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(54) Title: METHOD FOR SIMULTANEOUS DETECTION AND QUANTIFICATION OF LISTERIA MONOCYTOGENES, SALMONELLA SPP., AND SHIGA TOXIN-PRODUCING ESCHERICHIA COLI (STEC)

(57) Abstract: The invention refers to a method for simultaneous detection and quantification of Listeria monocytogenes, Salmonella spp. and Shiga toxin-producing Escherichia coli (STEC), from any kind of sample related to food production, including complex food matrices such as fish, meat, or fruit; or simple matrices such as water, or food contact surfaces. The method of invention allows specific quantification of the above-mentioned pathogens simultaneously thanks to specificity of designed primers for the qPCR reaction. It is also highly reliable because the method includes a system to adequately control the quantification of pathogens within the matrix. This system comprises the inoculation of samples with a known concentration of a transformed microorganism (host) carrying a chimeric sequence, acting as an internal control host for the whole process.

METHOD FOR SIMULTANEOUS DETECTION AND QUANTIFICATION OF *Listeria monocytogenes*, *Salmonella* spp., AND SHIGA TOXIN-PRODUCING *Escherichia coli* (STEC)

TECHNICAL FIELD OF THE INVENTION

This invention refers to a method for simultaneous detection and quantification of *Listeria monocytogenes*, *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC), from any kind of sample related with food production, including complex food matrices such as fish, meat, or fruit; or simple matrices such as water, or food contact surfaces.

BACKGROUND INFORMATION

Bacterial foodborne diseases produced by pathogenic bacteria or through their toxins are one of the main alimentary risks in food products, such as fruit, meat, or fish.

Among the most dangerous bacteria, due to the severity of their related diseases, are *L. monocytogenes*, *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC). For instance, after ingestion of contaminated food with STEC, the bacteria releases toxins known as Shiga toxins. These Shiga toxins provoke acute diarrhea, hemorrhagic colitis, and in some cases, acute kidney damage. *Salmonella* infections provoke vomiting, nausea and diarrhea. Listeriosis is a serious disease with symptoms ranging from febrile gastroenteritis to a severe invasive disease, and it especially affects pregnant women, children under 2 years, elders and immunocompromised people.

Therefore, food and sanitary regulations are strict to control and identify pathogens in food matrices. At the same time, since these microorganisms can survive within food processing, it is necessary to have exhaustive control of production processes, watching for possible contamination in food contact surfaces or utensils, in order to guarantee the safety of the final product.

Bacterial culture is the traditional method to identify and quantify foodborne pathogens in food. This method is based on identification of morphological, biochemical, physiological and immunological features from isolated bacterial colonies. However, even though this approach has been quite useful for many years, it has limitations such as being laborious and taking too long to obtain results. For example, the identification of any pathogen by culture method can take at least 4 days.

Time reduction for obtaining results thus becomes a critical aspect which directly impacts economics of agri-food industries. For example, it is commonly required to identify pathogens present in perishable food, so any delays in confirming food safety have important repercussions on storage costs while also reducing sales chances. Moreover, from a production process control perspective, rapid detection of possible contamination allows corrective actions to be taken on time, while also reducing possible cross contamination.

Furthermore, from a public health point of view, in the case of an outbreak; rapid identification also enables the control of sales of contaminated food (recall) and adequate treatment of affected people.

Rapidity has been accomplished through different molecular biology techniques, such as PCR, multiplex PCR and multiplex real-time PCR. Even though there are different PCR detection kits for these pathogens on the market, they are qualitative, in other words; they only screen for presence or absence of foodborne pathogens. The quantification of microorganisms present on a matrix at industrial level is relevant in order to evaluate sanitizing and safety protocols in the production process. However, there are no rapid quantitative methods available on the market. Additionally, it is still difficult to work with samples from food matrices using molecular biology tools, due to their complexity and the presence of inhibitors from the food which may affect or interfere with the results, giving false negatives. This situation offers an opportunity for developing rapid, simultaneous and

quantitative methods for foodborne pathogens, which is the application field of this invention.

Given the importance of foodborne pathogen detection and quantification, there are several disclosed methods directed at screening those microorganisms, however methods known from the prior art don´t have a system that ensures the effectiveness of the complete process, or only focus on one particular kind of complex matrix to be tested, while it remains unclear if said methods will work on other types of samples. In the following paragraphs, the closest prior art will be discussed:

Patent ES2540158 (B1) refers to a simultaneous screening method based on multiplex PCR for seven different pathogens, including *E. coli* O157:H7, *L. monocytogenes* and *Salmonella spp.* as targeted sequences. An internal control corresponding to chimeric DNA is exclusively used in the amplification stage. The present invention differs to this patent, in the PCR primers used, the internal control (not only used to evaluate the amplification stage) as well as in additional steps, which will be described later.

Scientific publication from Wei C. *et al.* (Simultaneous detection of *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella* by multiplex PCR in milk. 3 Biotech, Jan. 2018, 8: 76) points out simultaneous detection (non-quantitative) from milk through multiplex PCR. Also, in this publication, internal control is used only in the amplification stage.

International PCT publication WO2016164407 (A2) refers to simultaneous detection of pathogens including STEC, *L. monocytogenes* and *Salmonella* spp., through qPCR. An internal DNA control is used in the amplification stage, requiring a microorganism enrichment step of 24 hours at 37°C with an enrichment media prior to detection. Evidently, said enrichment step is performed to decrease false negative results, while artificially increasing pathogen concentration. Although this step makes pathogen detection easier; it makes impossible to determine original pathogen concentration of the actual food sample.

In conclusion, even where the prior art does provide simultaneous detection methods for the above mentioned three pathogens; there is still a need for a quantitative method which can be adaptable to different matrices, irrespective of its nature, like food, water or surfaces, and also allowing for a control of all the stages of the process in order to minimize the chances of obtaining false negatives. The internal control described has been designed to be included in the last part of the protocol to control the qPCR amplification reaction. However, they did not control the recovery of pathogens from the matrix, and the DNA extraction procedure.

The inventors have solved this technical issue with a new method including a control bacterium, which is added to the food sample to be analyzed before separating microorganisms from the matrix, and allowing for a control of all steps of the process; thus, decreasing the number of false negative results and allowing quantitative results to be obtained.

BRIEF DESCRIPTION OF FIGURES

Figure 1. Calibration curves for the quantification of the pathogens: a) *L. monocytogenes*, b) *Salmonella* spp. and c) STEC. All curves show Cq values versus each microorganism's concentration on samples (Log10 CFU/g or Log10 CFU/cm^2).

DETAILED DESCRIPTION OF THE INVENTION

The invention refers to a method for simultaneous detection and quantification of *Listeria monocytogenes*, *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC), from any kind of sample related to food production, including complex food matrices such as fish, meat, or fruit; or simple matrices such as water, or food contact surfaces.

