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DISSERTATION

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“Paving the way for molecular biological quantification of *Listeria monocytogenes* in food.”

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1. Summary

This work establishes a basis for reliable use of molecular biological methods for food pathogen detection with respect to *Listeria monocytogenes.* Real-time PCR (qPCR) was the detection method of choice. As a first step an existing conventional PCR assay for *L. monocytogenes* was extended to qPCR use by constructing and testing an appropriate probe design. A previous project dealing with PCR in food pathogen detection (FOOD-PCR I) resulted in a reliable conventional PCR assay targeting the *prfA* locus of *L. monocytogenes* (D'Agostino et al., 2004). This was extended here with a FAM (6-carboxyfluorescein) labelled minor groove binder Taqman-probe and an artificial internal amplification control (IAC). The resulting qPCR assay was thoroughly tested with special emphasis on the detection limit and specificity. A Poisson distribution based testing approach was used by analysis of a series containing a highly diluted DNA template (one copy, average). The positive/negative distribution within the samples after qPCR was compared with the expected distribution (Wang and Spadoro, 1998). Consequently, the detection limit of one copy could be determined experimentally, albeit the quantitative resolution was not tested in this range.

Subsequently, a rapid method, including the newly developed qPCR assay, was developed. In this way the qPCR assay was combined with a short enrichment protocol derived from the standard method (ISO 11290) using half Fraser broth for short enrichment. This resulted in a rapid protocol leading to qualitative results within 24 hours. With a specificity of 100% the method compared with the ISO 11290-1 standard method, with a relative accuracy of 96%, a relative specificity of 100%, and a relative sensitivity of 76.9%. Nevertheless, these results were biased by the usual condition of the long-term frozen food samples (Rossmanith et al., 2010a). See chapter 4.1.: Rossmanith et al. (2006).

Additionally, several DNA isolation/purification methods were tested in combination with the newly developed combined method to obtain maximum recovery of target DNA. The commercial NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) performed best and was therefore included in the protocol. See chapter 4.1.: Rossmanith et al. (2006).

Concurrently the possibility of direct quantification of the target bacteria can be investigated to obtain quantitative information. The influence of food matrices and enrichment media on qPCR performance with respect to inhibitory effects was investigated. In a first attempt the direct addition of food samples or food samples after selective enrichment to the PCR vial was tested. Chemical inhibition was not observed with one to the tenth diluted samples, with the exception of faeces and intestinal contents (data not shown). However, the direct approach was abandoned, as the volumes that are processable (≤ 0.5 µl of the original sample if diluted) are far too small in comparison to the required amount per sample of 25 g or ml foodstuff. Nevertheless, the direct application of food samples after selective enrichment to qPCR would be a practicable solution given a lack of chemical interference of the food matrix or the enrichment broth. However, investigations of the fluorescence characteristics of eleven most common enrichment media for food pathogens resulted in significant fluorescence in some cases when excited in the wavelength range of real-time PCR cycler optical path assignments. This was observed particularly for Fraser broth, the standard *L. monocytogenes* selective medium, on excitation with 492 nm, 517 nm and 535 nm, corresponding to the FAM, TET (6-carboxy-2',4,7,7'-tetrachlorofluorescein) and HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein) channels of the thermocycler. In conclusion, careful selection of the probe dyes is necessary to circumvent this problem. See Chapter 4.2.: Rossmanith et al. (2010b).

In a next step towards reliable molecular biology based food pathogen detection, the necessity of an analytical chain, consisting of sample preparation, DNA isolation/purification and qPCR

