 18 minutes, such as between 4 and 17 minutes, such as between 5 and 16 minutes, such as between 6 and 15 minutes, such as between 7 and 14 minutes, such as between 8 and 13 minutes, such as between 9 and 12 minutes, such as between 9 and 11 minutes, such as 10 minutes, such as 5 minutes.

In one embodiment, the incubation is performed at 37°C for 5 minutes. In some embodiments, the incubation is performed with shaking.

In some embodiments, the steps of resuspending the concentrated sample and incubating the resuspended sample at a temperature between 30 and 60°C for a time between 1 and 20 minutes are repeated. Thus in some embodiments, the method comprises a first step of resuspending the concentrated sample as described above; a first step of incubating the resuspended concentrated sample as described above; a second step of mixing or resuspending the concentrated sample as described above; and a second step of incubating the resuspended concentrated sample as described above.

Second concentration

The treated sample obtained in step iii) may then be concentrated to obtain a concentrated treated sample. This second concentration step is preferably performed directly, i.e. without transferring the treated sample to a new container.

The force and duration of centrifugation are preferably such that they allow separation of cellular material. In some embodiments, the centrifugation is carried out at a centrifugal force between 2000 and 5000 g, such as between 2500 and 4500 g, such as between 3000 and 4000 g, such as 3000 g or 4000 g. The duration of the centrifugation may vary depending on the force of centrifugation. In some embodiments, the duration of the centrifugation is between 1 and 10 minutes, such as between 2 and 9 minutes, such as between 3 and 8 minutes, such as between 4 and 7 minutes, such as between 4 and 6 minutes, such as 5 minutes. In a preferred embodiment, the centrifugation is performed at 3000 g for 5 minutes.

In some embodiments, at least part of the supernatant is discarded. Preferably, as much of the supernatant as possible is discarded. Because the volumes in this step are smaller than the volumes in the previous concentration step, the supernatant is preferably discarded by pipetting.

The concentrated treated sample is resuspended following the second concentration step. Suitable resuspension buffers depend on the method of detection used in the subsequent detection step. If the detection step is a nucleic acid amplification-based method, TE (Tris-EDTA) buffer (pH 8.0) is an example of a suitable buffer.

The volume in which the concentrated treated sample is resuspended likewise depends on the method of detection used in the subsequent step. If the detection step is a nucleic acid amplification-based method, volumes between 10 and 100 µL, such as between 20 and 90 µL, such as between 30 and 80 µL, such as between 40 and 70 µL, such as between 45 and 60 µL, such as 50 µL are suitable. In one embodiment, the concentrated treated sample is resuspended in 50 µL TE buffer by pipetting or vortexing.

The concentrated treated sample is then optionally transferred to a smaller container, such as a reagent tube. The reagent tube may have a capacity between 50 µL and 2 mL, such as 100 µL, such as 1 mL, such as 1.5 mL, such as 2 mL.

Cell lysis

In the next step of the present method, a step of cell lysis is performed on the concentrated treated sample. The step of cell lysis is such that it allows release of at least some of the intracellular materials, such as nucleic acid materials or intracellular proteins, to be released from the cells. Preferably, the intracellular materials thus released comprise at least one marker. If the sample comprises *Salmonella* cells, at least one *Salmonella* marker is released in this step.

In some embodiments, the cell lysis of step iv) is performed by incubation at a temperature between 90 and 100°C, such as between 91 and 99°C, such as between 92 and 99°C, such as between 93 and 99°C, such as between 94 and 99°C, such as between 95 and 99°C, such as between 96 and 99°C, such as between 97 and 99°C, such as 98°C, for a duration between 1 and 30 minutes, such as between 5 and 25

minutes, such as between 10 and 20 minutes, such as 15 minutes. In some embodiments, the sample is incubated for 10 min at 98°C. In other embodiments, the sample is incubated for 15 min at 98°C.

The step of cell lysis may be followed by an optional step of removing cellular debris from the lysate. Such debris can be removed by methods known in the art, such as centrifugation. The centrifugation may advantageously be performed at a temperature lower than room temperature in order to speed up the cooling of the sample. In some embodiments, the step of cell lysis further comprises a step of removing cellular debris by centrifugation for less than 10 minutes, such as less than 5 minutes, such as less than 4 minutes, such as less than 3 minutes, such as less than 2 minutes, preferably for 1 minute, wherein said centrifugation is performed at a temperature lower than 25°C, such as lower than 20°C, such as lower than 15°C, such as lower than 10°C, such as lower than 9°C, such as lower than 8°C, such as lower than 7°C, such as lower than 6°C, such as lower than 5°C, such as 4°C. In some embodiments, the centrifugation is carried out at a centrifugal force between 2000 and 5000 g, such as between 2500 and 4500 g, such as between 3000 and 4000 g, such as 3000 g. The duration of the centrifugation may vary depending on the force of centrifugation. In some embodiments, the duration of the centrifugation is between 1 and 10 minutes, such as between 2 and 9 minutes, such as between 3 and 8 minutes, such as between 4 and 7 minutes, such as between 4 and 6 minutes, such as 5 minutes. In a preferred embodiment, the centrifugation is performed at 3000 g for 1 minute.

In a preferred embodiment, the centrifugation is performed at 3000 g for 1 minute at a temperature of 4°C.

In other embodiments, the sample is placed on ice for cooling down, either prior to removing the cellular debris or after.

In embodiments where it is desirable to store the sample, the sample can be stored by methods known in the art. It can for example be placed in a freezer at -20°C until analysis or detection is performed. In such embodiments the step of removing the cellular debris may be performed once, e.g. before the storage step or after the storage step. The step of removing the cellular debris may also be performed twice, e.g. both before and after the storage step.