The invention allows specific quantification of the above-mentioned pathogens simultaneously thanks to specificity of designed primers for the qPCR reaction. It is also highly reliable because the method includes a system to adequately control the quantification of pathogens within the matrix. This system comprises the inoculation of samples with a known concentration of a transformed microorganism (host) carrying a chimeric sequence, acting as an internal control host for the whole process.

This way it is possible to maintain a precise control for the entire process and determine whether the result is valid or not. As previously established in the background section, the critical problem (unresolved until today) in determining the presence of these bacteria using molecular techniques, is the possibility for obtaining false negatives. Most methods for identifying these bacteria using PCR only control the last step of the process, which is the amplification of DNA (qPCR). There is lack of control in the first stages including separating bacteria from the sample and extraction of bacteria DNA. On the contrary, the present invention includes a **unique** control for all the stages which ensures pathogen collection from the matrix, DNA extraction and the qPCR reaction.

Accordingly, the invention consists in a method for simultaneous detection and quantification of *Listeria monocytogenes*, *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) from different matrices, including a host (like a bacterium) as internal control (I.C.) which ensures correct application of all process steps. It is important to note that the invention can be applied to detect and quantify 1 or simultaneously 2 or 3 of pathogens using the same internal control host. This invention then comprises the following steps:

(a) adding a known quantity of internal control host (I.C.) to the sample, wherein the internal control host carrying a polynucleotide comprising a chimeric polynucleotide sequence;

(b) assessing the sample for the detection and quantification of nucleic acids of internal control (I.C.) and *L. monocytogenes*, *Salmonella* spp., Shiga toxin-producing *Escherichia coli* (STEC), or combinations thereof;

(c) determining the pathogen concentration by standard curve analysis made for each of the pathogens;

(d) validating the process with the results for I. C. which must give a signal according to the concentration used in (a).

Regarding the I.C., a person skilled in the art should consider evident that selecting a microorganism species to be transformed does not represent a critical issue, thus any bacterial, archaea or yeast species available in the *prior art* can be used. However, the transformed bacterium species preferably used is an *Escherichia coli,* which does not produce Shiga toxins and is non-pathogenic.

Similarly, the nature of the chimeric sequence also does not affect the method of detection and quantification of the pathogens, while the sequence included would be artificial and not- homologous to any bacterial genomes under study. Conveniently for the method of invention, the inventors used a chimeric sequence constructed with a region with the sequence of one of the primers of the invention, separated by a region of 20 to 70 bp from a third region with the sequence of another of the primers of the invention. This way, the chimeric sequence incorporated in the control host carries a sequence of a forward primer selected from SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, and 17; a 20 to 70 bp central-linker region; and a complementary sequence to a reverse primer selected from SEQ ID No 2, 4, 6, 8, 10, 12, 14, 16 and 18. Conveniently both primers are chosen from two different pathogens. In a preferred embodiment of the invention, the central linker region have 26 bp, and its sequence is that defined in SEQ ID No. 19.

So, in a first embodiment, said chimeric polynucleotide sequence is selected from the combinations of any of SEQ ID No 1, 3, 5 with any complementary of SEQ ID No 8, 10, 12,

14, 16, 18 (i.e. SEQ ID No39, 40, 41, 42, 43 and 44) , wherein the central-linker region is SEQ ID No 19, i.e. SEQ ID No 21, 45 to 61.

In a second embodiment, said chimeric polynucleotide sequence is selected from the combinations of any of SEQ ID No 7, 9, 11 with any complementary of SEQ ID No 2, 4, 6, 14, 16, 18 (i.e. SEQ ID No 36, 37, 38, 42, 43 and 44), wherein the central-linker region is SEQ ID No 19, i.e. SEQ ID No 62 to 79.

In a third embodiment said chimeric polynucleotide sequence is selected from the combinations of any of SEQ ID No 13, 15, 17 with any complementary of SEQ ID No 2, 4, 6, 8, 10, 12 (i.e. SEQ ID No 36, 37, 38, 39, 40 and 41), wherein the central-linker region is SEQ ID No 19, i.e. SEQ ID No 20, 80 to 96.

Preferred examples of chimeric sequences that can be used according to this invention are SEQ ID No. 20 and 21. For instance, SEQ ID No. 20 is formed by a region which is equivalent to *Salmonella spp.*, the primer defined in SEQ ID No. 13, a 26 bp region sequence as defined in SEQ ID No. 19, and a region complementary to STEC, the primer defined in SEQ ID No. 8 (i.e. SEQ ID No 38). And SEQ ID No. 21 is formed by a region which is equivalent to *Listeria monocytogenes*, the primer defined in SEQ ID No. 1, a 26 bp region sequence as defined in SEQ ID No. 19, and a region complementary to *STEC.*, the primer defined in SEQ ID No. 10 (i.e. SEQ ID No 40).

The method of invention further comprises a step to separate microorganisms out of the sample. Wherein said step of microorganism separation is performed by filtration, centrifugation, sorting, magnetic beads or combinations thereof.

The method of invention further comprises a step of nucleic acids extraction from the sample.

The method of invention wherein said nucleic acids detection is performed by PCR, RT-PCR, qPCR, digital PCR, LinDA, DNA microarray, PCR coupled with high-throughput sequencing, PCR DGGE/TTGE, or combinations thereof.

Wherein said nucleic acids detection of *L. monocytogenes* is performed using primers comprising a sequence selected from SEQ ID No. 1 to 6, its derivatives, or combinations thereof; and when said nucleic acids detection of *L. monocytogenes* is performed by qPCR use probes comprising a sequence selected from SEQ ID No. 22 to 25, its derivatives, or combinations thereof.

Wherein said nucleic acids detection of STEC is performed using primers comprising a sequence selected from SEQ ID No. 7 to 12, its derivatives, or combinations thereof; and when said nucleic acids detection of STEC is performed by qPCR, use probes comprising a sequence selected from SEQ ID No. 26 to 29, its derivatives, or combinations thereof.

Wherein said nucleic acids detection of *Salmonella* spp. bacteria is performed using primers comprising a sequence selected from SEQ ID No. 13 to 18, its derivatives, or combinations thereof and when said nucleic acids of *Salmonella* spp. bacteria is performed by qPCR use probes comprising a sequence selected from SEQ ID No. 30 to 33, its derivatives, or combinations thereof.

Wherein said nucleic acids detection of IC is performed using as forward primer a sequence selected from SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, and 17, its derivatives, or combinations thereof; and as reverse primer a sequence selected from SEQ ID No 2, 4, 6, 8, 10, 12, 14, 16, and 18, its derivatives, or combinations thereof; and when said nucleic acids detection of IC is performed by qPCR use probes comprising a sequence selected from SEQ ID No. 34 to 35, its derivatives, or combinations thereof.

In the method of the invention (steps (a) to (d)), said (c) step comprises a step of determining *Listeria monocytogenes*, *Salmonella* spp. bacteria and or Shiga toxin-producing *Escherichia coli* (STEC) concentration in a sample, interpolating the Cq value obtained by qPCR obtained in step (b) on a standard curve for each pathogen *Listeria monocytogenes*,

Salmonella spp. bacteria and/or Shiga toxin producing *Escherichia coli* (STEC) concentration and validating it by the value for I.C. signal obtained in step (d).