detection was considered. Hence, a sample preparation method is proposed to circumvent the disadvantages of combined and direct detection and to create the optimal conditions for qPCR detection protocols. This sample preparation method should provide bacterial target cells as pure as possible to avoid the interference of residues from the food matrix with qPCR detection. Furthermore, the bacterial content of the original sample should be transferred to the DNA isolation/purification in its entirety. Previously developed sample preparation methods have in common bacterial targets that were separated from the more or less unmodified food matrix (Stevens and Jaykus, 2004a). These protocols usually have insufficient capacity in terms of sample size or insufficient recovery and often lack necessary reproducibility. To circumvent these problems a new approach has been chosen, bringing the food matrix into aqueous solution and separating the bacterial cells by centrifugation. A buffer containing a detergent and a chaotrope in a buffered solution was designed and tested. The resulting composition of 8 M urea and 1% sodium dodecyl sulphate (SDS) in 1% phosphate buffered saline (PBS) sufficiently reduced 6 g or 12 ml of several dairy products, egg, ice cream, meat, fish, chicken and blood. The resulting pellets of ≤ 200 µl could be further processed for DNA isolation/purification using the NucleoSpin® Tissue Kit. The relatively harsh incubation conditions consequential to the buffer composition allow for separation of Gram-positive bacteria, but their viability is not given after separation. The protocol was tested in combination with DNA isolation/purification using the NucleoSpin® Tissue Kit and the *prfA* qPCR assay. This resulted in a detection limit of 7.3 colony forming units (CFU) per ml of *L. monocytogenes* and a recovery of 29.9% after qPCR from ultra-high temperature treated (UHT) milk and raw milk. See Chapter 4.3.: Rossmanith et al. (2007).

During development of this new sample preparation method the crucial point in every step was the localization of the target organisms. As most target pathogens in food and clinical diagnostics are colourless and invisible, there is no possibility of pinpointing them within the

sample matrices. Consequently, it is necessary to simulate the bacterial targets from the beginning of the development of a new protocol to establish the necessary chemical and physical parameters. Chromogenic bacteria meet that prerequisite. The addition of coloured bacteria allowed for their localization in either the fat content, the water-soluble fraction or attached to the tube after the solubilization and centrifugation steps. Therefore development of the new sample preparation method was possible in an acceptable amount of time. Prior to this application of *Micrococcus roseus* in the developmental stage of the new method protocol, preliminary tests were performed to verify the comparability of the pigmented model bacteria with the actual target bacteria. See Chapter 4.4.: Rossmanith et al. (2010c).

1. Zusammenfassung

Das Thema dieser Arbeit war die Bearbeitung von grundlegenden Fragestellungen im Zusammenhang mit dem molekularbiologischen Nachweis von lebensmittelpathogenen Bakterien. Die Methode der Wahl war die Real-time Polymerase-Kettenreaktion (qPCR). Dieser Ansatz des molekularbiologischen Nachweises lebensmittelpathogener Bakterien sollte beispielhaft mit *Listeria monocytogenes* durchgeführt werden. In einem ersten Schritt sollte ein bereits publizierter *L. monocytogenes* spezifischer konventioneller PCR-Assay erweitert werden. Ein passendes Sondendesign sollte entwickelt werden um den Assay in einer qPCR Anwendung verwenden zu können. In einem früheren EU-Projekt (FOOD-PCR I) wurde ein *L. monocytogenes* spezifischer konventioneller PCR-Assay entwickelt und getestet, welcher auf der Amplifikation eines 274 bp Fragmentes des *prfA* Gens von *L. monocytogenes* basiert (D'Agostino et al., 2004). Der Assay wurde in dieser Arbeit um eine FAM (6-carboxyfluorescein) markierte Taq-man Sonde und eine künstliche interne Amplifikations-Kontrolle erweitert und anschließend wurde dieser qPCR-Assay sorgfältig getestet um die speziellen Anforderungen der Lebensmitteldiagnostik zu erfüllen. Die genaue Ermittlung des Detektionslimits und der Spezifität des Assays waren dabei Schwerpunkte. Das hierfür verwendete Testverfahren basierte auf der Poisson Verteilung von einzelnen DNA Molekülen in hoch verdünnter wässriger Lösung (Wang and Spadoro, 1998). Der Anteil positiver und negativer Proben wurde nach erfolgter qPCR mit dem Erwartungswert entsprechend der Poisson Verteilung verglichen und bewertet. Es konnte für den getesteten qPCR-Assay ein Detektionslimit von einer DNA Kopie experimentell nachgewiesen werden. Die quantitative Auflösung des Assays in diesem Bereich wurde nicht getestet.