Detection

The last step of the method is the detection step, where the presence of *Salmonella* in the lysate obtained in the previous step is confirmed or infirmed. The detection can be performed directly on at least a fraction of the lysate. The present method involves the detection of at least one *Salmonella* marker in the lysate, where the marker can be a marker allowing detection of *Salmonella*-specific DNA, proteins and/or RNA. The presence of at least one *Salmonella* marker in the lysate implies that at least one of the samples of the starting material comprised *Salmonella*.

The detection can be performed directly on the lysate. In other words, no specific nucleic acid extraction or purification is required besides the cell lysis step. Thus in some embodiments, the method does not comprise purification of nucleic acid, such as DNA, with a column, a resin, or an ethanol precipitation.

Several detection methods are known in the art which allow detection of the presence of *Salmonella* in the lysate.

Salmonella-specific DNA markers can be detected by methods including nucleic acid amplification such as real-time PCR amplification. In such embodiments the real-time PCR is designed to detect the presence of *Salmonella*-specific loci. Examples of such loci include, but are not limited to: *ttrRSBCA* locus, the *Inv*A locus, the *Sii* locus or the *Bcf*D locus. Other methods include LAMP multiplex ligation detection reaction (LDR), qPCR, microfluidic chip and ion-mobility spectrometry (IMS).

Other *Salmonella*-specific markers include *Salmonella*-specific proteins or peptides or fragments thereof, which can be detected by methods known in the art, including but not limited to: Western blot, ELISA, MALDI-TOF, mass spectrometry, GC/MS, LC/MS, NMR, IMS.

Yet other *Salmonella*-specific markers include *Salmonella*-specific transcripts, which can be detected by methods known in the art, such as RT-PCR or RT-qPCR, as well as high-throughput methods such as RNA microarrays.

The volume of lysate suitable for detecting the at least one *Salmonella* marker may vary depending of the type of detection performed.

In some embodiments, at least two *Salmonella* markers are detected, such as two markers, such as three markers, such as four markers, such as five markers or more.

In some embodiments, step v) comprises contacting a volume of the lysate with a real-time PCR composition comprising reagents suited for real-time amplification of a *Salmonella*-specific locus such as the *ttrRSBCA* locus, the *Inv*A locus, the *Sii* locus or the *Bcf*D locus, said volume of the concentrated lysate being between 5 and 50 µL, such as between 6 and 40 µL, such as between 7 and 40 µL, such as between 8 and 30 µL, such as between 8 and 20 µL, such as between 8 and 10 µL, such as 9 µL.

In one embodiment, one *Salmonella* marker is detected by real-time PCR, wherein the marker is the *ttrRSBCA* locus. It will be clear that the reaction mixture also contains reagents allowing the reaction to be performed, such as a DNA polymerase and a suitable buffer, deoxynuclotides dATP, dTTP, dCTP, dGTP and/or dUTP, a primer pair for amplifying the target locus, BSA, DMSO, salts such as magnesium chloride, glycerol, and reagents for internal control such as an internal amplification control probe and an international amplification control target. Suitable primer pairs are known in the art. For example, the *ttRSBCA* locus can be detected using the primers described in Josefsen et al., 2007.

Time-to-result

The present method allows rapid diagnostic of the presence of *Salmonella* in a lysate derived from at least one meat sample, with a time-to-result of 8 hours or less. In some embodiments, the time-to-result is 8 hours or less, such as 7.5 hours or less, such as 7 hours or less, such as 6.5 hours or less, such as 6 hours or less, such as 5.5 hours or less, such as 5 hours or less. The starting time in estimation the duration of the time-to-result is when the sample is added the enrichment broth. Before this starting time the sample may have been under transport from the sample collection location e.g. at an abattoir and/or the sample may have been cooled.

In some embodiments, steps ii) to iv) above, corresponding to concentrating the enriched sample, treating the concentrated sample with a protease and lysing said

treated sample, are performed in less than 2 hours, such as less than 1.5 hours, such as less than 1 hour. In some embodiments, step v) above, corresponding to detecting the presence of at least one *Salmonella* marker, is performed in 1 hour or less, such as 55 min or less, such as 50 min or less, such as 40 min or less.

In general, it is desirable in the field of meat production to have a time-to-result as short as possible. The time-to-result may to some extent depend on whether the sample is a pooled sample or an individual sample. Without being bound by theory, it appears that the method can be performed significantly faster when the sample is an individual sample than when it is a pooled sample. However, testing the same amount of samples by performing the present method on individual samples instead of pooled samples will usually require greater amount of reagents, thereby increasing the material expenses to some extent, in which case it may be more attractive to have a somewhat longer time-to-result, which is compensated for to some extent by savings on reagent costs. However, when slaughtering e.g. 350-700 pigs per hour in a line of an abattoir pooling the obtained meat samples from 10-25 carcasses is preferred especially for saving costs of reagents.

In some embodiments, the present method is performed on an individual sample and the time-to-result is 8 hours or less, such as 7.5 hours or less, such as 7 hours or less, such as 6.5 hours or less, such as 6 hours or less, such as 5.5 hours or less, such as 5 hours or less, such as 4.5 hours or less, such as 4 hours or less, such as 3.5 hours or less, such as 3 hours or less. In other embodiments, the present method is performed on a pooled sample and the time-to-result is 8 hours or less, such as 7.5 hours or less, such as 7 hours or less, such as 6.5 hours or less, such as 6 hours or less, such as 5.5 hours or less, such as 5 hours or less, such as 4.5 hours or less, such as 4 hours or less. The pooled sample may be any pooled sample as described herein, for example a pool of ten samples derived from ten different carcasses.