The method of invention allows work with different types of samples, like biological sample selected from meat, poultry, seafood, sausages, dairy, fruits, vegetables, ready to eat foods, beverages, or water, or surfaces.

The I.C. host is added to the sample at a concentration of 10^2-10^{10} cells or CFU per mL.

The polynucleotide to obtain an IC useful to detect and quantify *Listeria monocytogenes*, *Salmonella* spp., bacteria and/or Shiga toxin-producing *Escherichia coli* (STEC) in a sample, wherein said polynucleotide comprises:
a sequence of a forward primer selected from SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 and 17; a 26 bp central-linker region; and a complementary sequence to a reverse primer selected from SEQ ID No 2, 4, 6, 8, 10, 12, 14, 16 and 18 (i.e. SEQ ID No. 36 to 44). Wherein said central-linker region in a preferred embodiment is SEQ ID No. 19.

In a first embodiment, the polynucleotide is selected from the combinations of any of SEQ ID No 1, 3, 5 with any complementary of SEQ ID No 8,10, 12, 14,16, 18 (i.e. SEQ ID No39, 40, 41, 42, 43 and 44), wherein the central-linker region is SEQ ID No 19, i.e. SEQ ID No 21, 45 to 61.

In a second embodiment, the polynucleotide is selected from the combinations of any of SEQ ID No 7, 9, 11 with any complementary of SEQ ID No 2, 4, 6, 14, 16, 18 (i.e. SEQ ID No 36, 37, 38, 42, 43 and 44), wherein the central-linker region is SEQ ID No 19, i.e. SEQ ID No 62 to 79.

In a third embodiment, the polynucleotide is selected from the combinations of any of SEQ ID No 13, 15, 17 with any complementary of SEQ ID No 2, 4, 6, 8, 10, 12 (i.e. SEQ ID No 36,

37, 38, 39, 40 and 41), wherein the central-linker region is SEQ ID No 19, i.e. SEQ ID No 20, 80 to 96.

In a particularly preferred embodiment said polynucleotide chimeric comprises a sequence selected from a sequence selected from SEQ ID No 20 (formed by SEQ ID No 13, 19 and 8) or SEQ ID No. 21 (formed by SEQ ID No 1, 19 and 10).

The polynucleotide can be included in plasmid, cassette, episome, DNA construct, or combinations thereof, and is carried by a host selected from bacteria, archaea or yeast. Wherein said host is, transformed, edited or transfected with said polynucleotide. In one preferred embodiment said host is *Escherichia coli*.

In one preferred embodiment of the invention, *E. coli* K12 (host) is used for the construction of the internal control, then transformed with vector pGEM®-T Easy (Vector Systems-Promega Corporation) comprising a chimeric sequence as defined previously, and the transformants are selected using 100 µg/mL ampicillin.

As previously established, the sample to be assessed is inoculated with a previously known quantity of the internal control host corresponding to a bacterium (*E. coli* K-12) transformed with a vector carrying chimeric sequence. Preferably, adding a 10-200 µL aliquot of a bacterial suspension of 10^2-10^{10} CFU/mL.

As stated above, the internal control host (I.C.) is a bacterium transformed with the chimeric sequence, for the person skilled in the art it will be evident that there is a linear correlation between the concentration of the transformed bacteria and the chimeric sequence. So, depending on the stage of the process that is discussed, the term I.C. is used in this text to refer interchangeably to both terms, transformed control bacteria host or chimeric sequence.

The step referring to the microorganism's separation from the sample can be performed using any prior art methods depending on the nature of the sample. In one embodiment of the invention, separating all microorganisms from the sample is performed, for instance, from water only by filtration; from food by detachment washes using detergent solutions and then filtration; from surfaces by surface swabbing, resuspension in transport media and further filtration; and so on.

After microorganisms are collected, the DNA extraction step proceeds, which may be accomplished using any techniques available in the prior art. Then, DNA is resuspended in nuclease-free water.

Quantitative PCR (qPCR) for *L. monocytogenes*, *Salmonella* spp., STEC and the chimeric sequence is performed using DNA extracted from the samples. The qPCR result is validated in accordance with the Cq value for the chimeric sequence. In cases where the result is not consistent with the expected value, the measurement must be repeated.

In order to determine sample pathogen concentrations, it should be evident for a person skilled in this area, to construct a calibration curve or standard curve based on concentration standards known for each one of the pathogens to be assessed, versus the amplification cycle (Ct or Cq) related to qPCR technique. This way, the method of this invention provides Ct or Cq for each pathogen (*L. monocytogenes*, *Salmonella* spp. and STEC). Therefore, the concentration of each microorganism in the studied sample is determined by interpolating the obtained Ct value in the qPCR reaction in the standard curve for each pathogen.

Since the concentration of inoculated control bacteria host is previously known as well as their corresponding expected Cq value, the method can be validated by simply using this Cq value.

The following preferred embodiments of the invention are described merely as examples, without limiting any technical variants that a person skilled in the art may incorporate or modify, and which are therefore also included within the scope of the inventive concept claimed in this document.

EXAMPLES.

Example 1. Quantification of *Listeria monocytogenes*, *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) in Salmon and a comparison with the traditional method.

To establish the advantage of using the method of this invention, target pathogens *L. monocytogenes*, *Salmonella* spp. and STEC were determined in parallel by using the method of the present invention as well as by the traditional "gold standard" culture method, applied to samples inoculated with different quantities of these pathogens.

The traditional method consists of seeding the sample in different culture plates containing specific media for each bacterium for 24 to 48 hours and counting the colony-forming units (CFU), then performing identification tests such as conventional PCR for each microorganism.

1.1 Inoculation of pathogens using known concentrations and addition of I.C. (internal control host).

Ten raw fresh salmon samples, 100 g each, were inoculated with different concentrations of the 3 pathogens as described in Table 1, 2 and 3. Samples were processed with the method of this invention and the gold standard test (culture technique). Samples processed using the method of this invention were also inoculated with 25 µL of I.C. at a concentration of 10^2 CFU/mL.

I.C. was obtained through transformation of *E. coli* K12 with vector pGEM®-T Easy (Vector Systems - Promega Corporation) which carries a chimeric sequence defined as SEQ ID No. 31 designed with the reaction primers. To inoculate samples with I.C., this transformed bacterium was grown in LB (Luria-Bertani) broth plus 100 µg/mL ampicillin.

1.2 Conventional method (gold standard)

Quantification of *L. monocytogenes* was performed using the protocol described in the Bacteriological Analytical Manual (Hitchins, A., Jinneman, K., and Chen, Y. Chapter 10. Detection of *Listeria monocytogenes* in Foods and Environmental Samples, and Enumeration of *Listeria monocytogenes* in Foods, available on line). The quantification of *Salmonella* spp. was performed using the method described in: Brichta-Harhay DM, Arthur TM, Koohmaraie M, Enumeration of Salmonella from poultry carcass rinses via direct plating methods. Lett Appl Microbiol. 2008;46(2):186-191. doi:10.1111/j.1472-765X.2007.02289.x.