Basierend auf diesem qPCR-Assay wurde im folgenden eine kombinierte Anreicherungs/qPCR Methode entwickelt, welche einen Anreicherungsschritt in Fraser

Medium erfordert. Dies entspricht dem ersten Arbeitsschritt der Standardmethode zum Nachweis von *L. monocytogenes* (ISO 11290). Im Vergleich mit der Standardmethode konnte eine "relative accuracy" von 96%, eine „relative specifity" von 100% und eine „relative sensitivity" von 76.9% erreicht werden. Der letzte Wert wurde jedoch vom Zustand der Proben verzerrt, da diese zuvor über einen längeren Zeitraum (2 Jahre) bei -20°C gelagert worden waren (Rossmanith et al., 2010a). Siehe Kapitel 4.1.: Rossmanith et al. (2006).

Während der Entwicklung des kombinierten Anreicherungs/qPCR Protokolls wurden verschiedene DNA Isolations/Aufreinigungs-Methoden getestet, um ein Maximum an Ziel-DNA Ausbeute zu gewährleisten. Der kommerzielle NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) zeigte die beste Effizienz und wurde daher in das Protokoll übernommen. Siehe Kapitel 4.1.: Rossmanith et al. (2006).

Parallel dazu wurde die Möglichkeit der direkten quantitativen Analyse aus dem Lebensmittel ohne vorhergehende Anreicherung untersucht. Der Einfluss der Lebensmittelmatrix und der verwendeten Anreicherungsmedien auf die Effizienz der qPCR Reaktion wurde untersucht. In einem ersten Ansatz wurde der Einfluss verschiedener direkt in das PCR-Röhrchen eingebrachter Lebensmittelmatrices getestet. Dabei konnte bei einer Verdünnung von eins zu zehn außer bei Kot und Innereien (Muschel) keine signifikante Inhibition festgestellt werden (Resultate nicht präsentiert). Dieser direkte Ansatz wurde jedoch wieder aufgegeben, da die direkt einsetzbare Probenmenge (ca. 0.5 µl) im Vergleich zu den geforderten 25 g Lebensmittelmatrix zu gering ist. Im Gegensatz dazu erscheint die Zugabe von bakteriellen Zielzellen direkt aus der Anreicherung sinnvoll, da die Lebensmittelmatrix in diesem Fall keinen signifikanten Einfluss auf die Effizienz der Amplifikations-Reaktion hat. Um einen möglichen Einfluss der Anreicherungsmedien ausschließen zu können, wurden elf der gebräuchlichsten Anreicherungsmedien in Hinblick auf ihre Fluoreszenzeigenschaften getestet. Inhibitorische Effekte auf die PCR sind für Fraser Anreicherungsmedium in der

Literatur zu finden, dennoch wurde bei eins zu zehn Verdünnung der Anreicherungsmedien in der vorliegenden Studie kein solcher Effekt beobachtet (Daten nicht gezeigt). Die Fluoreszenzeigenschaften der Medien wurden mit den entsprechenden Wellenlängen des qPCR Thermocyclers getestet und für das *L. monocytogenes* Fraser-Selektiv-Medium konnte eine signifikante Fluoreszenz in den entsprechenden Wellenlängen der Farbstoffe FAM, TET (6-carboxy-2',4,7,7'-tetrachlorofluorescein) und HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein) beobachtet werden (492 nm, 517 nm und 535 nm). Die entsprechende Wahl des Sondenfarbstoffes ist daher wichtig, wenn *L. monocytogenes* direkt aus Anreicherungsmedien mittels qPCR nachgewiesen werden soll. Siehe Kapitel 4.2.: Rossmanith et al. (2010b).

Ein weiterer Schritt in Richtung zuverlässiger molekularbiologischer Nachweisverfahren für die Detektion lebensmittelpathogener Bakterien ist letztendliche die Berücksichtigung der Notwendigkeit einer analytischen Kette, bestehend aus Probenvorbereitung, DNA Isolation/Aufreinigung und qPCR Detektion.