Importantly, and as shown in the examples, it appears that it is the combination of the relatively high incubation temperature (between 40°C and 44°C) and the treatment described herein (in particular the steps of protease treatment and cell lysis) which allow for such short time-to-results. Examples 2 and 3 show that a standard protocol known in the art (Löfström, Hansen et al., 2012) combined with incubation at high

temperature does not generate any detectable signal from individual or pooled samples when the incubation time is short (for example limited to 4.5 hours).

The inventors also tested that incubation at a temperature of 37°C, as is customary in the art, combined with the treatment described above (in particular the steps of protease treatment and cell lysis) likewise does not allow for generation of a signal when the incubation time is short.

Limit of detection

The present method allows detection of 1 cell in the at least one meat sample, such as 1 CFU in said at least one meat sample. In some embodiments, the present method allows detection of 1 CFU/10 g meat sample or less, such as 1 CFU/15 g meat sample or less, such as 1 CFU/20 g meat sample or less, such as 1 CFU/25 g meat sample or less. For individual meat samples, the present method allows detection of 1 CFU/10 g meat sample or less, such as 1 CFU/15 g meat sample or less, such as 1 CFU/20 g meat sample or less, such as 1 CFU/25 g meat sample or less. For pooled meat samples, the present method allows lower detection limits. For example, when 10 samples are pooled, the detection limit is 1 CFU/10/15 g (1 CFU/150 g) meat sample or less, such as 1 CFU/10/20 g (1 CFU/200 g) meat sample or less, such as 1 CFU/10/25 g (1 CFU/250 g) meat sample or less.

Kit

In another aspect, the invention relates to a kit for performing the method of the invention, said kit comprising:

- a protease or a protease mixture such as bacterial alkaline protease and/or serine endopeptidase from *Bacillus licheniformis*;
- enrichment broth powder;
- reagents for detecting the presence of at least one *Salmonella* marker such as a real-time composition for detecting a *Salmonella*-specific locus such as the *ttrRSBCA* locus, the *Inv*A locus, the *Sii* locus or the *Bcf*D locus;
- instructions for performing said method; and
- optionally, at least one Stomacher bag with a filter with a mesh size of 200-300 µm and tubes for performing the method.

The at least one Stomacher bag may be at least two Stomacher bags, such as at least three Stomacher bags, such as at least four Stomacher bags, such as at least five Stomacher bags. In a specific embodiment, the at least one Stomacher bag is 96 stomacher bags.

The reagents may be provided in the form of a mastermix, to which the lysate can be added directly.

Examples

Example 1

As meat is the greatest source of *Salmonella* it is important to detect the pathogen and reduce its spread. The current methods include a whole day of incubation, which means that the abattoirs need two working shifts to cover the *Salmonella* detection. In this example a rapid detection method for *Salmonella* in meat samples was demonstrated, consisting of a short enrichment in buffered peptone water, a real-time PCR compatible sample pre-treatment, based on filtration and enzymatic digestion, combined with lysis by boiling DNA extraction and a fast cycling real-time PCR to obtain test results within one working shift at abattoirs.

Introduction

Salmonella is an important foodborne pathogen, responsible for 30 % of outbreaks in the United States and one of the most frequently detected causative agents in foodborne outbreaks the European Union (EU) . Although no longer the main source of *Salmonella*, pork meat remains an important vehicle for salmonellosis in Europe and is being controlled by sampling at critical control points during production. The international standard for analysis of these samples is the culture based reference method ISO6529:2002, but in Scandinavian countries the Nordic committee on Food Analysis method 187 from 2007 is applied (NMKL 187, 2007) which is comparable to ISO 6579:2002/Amd.1:2007(E) but approved for detection of *Salmonella* in foods, feces and materials from primary animal production. Previously a same-day real-time PCR method has been validated against NMKL 187, 2007 (Löfström et al., 2012) but this method was based on 12 hours of incubation, requiring the abattoirs to use two working shifts for the detection. There is therefore a great economical incitement for an

even faster method as this could allow the abattoirs to finish the test within one working shift and release the meat for downstream processing within the same day.

Sample material

Experiments were performed on pork meat (loin cut with skin, tenderloin and shoulder cut) obtained from local supermarkets, which was cut into portions of 25 ± 1 g using sterile scalpels. For validation of the method meat from pig carcasses naturally contaminated with *Salmonella* were provided by Danish meat producers.

Bacterial strains and preparation of inoculum

The *Salmonella* strains (*S. enterica* serovar Typhimurium DT 193 (ref nr. 4984 PX), *S.* Dublin (ref nr. 4983 PX) and *S.* Derby (ref nr.4985 PX) were obtained from the Danish Meat Research Institute (DMRI, Høje Taastrup, Denmark) and stored in Protect Multipurpose Microorganism Preservation System (Technical Service Consultants Ltd, Lancashire, UK) containing 20% glycerol as cryo-protectant. Strains were revived on Tryptone Soy Agar with Sheep Blood (TSASB, Oxoid, Greve, Denmark) and isolated on Xylose Lysine Deoxycholate agar (XLD agar, Oxoid). Incubation of *Salmonella* was performed at 37 ± 1°C for 18 ± 2 hours.