There is no gold standard method for STEC quantification.

1.3 Method of the invention

For separation of bacteria from the matrix a detergent solution was added to samples. Samples resuspended in detergent solution were filtered using a sterile gauze, and then the filtrate was passed through a 0,45 µm pore diameter nitrocellulose filter, to retain bacteria. Filtration was performed using a vacuum pump.

The Nitrocellulose filter, containing the bacteria, was recovered using sterile tweezers and then put in a 2 mL centrifuge tube in order to proceed with DNA sample extraction.

Then, bacteria DNA extraction was performed through standard extraction technology using phenol:chloroform. This protocol allows lysing bacteria through enzymatic (lysozyme and proteinase) and chemical action (sodium dodecyl sulfate or SDS). Then the DNA is finally resuspended in nuclease-free water or a TE (Tris-EDTA) buffer.

Once the DNA is obtained, qPCR is performed for detection of each pathogen and the chimeric sequence. For this 1 µL of resuspended DNA was used for each reaction, in triplicate.

Primers SEQ ID No. 1 and 2 were used for *L. monocytogenes*; primers SEQ ID No. 9 and 10 for STEC; primers SEQ ID No. 15 and 16 for *Salmonella* spp. and primers SEQ ID No. 1 and 10 for the chimeric sequence (I.C.). The probes used for detection of *L. monocytogenes* (SEQ ID No. 24), *Salmonella* spp. (SEQ ID No. 33), STEC (SEQ ID No. 26) and IC (SEQ ID No. 34).

After the qPCR reaction, a Cq value was obtained for each pathogen and the I.C. The concentration of each pathogen was estimated in CFU/g of salmon using a standard curve previously constructed (Cq v/s CFU/g, as shown in Figure 1). Lower Cq means a higher concentration of each pathogen expressed as CFU/g.

The results obtained using the method of the present invention are shown in Table 1 for *L. monocytogenes*, Table 2 for *Salmonella* spp. and Table 3 for STEC. The tables include the concentration of each microorganism added (Inoculum) to each sample, and the results obtained applying a *Gold standard* test. In addition, tables show the Cq value obtained for the I.C. In order to validate the assay, the Cq value for the IC must be in a 28 to 33.5 range.

Table 1. Comparative results for quantification of *L. monocytogenes* from salmon using the method of the present invention and the *Gold standard* test.

Sample ID	**Log_{10} CFU/g Inoculum**	**Log_{10} CFU/g Gold Standard**	**Log_{10} CFU/g Invention**	**Cq Internal Control**
S1	NI*	<0.6**	<1.59**	29.2
S2	NI*	<0.6**	<1.59**	29.5
S3	NI*	<0.6**	<1.59**	30.9
S4	2.1	<0.6**	<1.59**	30.4

S5	2.2	2.0	2.02	29.5
S6	2.3	2.5	1.85	29.4
S7	3.0	1.9	2.48	29.2
S8	3.2	<0.6**	3.06	30.4
S9	3.2	2.7	3.00	30.6
S10	3.3	3.5	2.88	29.7

NI*: non-inoculated. ** Detection limit of each method.

Table 2. Comparative results for quantification of *Salmonella* spp. from salmon using the method of the present invention and the *Gold standard* test.

Sample ID	**Log_{10} CFU/g Inoculum**	**Log_{10} CFU/g Gold Standard**	**Log_{10} CFU/g Invention**	**Cq Internal Control**
S1	NI*	<0.6**	<1.76**	29.2
S2	NI*	<0.6**	<1.76**	29.5
S3	NI*	<0.6**	<1.76**	30.9
S4	0.5	<0.6**	<1.76**	29.8
S5	0.9	<0.6**	<1.76**	30.4
S6	2.47	1.4	1.76	29.5
S7	2.54	1.4	2.05	29.2
S8	3.48	2.3	2.52	30.6
S9	3.60	1.4	2.69	30.4
S10	3.88	2.3	3.28	29.7

NI*: non-inoculated. ** Detection limit of each method.

Table 3. Comparative results for quantification of STEC from salmon using the method of the present invention. There is no *Gold Standard* test for quantifying this pathogen from salmon samples.

Sample	**Log_{10} CFU/g**	**Log_{10} CFU/g**	**Log_{10} CFU/g**	**Cq**

ID	Inoculum	Gold Standard	Invention	Internal Control
S1	NI*	NA**	<1.64***	29.2
S2	NI*	NA**	<1.64***	29.5
S3	NI*	NA**	<1.64***	30.9
S4	0	NA**	<1.64***	29.8
S5	0.2	NA**	<1.64***	30.4
S6	2.0	NA**	1.72	29.2
S7	2.6	NA**	<1.64***	29.5
S8	3.2	NA**	2.71	29.7
S9	3.4	NA**	2.91	30.4
S10	3.6	NA**	2.48	30.6

NI*: non-inoculated; **NA: gold standard quantification method is not available; *** Detection limit

When comparing the results obtained for *L. monocytogenes, Salmonella* spp., and STEC using the method of this invention, they show similar quantification limits (Log_{10}1.59, Log_{10}1.76, and Log_{10}1.64 CFU/g respectively). The method of the present invention showed a ten-fold higher quantification limit compared to the limit of the gold standard method. For the STEC quantification, there is no standard protocol available to compare with our results and we compared our results with the inoculum added to the samples.

The results indicate that, when applying the method of this invention, most of the concentration values obtained are closer to the inoculum added to the sample, than those obtained through the gold standard method.

Considering the time taken since the sample processing initiated, the method of this invention took only 8 hours, while for the traditional test 2 days were needed to get results. Accordingly, the method of the invention delivers similar results to those obtained using gold standard tests in a substantially shorter period of time.

Example 2. Quantification of *Listeria monocytogenes*, *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) in the transport media used for the surface testing system.

There are different sampling protocols for the microbiological analyses of surfaces, which are standardized. Among these protocols, there is sample collection through a sterile swab that is repeatedly rubbed (in different directions) over a 100 cm^2 area. Afterwards, this swab is submerged in Letheen transport media.

2.1 Inoculation of pathogens using known concentrations and addition of I.C. (internal control host).

In order to contrast results using the method of this invention with the gold standard test, swabs submerged in Letheen buffer (20 mL) were inoculated with different pathogen concentrations to be analyzed as detailed in Tables 4, 5 and 6. Afterwards, 10 mL of inoculated Letheen buffer was processed using the method of the present invention, while another 10 mL was analyzed through the gold standard method. Samples processed using the method of this invention were also inoculated with 25 µL of I.C. at a 10^2 CFU/mL concentration.

2.2 Gold standard method.

Similarly, as explained in example 1.2, quantification of pathogens *L. monocytogenes*, and *Salmonella* spp. present in surface samples were processed by the protocols described in the Bacteriological Analytical Manual (gold standard tests) and by Brichta-Harhay, et al (2008).