Artificial contamination

The culture for artificial contamination was prepared by transferring 3-4 colonies of each of the *Salmonella* isolates into a separate tube containing 4 ml of Nutrient broth (8.5 g sodium chloride (AppliChem A1371,9025), 20 g Nutrient Broth (Becton Dickinson 234000) and 1 L demineralized water, pH 6.6-7.0) and incubated for 37 ± 1°C 1-2 h without shaking. To produce the pooled culture of *Salmonella* used for artificial contamination, the three cultures were mixed by using equal volumes of each strain. This pooled culture was then kept at 2-5 °C while the number of CFUs was determined by plating dilution series in sterile saline solution (0.9 % NaCl) on TSASB plates (Oxoid) which were incubated at 37 ± 1°C for 18 ± 2 hours before counting the CFUs. To ensure that the meat used for artificial contamination experiments was not naturally contaminated with *Salmonella*, a 25 g portion was analysed using the combination of enrichment, lysis by boiling and real-time PCR as previously described (Löfström et al. 2012). For the artificial contamination experiment, portions of pork meat, weighing 25 ± 1 g, was transferred to sterile filter bags (pore size < 250 um, BagPage® model R, Interscience, Saint Nom, France) and spiked with droplets of diluted *Salmonella* culture

directly on the meat. The meat was then incubated over night at 2-5°C to simulate cold stressed conditions.

Sample pre-treatment

Enrichment: Buffered Peptone Water (BPW) (Oxoid) with 0.5 % Tween20 (Molecular grade, Sigma P9416) was preheated to 45°C and 60 ml added to each filter bag containing 25 ± 1 g of artificially or naturally contaminated meat. The meat pieces were gently separated by pressing on the outside of the bag to ensure that the entire surface of the meat was exposed to the liquid and the bags were incubated at 41.5°C for 3 h ± 5 min in an incubator without shaking.

Removal of meat matrix components: After the enrichment 50 ml of the culture was collected by pouring from the filtered side of the bag, centrifuged for 5 min at 3000 × *g* and the supernatant discarded. The pellet was resuspended in 1 ml of Phosphate Buffered Saline (PBS), transferred to a 1.5 ml Eppendorf tube, and 100 µl of alkaline protease (food grade, 580000-650000 units/g) was added. The suspension was then mixed by vortexing 20 s and incubated at 37˚C for 5 min in a heating block followed by another 20 s vortexing and incubation for 5 min at 37°C. The tube was then centrifuged for 5 min at 3000 × *g*, the supernatant was discarded by pipetting and 50 µl 1 × TE buffer was added to the pellet followed by mixing until the pellet was fully dissolved.

DNA extraction

The pretreated samples were lyzed at 98˚C for 15 min in a heating block. The samples were then centrifuged for 1 min at 3000 × *g*. Samples were stored at - 20˚C until PCR analysis, where 9 µl was used as template in PCR.

Real-time PCR analysis

For the specific detection of *Salmonella* in meat samples, a TaqMan real-time PCR method, targeting a region within the *ttrRSBCA* locus (Malorny et al. 2004), was performed on an StepOnePlus (Life Technologies) using the fast cycling option (20 s initial denaturation at 95°C and 40 cycles of 95°C for 1 s and 60°C for 20 s) and a PCR master mix as previously described (Josefsen et al. 2007). Fluorescence measurements (i.e. FAM for the *Salmonella* target probe and VIC for the internal amplification control (IAC)) were obtained online and analyzed with the StepOne™ software (version 2.0). The baseline was set manually for each experiment with

baseline start around cycle 5-6 and baseline end two cycles before the threshold cycle (Ct) of the amplification curve for the positive control. The threshold was assigned based on evaluation of the curves of multiple experiments to 1000 dR for the *Salmonella* target probe and 300 dR for the IAC probe.

In every PCR analysis an internal amplification control (IAC) was included in all samples to detect false negative PCR results due to e.g. PCR inhibition. Furthermore, a positive control with *Salmonella* Typhimurium DNA (in a concentration of approximately 0.005 ng) as template was included, as well as a non-template control (NTC, containing only the PCR mix and 9 µl PCR grade water) and a negative DNA control (*Escherichia coli* in a concentration of approximately 5 ng). For each biological replicate two PCR replicates were tested.

Reference method for comparative study

The culture based reference method used for the comparative study was the Nordic Committee on Food Analysis (NMKL) *Salmonella* method no. 187, 2007 for Detection in foods, faeces and materials from primary animal production using MSRV. This method is comparable to the ISO 6579:2002/Amd.1:2007(E) (ISO 6579/A1), with the difference that ISO 6579/A1 is not recommended for detection of *Salmonella* in other matrices than animal feces and in environmental samples from the primary production stage.

Comparative study

To evaluate the performance of the new fast protocol it was tested in parallel with the culture based reference method according to the NordVal guidelines as previously described (Löfström et al., 2009). For the artificially contaminated samples, three concentration levels were tested (negative (n =30), artificially contaminated with 1-10 CFU/sample (n =30), and with 10-100 CFU/sample (n = 15)) using both the fast PCR protocol and the NMKL187 method. All samples of artificially contaminated with 1-10 CFU/sample were confirmed (see below) to check for false negatives. The naturally contaminated samples were tested by first cutting the meat into smaller pieces and mixing it to obtain a more even distribution of *Salmonella*, before the samples were divided into two portions, one to be tested by the fast protocol and one to be analyzed using the reference method. All negative samples with the fast protocol were confirmed to check for false negatives.

Confirmation of negative samples

Confirmation of negative results from tests of the fast protocol were performed similar to the reference method, by adding 225 ml fresh pre-warmed (37°C) BPW to the filter bags containing the meat from the enrichment step of the fast protocol. The bags were then incubated at 37°C for 18 ± 1 h. Following incubation 100 µl was distributed in three droplets on an MSRV plate and analyzed according to the NMKL187 and/or 1 ml was used for lysis by boiling and subsequent real-time PCR analysis as previously described (Löfström et al., 2010).

Data analysis

Statistics in optimization experiments: In order to confirm that the findings were of statistical significance, confidence intervals (CI) ($\alpha = 0.05$) were compared. If no overlap was found, the values were found to be statistically significantly different.

Variation in biological and PCR replicates during optimization: For the optimization three biological replicates and two PCR replicates per biological replicate were generally used. A PCR replicate was considered an outlier if its Ct value differed from the other replicates representing the same sample type with more than 3 cycles. Generally all amplification curves crossing the threshold was considered positive (also samples with Ct values between 36-40).

Limit of detection (LOD_{50}): was calculated according to the NordVal guidelines, using a Spearman-Kärber-based method.

Validation of protocol on artificially and naturally contaminated samples

To validate the performance of the final fast protocol (Figure 1), a comparative study was undertaken using artificially contaminated meat samples with different cuts of pork meat. The fast protocol was compared to the culture reference method (Table 1).

Table 1 – Results from the comparative validation of the fast protocol against the NMKL 187 2007 reference method.

Sample type	Inoculation level (CFU/25 g)[a]	No. of samples	Total no. of samples (N)[b]	PA[c]	NA	FN[d]	TP	FP	AC (%)	SE (%)	SP (%)	κ
Loin cut with skin	0	10	25	11	10	3	1	0	84.0	85.7	100.0	0.86
	1-10	10										
	10-100	5										
Tenderloin	0	10	25	13	10	1	0	1	96.0	92.9	90.9	0.92
	1-10	10										
	10-100	5										
Shoulder cut	0	10	25	14	10	1	0	0	96.0	93.3	100.0	0.92
	1-10	10										
	10-100	5										
TOTAL artificially contami-nated			75	38	30	5	1	1	92.0	90.7	96.8	0.84
Tongue (paired homoge-nization)	Naturally contami-nated	50	50	14	16	10	10	0	60.0	100.0	100.0	0.20
Tongue (pooled homoge-nization	Naturally contami-nated	15	15	15	0	0	0	0	100.0	100.0	100.0	1.00
TOTAL naturally contami-nated			65	29	16	10	10	0	69.2	100.0	100.0	0.41
TOTAL all			140	67	46	15	11	1	81.4	95.1	97.9	0.64

[a]Level of *Salmonella* contamination in naturally contaminated samples was not determined.

[b]Total no. of samples per sample type, N = PA + NA + FN + TP + FP.

[c]PA: positive agreement (positive result by both methods), NA: negative agreement (negative result by both methods), FN: false negative (positive by NMKL187, negative by fast protocol), TP: true positive (confirmed positive result by fast protocol, negative by NMKL187), FP: false positive (negative result by NMKL and positive by fast protocol but not confirmed).

[d]False negative according to NordVal definition, but confirmation revealed that with the exception of one

tongue samples, all of these were true negatives

The relative specificity of the fast protocol compared to the reference method was 100 % as neither of the tests generated false positives when tested on *Salmonella* negative meat. When tested at low level artificially contaminated meat (1-10 CFU), one sample of tenderloin was found to be false positive according to the NordVal definition which affected the specificity for this sample type. However, subsequent culture confirmed the results of the fast protocol and the discrepancy was attributed to the low contamination level.

The limit of detection for the fast protocol was found to be 8.8 CFU/sample versus 7.7 CFU/sample for the reference method.

Conclusion

The method was tested in a comparative study against the culture based method NMKL187:2007, comparable to ISO 6579:2002/Amd.1:2007(E) on samples of raw pork meat (n = 140) artificially contaminated with 0 CFU, 1-10 CFU and 10-100 CFU *Salmonella* and naturally contaminated. Results showed good agreement (Cohen's Kappa = 0.64) between the fast protocol and the reference method, with relative accuracy, sensitivity and sensitivity of 81.4 %, 95.1 % and 97.9 % respectively. The correlation was found to be higher for artificially contaminated samples (Kappa = 0.92) than for naturally contaminated (Kappa = 0.41) due to uneven distribution of Salmonella in these samples. The limit of detection (LOD_{50}) was found to be 8.8 CFU/sample and 7.7 CFU/sample for the fast method and the reference method respectively.

In conclusion we show that the present method performs as well as a reference method with comparable detection limit.

Example 2

Fresh pig meat samples purchased in retail stores were spiked with < 5 CFU *S. typhimurium* for 4.5 hours at 41.5°C. Single samples of 25 g meat were treated as follows: enrichment in 100 mL buffered peptone water pre-heated at 45°C and incubated at 41.5°C for 4.5 h. The enriched samples were then treated either using the boiling protocol described in Löfström, Hansen et al., 2012 or using the method

described herein, i.e. comprising a protease treatment and a lysis step instead of DNA extraction. For each protocol, real-time PCR was performed using two different platforms. A FAM reporter fluorescence probe was used. The PCR signal for the internal control was negative for the samples of the standard method, while the signal obtained with the present method was in the expected range of 28-30. The results are shown in table 2:

Table 2

	Standard method (Löfström, Hansen et al., 2012)		Present method	
	PCR machine 1	PCR machine 2	PCR machine 1	PCR machine 2
1	No Ct	No Ct	22.3	19.71
2	No Ct	No Ct	22.34	19.31
3	38.18	No Ct	20.87	18.5

The results show that incubation at 41.5°C in pre-heated BPW for 4.5 h combined with a traditional method such as boiling as described in Löfström et al. does not result in a detectable signal ("no Ct") in 5 of the 6 samples. In contrast, such incubation conditions were sufficient to generate a detectable signal using the present method comprising a protease treatment and a lysis step.

In conclusion, a short incubation (4.5 h) at increased temperature without using a protease treatment and a lysis step as described herein does not enable detection of *Salmonella* in a 25 g meat sample.

Example 3

In the present example, the same experiment was performed as in example 2, with the exception that each of the samples was a pooled composite of 10 meat samples (250 g in total).