There is no gold standard method for STEC quantification, so quantification of this pathogen was determined only using the method of the present invention.

2.3 Method of the invention

Separation of bacteria from media: The Letheen broth was filtrated through a 0,45 µm pore diameter nitrocellulose filter, which is capable of retaining these bacteria. Filtration was performed using a vacuum pump.

The filter, containing the bacteria, was recovered using sterile tweezers and then put in a 2 mL centrifuge tube.

Then, DNA extraction of the bacteria present in the filter was performed through standard extraction technology using phenol:chloroform, in accordance with the details of the previous example. The DNA was resuspended in nuclease-free water or a TE (for Tris-EDTA) buffer.

Once the DNA is obtained, qPCR is performed for detection of each pathogen and the chimeric sequence. 1 µL of suspended DNA was used for each reaction, in triplicate.

Primers SEQ ID No. 3 and 4 were used for *L. monocytogenes*; primers SEQ ID No. 7 and 8 for STEC; primers SEQ ID No. 13 and 14 for *Salmonella* spp. and primers SEQ ID No. 13 and 8 for the chimeric sequence (I.C.). The probes used for detection of *L. monocytogenes* (SEQ ID No. 25), *Salmonella* spp. (SEQ ID No. 32), STEC (SEQ ID No. 27) and IC (SEQ ID No. 35).

After the qPCR reaction, a Cq value was obtained for each pathogen and the I.C. The concentration of each pathogen was estimated in CFU/cm^2 of the surface using a standard curve previously constructed (Cq v/s CFU/cm^2, as shown in Figure 1).

The results after using the method of the invention are shown in Table 4 for *L. monocytogenes*, in Table 5 for *Salmonella* spp. and in Table 6 for STEC. The tables include the concentration of each microorganism added (Inoculum) to each sample, and the results obtained applying a Gold standard test. In addition, the tables show the Cq value obtained

for the I.C. in order to validate the assay, the Cq value for the IC must be in a 28 to 33.5 range.

Table 4. Comparative quantification results of *L. monocytogenes* from surface samples using the method of the present invention and the *Gold standard* test.

Sample ID	Log_{10} CFU/cm^2 Inoculum	Log_{10} CFU/cm^2 Gold Standard	Log_{10} bacterium/cm^2 Invention	Cq Internal Control
S1	NI	< 0**	<0.98**	28.55
S2	NI	< 0**	<0.98**	28.39
S3	NI	< 0**	<0.98**	28.66
S4	0.95	2.51	<0.98**	32.33
S5	0.95	2.54	<0,98**	28.64
S6	0.95	1.18	<0.98**	28.52
S7	1.95	2.20	2.27	32.08
S8	1.95	2.08	2.28	28.20
S9	1.95	3.48	2.25	28.54
S10	2.95	3.00	3.35	28.57
S11	2.95	3.73	3.27	33.52
S12	2.95	3.69	3.13	28.92

NI*: non-inoculated. ** Detection limit (corresponds to 1 CFU/cm^2 for the Gold Standard method)

Table 5. Comparative quantification results of *Salmonella* spp. from surface samples using the method of the present invention and the *Gold standard* test.

Sample ID	Log_{10} CFU/cm^2 Inoculum	Log_{10} CFU/cm^2 Gold Standard	Log_{10} bacterium/cm^2 Invention	Cq Internal Control
S1	NI*	< 0**	<1.16**	28.55
S2	NI*	< 0**	<1.16**	28.39

S3	NI*	< 0**	<1.16**	28.66
S4	0.60	2.75	<1.16**	32.33
S5	0.60	3.51	<1.16**	28.64
S6	0.60	1.26	<1.16**	28.52
S7	1.60	2.28	1.16	32.08
S8	1.60	1.72	1.18	28.20
S9	1.60	3.71	<1.16**	28,54
S10	2.60	2.83	2.04	28.57
S11	2.60	4.00	1.99	33.52
S12	2.60	4.04	2.00	28.92

NI*: non-inoculated. ** Detection limit (corresponds to 1CFU/cm^2 for the Gold Standard method)

Table 6. Comparative quantification results of STEC from surface samples using the method of the present invention and the Gold standard test.

Sample ID	**Log_{10} CFU/cm^2 Inoculum**	**Log_{10} CFU/cm^2 Gold Standard**	**Log_{10} bacterium/cm^2 Invention**	**Cq Internal Control**
S1	NI*	NA**	<1.04***	28.55
S2	NI*	NA**	<1.04***	28.39
S3	NI*	NA**	<1.04***	28.66
S4	0.48	NA**	<1.04***	32.33
S5	0.48	NA**	<1.04***	28.64
S6	0.48	NA**	<1.04***	28.52
S7	1.48	NA**	2.17	32.08
S8	1.48	NA**	2.24	28.20
S9	1.48	NA**	2.09	28.54
S10	2.48	NA**	3.11	28.57

S11	2.48	NA**	3.08	33.52
S12	2.48	NA**	3.05	28.92

NI*: non-inoculated. NA**: gold standard quantification method is not available; *** Detection limit

Results show that it is possible to quantify *L. monocytogenes*, *Salmonella* spp., and STEC using the method of the invention at concentrations higher than $Log_{10}0.98$, $Log_{10}1.16$ and $Log_{10}1.04$ CFU/cm^2 respectively, that corresponds to the detection limit of the method for this matrix.

Additionally, values determined by the method of this invention are closer to the concentration inoculated to the surface sample, compared with those quantified by the gold standard test.

Internal control was successfully detected using the method of this invention for all samples, as expected, and the Cq values for this IC fluctuated between 28 and 33.5 as predicted for this I.C.

On the other hand, it is important to highlight that using the method of the present invention the results were obtained 8 hours after the start of the sample processing, compared to more than 2 days required for the gold standard method. Moreover, the method of the present invention quantifies three pathogens simultaneously while the gold standard processes each one separately.

Claims

1. A method for simultaneous or independent detection and quantification of *Listeria monocytogenes*, *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) in a sample comprising the following steps:
   a) adding a known quantity of internal control host (I.C.) to the sample, wherein the internal control host carrying a polynucleotide comprising a chimeric polynucleotide sequence;
   b) assessing the sample for the detection and quantification of nucleic acids of internal control (I.C.) and *L. monocytogenes*, *Salmonella* spp., Shiga toxin-producing *Escherichia coli* (STEC), or combinations thereof;
   c) determining the pathogen concentration by standard curve analysis made for each of the pathogens;
   d) validating the process with the results for I. C. which must give a signal according to the concentration used in (a).

2. The method according to claim 1, wherein the internal control host is selected from bacteria, archaea or yeast.

3. The method of claim 2, wherein the said host is *Escherichia coli*.

4. The method of claim 1, wherein said chimeric polynucleotide sequence comprises: a sequence of a forward primer selected from SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, and 17; a 20 to 70 bp central-linker region; and a complementary sequence to a reverse primer selected from SEQ ID No 2, 4, 6, 8, 10, 12, 14, 16 and 18.