The results are shown in table 3.

Table 3

	Standard method (Löfström, Hansen et al., 2012)		Present method	
	PCR machine 1	PCR machine 2	PCR machine 1	PCR machine 2
10	No Ct	No Ct	26.81	22.49
11	No Ct	No Ct	27.58	24.19
12	No Ct	No Ct	23.57	20.63

The results show that for pooled samples too, the present method allows detection of *Salmonella* after incubation for 4.5 hours while the standard method does not.

Example 4

One possible protocol for analyzing individual samples is outlined below. The same protocol can be adapted for pooled samples. For example for 10 pooled samples, the protocol is modified as follows: the enrichment time is 4.5 h, and the decanted volume of enriched BPW is 2 mL (instead of 50 mL).

Enrichment:

- 25 g sample + 60 mL BPW with 0.5% Tween20 (45°C) or a pooled sample of 10x25 g sample + 60 mL BPW with 0.5% Tween20 (45°C)
- Incubation 3 h at 41.5°C for single samples or 4.5 h at 41.5°C for pooled samples

Sample preparation:

Removal of meat components

- Decantation of 50 mL from filter bags with single samples or 2 mL from filter bags with pooled samples
- Centrifugation 5 min at 3000 x g, removal of supernatant
- Resupension in 1 mL PBS and 100 µL alkaline protease
- Vortex 20 s and incubation 5 min at 37°C (perform twice)
- Centrifugation 5 min at 3000 x g, removal of supernatant

DNA extraction

- Resuspension in 50 µL TE buffer
- Lysis by boiling 15 min at 98°C
- Centrifugation 1 min at 3000 x g

Real-time PCR analysis

- PCR setup (StepOnePlus, 16 µL master mix, 9 µL template)
- Fast cycling PCR (95°C, 20 s, 40 cycles: 95°C, 1 s, 60°C, 20 s)
- Data analysis

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Claims

1. A method for detection of *Salmonella* in at least one meat sample, said method comprising the steps of:

i) enriching the at least one meat sample by incubating said at least one meat sample in pre-heated enrichment broth such as buffered peptone water optionally comprising a surfactant such as polyoxyethylene (20) sorbitan monolaurate, wherein the incubation time is less than 6 hours and the incubation temperature is between 40°C and 44°C, thereby obtaining an enriched sample;

ii) concentrating at least part of said enriched sample, thereby obtaining a concentrated sample;

iii) treating said concentrated sample with a protease, thereby obtaining a treated sample, and concentrating said treated sample, thereby obtaining a concentrated treated sample;

iv) lysing said concentrated treated sample, thereby obtaining a lysate;

v) detecting the presence of at least one *Salmonella* marker in the lysate;

wherein the time-to-result of said method is less than 8 hours.

2. The method of claim 1, wherein the at least one meat sample is an individual meat sample or a pooled meat sample of individual meat samples, wherein each individual meat sample has a weight of between 2 and 100 g, such as between 2.5 and 90 g, such as between 5 and 80 g, such as between 10 and 70 g, such as between 15 and 50 g, such as between 20 and 40 g, such as between 20 and 30 g, such as between 21 and 29 g, such as between 22 and 28 g, such as between 23 and 27 g, such as between 24 and 26 g, such as 25 g, 10 g or 15 g.

3. The method of any one of the preceding claims, wherein step i) is performed by incubating said sample with a volume of pre-heated enrichment broth, wherein said volume is between 20 and 100 mL, such as between 30 and 90 mL, such as between 40 and 80 mL, such as between 50 and 70 mL, such as 60 mL.

4. The method of any one of the preceding claims, wherein step i) is performed by incubating said sample with a volume of pre-heated enrichment broth further

comprising between 0.1% (volume/volume) and 1.0% of a surfactant such as polyoxyethylene (20) sorbitan monolaurate, such as 0.1%, such as 0.2%, such as 0.3%, such as 0.4%, such as 0.5%, such as 0.6%, such as 0.7%, such as 0.8%, such as 0.9%, such as 1%, preferably 0.5% of a surfactant such as polyoxyethylene (20) sorbitan monolaurate.

5. The method of any one of the preceding claims, wherein said pre-heated enrichment broth is at a temperature between 40°C and 50°C, such as 40°C, such as 41°C, such as 41.5°C, such as 42°C, such as 43°C, such as 44°C, such as 45°C, such as 46°C, such as 47°C, such as 48°C, such as 49°C, such as 50°C, preferably at 45°C.

6. The method of any one of the preceding claims, wherein the sample is incubated at a temperature supporting growth of *Salmonella*, such as 40°C, such as 40.5°C, such as 41°C, such as 41.5°C, such as 42°C, such as 42.5°C, such as 43°C, such as 43.5°C, such as 44°C, preferably 41.5°C.

7. The method of any one of the preceding claims, wherein the incubation time of step i) is between 2 and 6 hours, such as between 2.5 hours and 5 hours, such as between 2 hours 45 minutes and 4 hours 30 minutes, such as between 2 hours 50 minutes and 4 hours, such as between 2 hours 55 minutes and 3.5 hours, such as 3 hours.

8. The method of any one of the preceding claims, wherein step i) is carried out without shaking.

9. The method of any one of the preceding claims, wherein step ii) is carried out with 50 mL of enriched sample.

10. The method of any one of the preceding claims, wherein step ii) is carried out by centrifugation, thereby obtaining a pellet and a supernatant.

11. The method of any one of the preceding claims, wherein the centrifugation is carried out for less than 10 minutes, such as less than 9 minutes, such as less than 8 minutes, such as less than 7 minutes, such as less than 6 minutes, such

as less than 5 minutes, such as less than 4 minutes, such as less than 3 minutes, such as less than 2 minutes.

12. The method of any one of the preceding claims, wherein the centrifugal force is between 1000 and 5000 g, such as between 1500 and 4500 g, such as between 2000 and 4000 g, such as between 2500 and 3500 g, such as at 3000 g.

13. The method of any one of the preceding claims, wherein the centrifugation is carried out at 3000 g for 5 minutes.

14. The method of any one of the preceding claims, wherein step ii) further comprises the steps of removing the supernatant, adding buffer to the pellet and resuspending the pellet.

15. The method of to any one of the preceding claims, wherein the buffer is phosphate buffered saline (PBS).

16. The method of any one of the preceding claims, wherein the volume of phosphate saline buffer added to the pellet is less than 5 mL, such as less than 4 mL, such as less than 3 mL, such as less than 2 mL, such as 1 mL.

17. The method of any one of the preceding claims, wherein the added volume of protease and buffer is between 2 and 10 mL, such as between 2.5 and 9 mL, such as between 3 and 8 mL, such as between 3.5 and 7 mL, such as between 4 and 6.5 mL, such as between 4.5 and 6 mL, such as between 5 and 5.5 mL.

18. The method of any one of the preceding claims, wherein the protease of step iii) is a protease or a protease mixture such as bacterial alkaline protease and/or serine endopeptidase from *Bacillus licheniformis*, provided in a treatment mixture, the treatment mixture further comprising a buffer such as PBS.

19. The method of any one of the preceding claims, wherein the volume of the protease relative to the volume of buffer in the treatment mixture is between 5 and 20%, such as between 5 and 15%, such as between 6 and 14%, such as

between 7 and 13%, such as between 8 and 12%, such as between 9 and 11%, such as 10%.

20. The method of any one of the preceding claims, wherein the treatment mixture comprises 500 µL protease and 5 mL buffer.

21. The method of any one of the preceding claims, wherein step iii) further comprises a step of mixing the treatment mixture and the concentrated sample.

22. The method of any one of the preceding claims, wherein the step of mixing the treatment mixture and the concentrated sample is performed by vortexing for less than 1 minute, such as less than 50 seconds, such as less than 40 seconds, such as less than 30 seconds, such as 20 seconds.

23. The method of any one of the preceding claims, wherein step iii) is performed by mixing the treatment mixture and the concentrated sample and incubating at a temperature between 30 and 60°C, such as between 31 and 41°C, such as between 32 and 40°C, such as between 33 and 39°C, such as between 34 and 39°C, such as between 35 and 38°C, such as between 36 and 38°C, such as at 37°C.

24. The method of any one of the preceding claims, wherein the incubation time of step iii) is between 1 and 20 minutes, such as between 2 and 19 minutes, such as between 3 and 18 minutes, such as between 4 and 17 minutes, such as between 5 and 16 minutes, such as between 6 and 15 minutes, such as between 7 and 14 minutes, such as between 8 and 13 minutes, such as between 9 and 12 minutes, such as between 9 and 11 minutes, such as 10 minutes.

25. The method of any one of the preceding claims, wherein concentrating the treated sample is carried out by centrifugation, thereby obtaining a pellet and a supernatant.

26. The method of any one of the preceding claims, wherein at least part of the supernatant is discarded and wherein the pellet is resuspended in a volume of resuspension buffer between 10 and 100 µL, such as between 20 and 90 µL,

such as between 30 and 80 µL, such as between 40 and 70 µL, such as between 45 and 60 µL, such as 50 µL.

27. The method of any one of the preceding claims, wherein centrifugation is carried out on the treated sample for less than 10 minutes, such as less than 9 minutes, such as less than 8 minutes, such as less than 7 minutes, such as less than 6 minutes, such as less than 5 minutes, such as less than 4 minutes, such as less than 3 minutes, such as less than 2 minutes.

28. The method of any one of the preceding claims, wherein centrifugation is carried out on the treated sample at a centrifugal force between 1000 and 5000 g, such as between 1500 and 4500 g, such as between 2000 and 4000 g, such as between 2500 and 3500 g, such as at 3000 g.

29. The method of any one of the preceding claims, wherein centrifugation is carried out on the treated sample at 3000 g for 5 minutes.

30. The method of any one of the preceding claims, wherein the cell lysis of step iv) is performed by incubation of the concentrated treated sample at a temperature between 90 and 100°C, such as between 91 and 99°C, such as between 92 and 99°C, such as between 93 and 99°C, such as between 94 and 99°C, such as between 95 and 99°C, such as between 96 and 99°C, such as between 97 and 99°C, such as 98°C.

31. The method of any one of the preceding claims, wherein the cell lysis of step iv) is performed by incubation of the concentrated treated sample for a duration between 1 and 30 minutes, such as between 5 and 25 minutes, such as between 10 and 20 minutes, such as 15 minutes.

32. The method of any one of the preceding claims, wherein the step of cell lysis further comprises a step of removing cellular debris by centrifugation for less than 10 minutes, such as less than 5 minutes, such as less than 4 minutes, such as less than 3 minutes, such as less than 2 minutes, preferably for 1 minute.

33. The method of any one of the preceding claims, wherein the step of removing cellular debris by centrifugation is performed at a temperature lower than 25°C, such as lower than 20°C, such as lower than 15°C, such as lower than 10°C, such as lower than 9°C, such as lower than 8°C, such as lower than 7°C, such as lower than 6°C, such as lower than 5°C, such as 4°C.

34. The method of any one of the preceding claims, wherein the step of removing cellular debris by centrifugation is performed at a centrifugal force between 1000 and 5000 g, such as between 1500 and 4500 g, such as between 2000 and 4000 g, such as between 2500 and 3500 g, such as at 3000 g.