5. The method according to claim 4 wherein the complementary reverse sequence is selected from SEQ ID No.36 to 44.

6. The method according to claim 4, wherein said central-linker region is SEQ ID No. 19.

7. The method according to claim 4, wherein said chimeric polynucleotide sequence is selected from the combinations of any of SEQ ID No 1, 3, 5 with any complementary of SEQ ID No 8, 10, 12, 14, 16, 18, wherein the central-linker region is SEQ ID No 19.

8. The method according to claim 7, wherein the full length sequence of said chimeric polynucleotide sequence is selected from SEQ ID No 21, 45 to 61.

9. The method according to claim 4, wherein said chimeric polynucleotide sequence is selected from the combinations of any of SEQ ID No 7, 9, 11 with any complementary of SEQ ID No 2, 4, 6, 14, 16, 18, wherein the central-linker region is SEQ ID No 19.

10. The method according to claim 9, wherein the full length sequence of said chimeric polynucleotide sequence is selected from SEQ ID No 62-79.

11. The method according to claim 4, wherein said chimeric polynucleotide sequence is selected from the combinations of any of SEQ ID No 13, 15, 17 with any complementary of SEQ ID No 2, 4, 6, 8, 10, 12, wherein the central-linker region is SEQ ID No 19.

12. The method according to claim 11, wherein the full length sequence of said chimeric polynucleotide sequence is selected from SEQ ID No 20, 80-96.

13. The method of claim 2, wherein said host is a transformed, edited or transfected host carrying the polynucleotide comprising the chimeric sequence selected from SEQ ID No. 20, 21, 44 to 96.

14. The method of claim 1, wherein said method further comprises a step to separate microorganisms out of the sample.

15. The method of claim 14, wherein said step of microorganism separation is performed by filtration, centrifugation, sorting, magnetic beads or combinations thereof.

16. The method of claim 15, wherein said method further comprises a step of nucleic acids extraction from the sample.

17. The method of claim 1, wherein said nucleic acids detection is performed by PCR, RT-PCR, qPCR, digital PCR, LinDA, DNA microarray, PCR coupled with high-throughput sequencing, PCR DGGE/TTGE, or combinations thereof.

18. The method of claim 17, wherein for said nucleic acids detection of *L. monocytogenes* is performed using primers comprising a sequence selected from SEQ ID No. 1 to 6, its derivatives, or combinations thereof.

19. The method of claim 18, wherein said nucleic acids detection of *L. monocytogenes* is performed by qPCR using probes comprising a sequence selected from SEQ ID No. 22 to 25, its derivatives, or combinations thereof.

20. The method of claim 17 wherein for said nucleic acids detection of STEC is performed using primers comprising a sequence selected from SEQ ID No. 7 to 12, its derivatives, or combinations thereof.

21. The method of claim 20, wherein said nucleic acids detection of STEC is performed by qPCR using probes comprising a sequence selected from SEQ ID No. 26 to 29, its derivatives, or combinations thereof.

22. The method of claim 17, wherein for said nucleic acids detection of *Salmonella*-type bacteria is performed using primers comprising a sequence selected from SEQ ID No. 13 to 18, its derivatives, or combinations thereof.

23. The method of claim 22, wherein said nucleic acids of *Salmonella* spp. bacteria is performed by qPCR using probes comprising a sequence selected from SEQ ID No. 30 to 33, its derivatives, or combinations thereof.

24. The method of claim 1, wherein said c) step comprises a step of determining *Listeria monocytogenes*, *Salmonella* spp. bacteria and or Shiga toxin-producing *Escherichia coli* (STEC) concentration in a sample interpolating the Cq value, obtained by qPCR in step b), on a standard curve for each pathogen *Listeria monocytogenes*, *Salmonella* spp. bacteria and/or Shiga toxin producing *Escherichia coli* (STEC) concentration and validating it by the value for I.C. signal obtained in step d).

25. The method of claim 24, wherein said nucleic acids detection of IC is performed using as forward primer a sequence selected from SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, and 17, its derivatives, or combinations thereof; and as reverse primer a sequence selected from SEQ ID No 2, 4, 6, 8, 10, 12, 14, 16, and 18, its derivatives, or combinations thereof.

26. The method of claim 25, wherein said nucleic acids detection of IC is performed by qPCR using probes comprising a sequence selected from SEQ ID No. 34 to 35, its derivatives, or combinations thereof.

27. The method of claim 1, wherein said sample is a biological sample selected from meat, poultry, seafood, sausages, dairy, fruits, vegetables, ready to eat foods, beverages, water or surfaces.

28. The method of claim 1, wherein the I.C. host is added at a concentration of 10^2-10^{10} cells or CFU per mL.

29. A polynucleotide to obtain an IC useful to detect and quantify *Listeria monocytogenes*, *Salmonella* spp., and/or Shiga toxin-producing *Escherichia coli* (STEC) in a sample, wherein said polynucleotide comprises:
a sequence of a forward primer selected from SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15 and 17; a 26 bp central-linker region; and a complementary sequence to a reverse primer selected from SEQ ID No 2, 4, 6, 8, 10, 12, 14, 16 and 18.

30. The polynucleotide according to claim 29 wherein the complementary reverse sequence is selected from SEQ ID No.36 to 44.

31. The polynucleotide of claim 29, wherein said central-linker region is SEQ ID No. 19.

32. The polynucleotide of claim 29 wherein said polynucleotide is selected from the combinations of any of SEQ ID No 1, 3, 5 with any complementary of SEQ ID No 8, 10, 12, 14, 16, 18, wherein the central-linker region is SEQ ID No 19.

33. The polynucleotide according to claim 32 wherein the full length sequence of said polynucleotide sequence is selected from SEQ ID No 21, 45 to 61.

34. The polynucleotide of claim 29, wherein said polynucleotide is selected from the combinations of any of SEQ ID No 7, 9, 11 with any complementary of SEQ ID No 2, 4, 6, 14, 16, 18, wherein the central-linker region is SEQ ID No 19.

35. The polynucleotide according to claim 34, wherein the full length sequence of said polynucleotide sequence is selected from SEQ ID No 62-79.

36. The polynucleotide of claim 29, wherein said polynucleotide is selected from the combinations of any of SEQ ID No 13, 15, 17 with any complementary of SEQ ID No 2, 4, 6, 8, 10, 12, wherein the central-linker region is SEQ ID No 19.

37. The polynucleotide according to claim 36, wherein the full length sequence of said polynucleotide sequence is selected from SEQ ID No 20, 80-96.

38. The polynucleotide according to claim 31 wherein said polynucleotide chimeric comprises a sequence selected from SEQ ID No 20 , 21, 44 to 96.

39. The polynucleotide according to claim 31, wherein said polynucleotide is included in plasmid, cassette, episome, DNA construct, or combinations thereof.

40. The polynucleotide according to claim 29 wherein said polynucleotide is carried by a host selected from bacteria, archaea or yeast.

41. The polynucleotide of claim 40, wherein said host is, transformed, edited or transfected with said polynucleotide.

42. The polynucleotide according to claim 31 wherein said host is *Escherichia coli.*

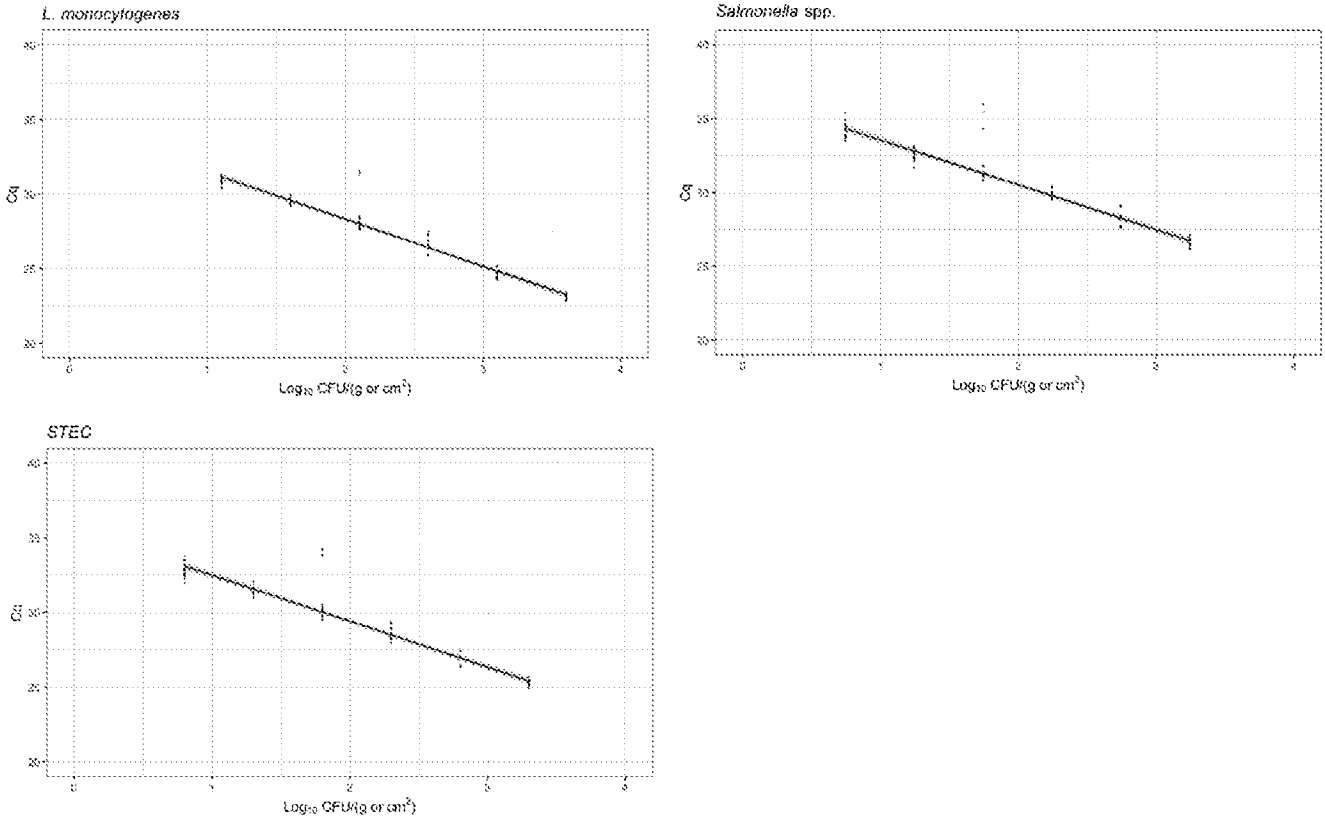

L. monocytogenes
Cq
Log10 CFU/(g or cm2)
Salmonella spp.
Cq
Log10 CFU/(g or cm2)
STEC
Cq
Log10 CFU/(g or cm2)

Figure 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/054614

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07H 21/04; C12N 15/09; C12N 15/10; C12N 15/11; C12P 19/34; C12Q 1/34 (2021.01)
CPC - C12Q 1/6851; C12Q 1/689; C12Q 2521/327; C12Q 2549/101; C12Q 2563/107 (2021.08)

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)
see Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2019/0183130 A1 (ARCIS BIOTECHNOLOGY HOLDINGS LIMITED) 20 June 2019 (20.06.2019) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42
A	CARLONI et al. "Rapid and simultaneous detection of Salmonella spp., Escherichia coli O157, and Listeria monocytogenes by magnetic capture hybridization and multiplex real-time PCR," Folia Microbiologica, 25 May 2018 (25.05.2018), Vol. 63, Iss. 6, Pgs. 735-742. entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42
A	CN 101928773 B (INSTITUTE OF ENVIRONMENT & HEALTH ACADEMY OF MILITARY MEDICAL SCIENCES PLA) 29 August 2012 (29.08.2012) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42
A	US 2007/0020640 A1 (MCCLOSKEY et al) 25 January 2007 (25.01.2007) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42
A	US 2016/0319294 A1 (MONSANTO TECHNOLOGY LLC et al) 03 November 2016 (03.11.2016) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42
A	CN 102676647 B (NORTHEAST AGRICULTURAL UNIVERSITY) 28 August 2013 (28.08.2013) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42
A	US 2011/0223598 A1 (OPDYKE et al) 15 September 2011 (15.09.2011) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42
A	US 2018/0258467 A1 (POLYSKOPE LABS) 13 September 2018 (13.09.2018) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 October 2021	NOV 24 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Harry Kim Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/054614

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009/0119022 A1 (TIMBERLAKE et al) 07 May 2009 (07.05.2009) entire document	1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/054614

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1, 10, 20-24, 26-28, 34, and 45-96 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/054614

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4, 6-8, 13-17, 20, 24-29, 31-33, 38-42

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/054614

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-42 are drawn to methods for simultaneous detection and quantification of Listeria monocytogenes, Salmonella spp. and Shiga toxin-producing Escherichia coli (STEC), and polynucleotides comprising the same.

The first invention of Group I+ is restricted to an internal control comprising a chimeric polynucleotide and a PCR probe, where the chimeric polynucleotide selected to be SEQ ID NO:21, where SEQ ID NO:21 further comprises forward primer SEQ ID NO:1, reverse primer SEQ ID NO:10, and linker SEQ ID NO:19; and a PCR probe selected to be SEQ ID NO:34, and methods and polynucleotides comprising the same. It is believed that claims 1-4, 6-8, 13-17, 20, 24-29, 31-33, and 38-42 read on this first named invention and thus these claims will be searched without fee to the extent that they read on SEQ ID NOs: 1, 10, 21, and 34.