35. The method of any one of the preceding claims, wherein the lysate is optionally stored at -20°C prior to step v).

36. The method of any one of the preceding claims, wherein step v) comprises contacting a volume of the lysate with a real-time PCR composition comprising reagents suited for real-time amplification of a *Salmonella*-specific locus such as the *ttrRSBCA* locus, the *Inv*A locus, the *Sii* locus or the *Bcf*D locus.

37. The method of any one of the preceding claims, wherein step v) is performed on a sample having a volume between 5 and 50 µL, such as between 6 and 40 µL, such as between 7 and 40 µL, such as between 8 and 30 µL, such as between 8 and 20 µL, such as between 8 and 10 µL, such as 9 µL.

38. The method of any one of the preceding claims, wherein step v) comprises contacting at least part of the concentrated lysate with a real-time PCR composition comprising reagents suited for real-time amplification of the *ttrRSBCA* locus.

39. The method of any one of the preceding claims, wherein the time-to-result is less than 8 h, such as less than 7 h, such as less than 6 h, such as less than 5 h, such as less than 4 h, such as less than 3 h.

40. The method of any one of the preceding claims, wherein steps ii) to v) are performed in less than 2 hours, such as less than 1.5 hours, such as less than 1 hour.

41. The method of any one of the preceding claims, wherein step v) is performed in 1 hour or less, such as 55 min or less, such as 50 min or less, such as 45 min or less, such as 40 min or less.

42. The method of any one of the preceding claims, wherein the at least one meat sample is an individual meat sample and the method has a sensitivity of detection of 1 cell in said at least one meat sample, such as 1 CFU/10 g meat sample or less, such as 1 CFU/15 g meat sample or less, such as 1 CFU/20 g meat sample or less, such as 1 CFU/25 g meat sample or less.

43. The method of any one of the preceding claims, wherein the at least one meat sample is a pooled sample of n individual meat samples, and the method has a sensitivity of detection of 1 cell in said at least one meat sample, such as 1 CFU/n/10 g meat sample or less, such as 1 CFU/ n/15 g meat sample or less, such as 1 CFU/ n/20 g meat sample or less, such as 1 CFU/ n/25 g meat sample or less, and where n is an integer greater than 1, such as 2, such as 3, such as 4, such as 5, such as 6, such as 7, such as 8, such as 9, such as 10 or more.

44. The method of any one of the preceding claims, wherein the method can be performed on pooled samples.

45. A kit for performing the method of any one of claims 1 to 44, said kit comprising:

- a protease or a protease mixture such as bacterial alkaline protease and/or serine endopeptidase from *Bacillus licheniformis*;
- enrichment broth powder;
- reagents for detecting the presence of at least one *Salmonella* marker such as a real-time composition for detecting a *Salmonella*-specific locus such as the *ttrRSBCA* locus, the *Inv*A locus, the *Sii* locus or the *Bcf*D locus;
- instructions for performing said method; and

- optionally, at least one Stomacher bag with a filter with a mesh size of 200-300 µm and tubes for performing the method.

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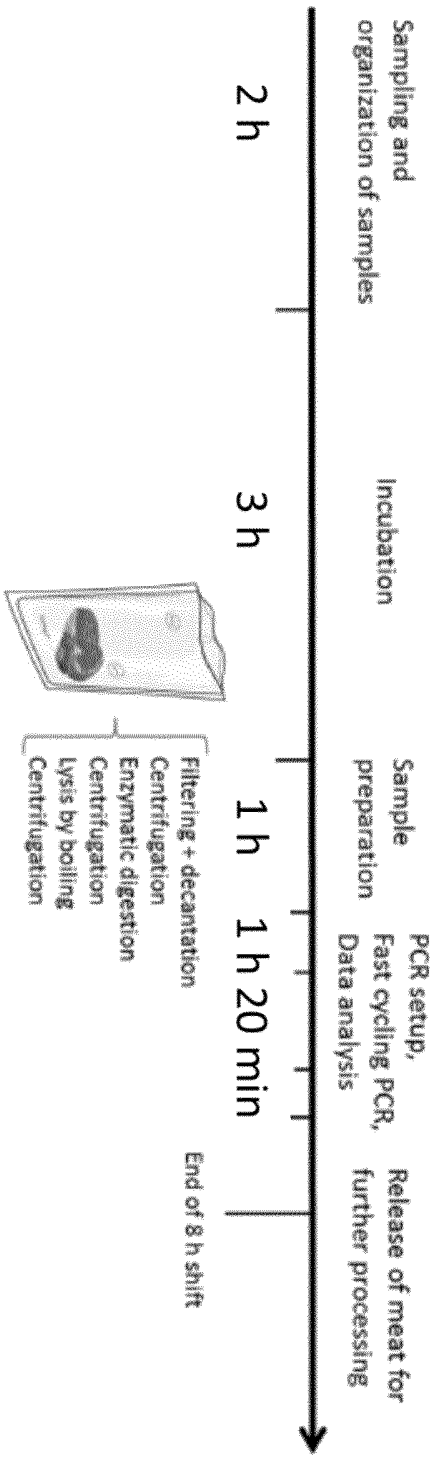
FIGURE 1


Sampling and organization of samples
2 h
Incubation
3 h
Sample preparation
1 h
PCR setup, Fast cycling PCR, Data analysis
1 h 20 min
Release of meat for further processing
Filtering + decantation
Centrifugation
Enzymatic digestion
Centrifugation
Lysis by boiling
Centrifugation
End of 8 h shift

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/077234

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 January 2016	09/02/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lapopin, Laurence

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/077234

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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