Applicant is invited to elect additional forward primers, reverse primers, chimeric polynucleotides, probes, and their respective, corresponding, SEQ ID NOs to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be an internal control comprising a chimeric polynucleotide and a PCR probe, and a forward primer and a reverse primer for Listeria monocytogenes, where the chimeric polynucleotide selected to be SEQ ID NO:21, where SEQ ID NO:21 further comprises forward primer SEQ ID NO:1, reverse primer SEQ ID NO:10, and linker SEQ ID NO:19; and a PCR probe selected to be SEQ ID NO:34, and the Listeria monocytogenes forward primer is selected to be SEQ ID NO:1 and the Listeria monocytogenes reverse primer is selected to be SEQ ID NO:2, and methods and polynucleotides comprising the same. Additional forward primers, reverse primers, chimeric polynucleotides, probes, and their respective, corresponding, SEQ ID NOs will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for simultaneous or independent detection and quantification of Listeria monocytogenes, Salmonella spp. and Shiga toxin-producing Escherichia coli (STEC) in a sample requiring the selection of alternative chimeric polynucleotides where "chimeric polynucleotide sequence comprises: a sequence of a forward primer selected from SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, and 17; a 20 to 70 bp central-linker region; and a complementary sequence to a reverse primer selected from SEQ ID No 2, 4, 6, 8, 10, 12, 14, 16 and 18," and probes where "said nucleic acids detection of STEC is performed by qPCR using probes comprising a sequence selected from SEQ ID No. 26 to 29, its derivatives, or combinations thereof."

Additionally, even if Groups I+ were considered to share the technical features of a method for simultaneous or independent detection and quantification of Listeria monocytogenes, Salmonella spp. and Shiga toxin-producing Escherichia coli (STEC) in a sample comprising the following steps: a) adding a known quantity of internal control host (L.C.) to the sample, wherein the internal control host carrying a polynucleotide comprising a chimeric polynucleotide sequence; b) assessing the sample for the detection and quantification of nucleic acids of internal control (L.C.) and L. monocytogenes, Salmonella spp., Shiga toxin-producing Escherichia coli (STEC), or combinations thereof; c) determining the pathogen concentration by standard curve analysis made for each of the pathogens; d) validating the process with the results for I. C. which must give a signal according to the concentration used in (a); a polynucleotide to obtain an IC useful to detect and quantify Listeria monocytogenes, Salmonella spp., and/or Shiga toxin-producing Escherichia coli (STEC) in a sample, wherein said polynucleotide comprises: a sequence of a forward; a 26 bp central-linker region; and a complementary sequence to a reverse primer. However, these shared technical features do not represent a contribution over the prior art.

Specifically, "Rapid and simultaneous detection of Salmonella spp., Escherichia coli O157, and Listeria monocytogenes by magnetic capture hybridization and multiplex real-time PCR," to Carloni et al. discloses a method for simultaneous or independent detection and quantification of Listeria monocytogenes, Salmonella spp. and Shiga toxin-producing Escherichia coli (STEC) in a sample (methods for pathogen detection and quantification is very advantageous in diagnosis of human pathogens in several applications ... multiplexed detection and quantification of three significant foodborne pathogenic species (Escherichia coli O157, Salmonella spp., and Listeria monocytogenes), Abstract) comprising the following steps: b) assessing the sample for the detection and quantification of nucleic acids of internal control (L.C.) and L. monocytogenes, Salmonella spp., Shiga toxin-producing Escherichia coli (STEC), or combinations thereof (following the manufacturer's instructions. The Multipathogen FLUO kit could be used for the simultaneous identification of Salmonella spp., L. monocytogenes, and E. coli O157 using dual-labelled probes strategy. Salmonella spp. amplification is detected in Green channel (Ex 495 to Em 520 nm), L. monocytogenes in Red channel (Ex 647 to Em 667 nm), E. coli O157 in Orange channel (Ex 590 to Em 610 nm) and Internal Amplification Control (IAC) in Yellow channel (Ex 538 to Em 554 nm). The internal control in the amplification kit gives assurance of reliability of negative results, Pg. 2, right-hand column, third paragraph); c) determining the pathogen concentration by standard curve analysis made for each of the pathogens (following the manufacturer's instructions. The Multipathogen FLUO kit could be used for the simultaneous identification of Salmonella spp., L. monocytogenes, and E. coli O157 using dual-labelled probes strategy. Salmonella spp. amplification is detected ... automatically calculates the standard curve. A linear regression line was plotted, and the resulting equation was used to calculate the log10 GU for the determination of sensitivity, standard curve, and amplification efficiency, Pg. 2, right-hand column, third paragraph); d) validating the process with the results for I. C. which must give a signal according to the concentration used in (a) ([t]he internal control in the amplification kit gives assurance of reliability of negative results, Pg. 2, right-hand column, third paragraph; to compare the sensitivity of the mMCH with respect to the MCH, DNA of each pathogen was extracted from bacterial pellets using its specific probe-conjugated nanoparticles (MCH) or the mixture of the three types of conjugated nanoparticles, Pg. 5, left-hand column, first paragraph); a polynucleotide to obtain an IC useful to detect and quantify Listeria monocytogenes, Salmonella spp., and/or Shiga toxin-producing Escherichia coli (STEC) in a sample (methods for pathogen detection and quantification is very advantageous in diagnosis of human pathogens in several applications ... multiplexed detection and quantification of three significant foodborne pathogenic species (Escherichia coli O157, Salmonella spp., and Listeria monocytogenes), Abstract; following the manufacturer's instructions. The Multipathogen FLUO kit could be used for the simultaneous identification of Salmonella spp., L. monocytogenes, and E. coli O157 using dual-labelled probes strategy. Salmonella spp. amplification is detected

in Green channel (Ex 495 to Em 520 nm), L. monocytogenes in Red channel (Ex 647 to Em 667 nm), E. coli O157 in Orange channel (Ex 590 to Em 610 nm) and Internal Amplification Control (IAC) in Yellow channel (Ex 538 to Em 554 nm). The internal control in the amplification kit gives assurance of reliability of negative results, Pg. 2, right-hand column, third paragraph).

Further US 2011/0223598 A1 to Opdyke et al. teaches a) adding a known quantity of internal control host (L.C.) to the sample (includes this type of internal control. The number of copies of the internal control is limited so as not to interfere with efficient amplification of Salmonella Typhimurium, Para. [0115]; 15 copies of internal amplification control plasmid, Para. [0142]; providing a sample to be tested for the presence of a Salmonella target RNA, Para. [0015]), wherein the internal control host carrying a polynucleotide comprising a chimeric polynucleotide sequence (includes this type of internal control. The number of copies of the internal control is limited so as not to interfere with efficient amplification of Salmonella Typhimurium, Para. [0115]; 15 copies of internal amplification control plasmid, Para. [0142]; CataCleave probe has a chimeric structure where the 5' and 3' ends of the probe are constructed of DNA and the cleavage site contains RNA, Para. [0078]); wherein said polynucleotide comprises: a sequence of a forward; and a complementary sequence to a reverse primer (800 micro M Salmonella-Forward primer, 800 micro M Salmonella-reverse primer, Para. [0132]).

Further, US 2007/0020640 A1 to Mccloskey et al. teaches a 26 bp central-linker region (the 26 nt linker, Para. [0016]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.