Daher wurde eine neue Probenvorbereitungsmethode entwickelt, um die Nachteile bestehender Methodik zu vermeiden: Unzureichende Ausbeute, zu kleine Probenvolumina und fehlende Reproduzierbarkeit der Ergebnisse. Die Methode sollte bakterielle Zielkeime in möglichst reiner Form gewährleisten, um den Einfluss inhibitorischer Substanzen aus dem Lebensmittel auf den qPCR Nachweis ausschließen zu können. Des Weiteren sollte die Gesamtheit der in der Lebensmittelprobe enthaltenen bakteriellen Zielkeime in die DNA Isolation/Aufreinigung überführt werden. Bestehende Protokolle basieren auf der Trennung der Zielkeime von der weitestgehend unveränderten Lebensmittelmatrix. Bei der hier vorgestellten Methode wird im Gegensatz dazu die Lebensmittelmatrix in einem wäßrigen Lösungssystem aufgelöst und die Zielkeime anschließend mittels Zentrifugation abgetrennt. Ein Lysispuffer wurde enwickelt, welcher ein Detergens und eine chaotrope Substanz in einer

gepufferten Lösung beinhaltet. In der endgültigen Zusammensetzung mit 8 M Harnstoff, 1% Natrium-dodecyl-sulphat (SDS) und Phosphat gepufferter Saline (PBS) konnten 6 g oder 12 ml verschiedener Milchprodukte, Ei, Eiscreme, Fleisch, Fisch, Geflügel und Blut auf ≤ 200 µl Probenvolumen reduziert werden. Dies ist ausreichend um die Proben anschließend mit herkömmlichen kommerziellen DNA Isolations/Aufreinigungsmethoden prozessieren zu können (z.B. NucleoSpin® Tissue Kit). Es konnten aufgrund der harschen Inkubationsbedingungen, welche zur Lyse der Lebensmittelmatrix notwendig waren, in diesem ersten Entwicklungsschritt ausschließlich Gram positive Bakterien mit eingeschränkter Lebensfähigkeit extrahiert werden. Das gesamte Protokoll wurde mit dem NucleoSpin® Tissue Kit für die DNA Isolation/Aufreinigung und dem oben genannten *prfA* qPCR-Assay getestet. Das ermittelte Detektionslimit war 7.3 Kolonie-bildende Einheiten (KBE) *L. monocytogenes* pro ml und die durchschnittliche Wiederfindungsrate belief sich auf 29.9%. Siehe Kapitel 4.3.: Rossmanith et al. (2007).

Ein kritischer Punkt während der Entwicklung der neuen Probenvorbereitungsmethode war das Auffinden der präsumptiven Zielkeime. Da lebensmittelpathogene Bakterien farblos und daher in der Probe unsichtbar sind, war es notwendig sie während der Entwicklung durch farbige Indikatoren zu ersetzen. Die Untersuchung einer so großen Anzahl an zu testenden physikalischen und chemischen Parametern wäre in akzeptabler Zeit und mit den vorhandenen Ressourcen nicht möglich gewesen, hätte jede Probe einzeln mit molekularbiologischen oder mikrobiologischen Methoden auf den Verbleib der künstlich zugesetzten Zielkeime untersucht werden müssen. Daher wurden der chromogene Gram positive Bakterienstamm *Micrococcus roseus* und der Gram negative Stamm *Serratia marcescens* verwendet, welche zuvor auf die notwendige physikalische und chemische Ähnlichkeit mit den tatsächlichen Zielkeimen untersucht worden waren. Siehe Kapitel 4.4.: Rossmanith et al. (2010c).

2. Question

The underlying questions of this work are associated with the rising importance of fast and reliable detection methods for food pathogens. Additionally, these methods should offer quantitative value, as demanded by legal authority. Consequently, a promising approach is support of existing microbiological methods with molecular biological methods, particularly PCR. Several attempts to use conventional PCR for this task have been published in the past (Border et al., 1990; Bubert et al., 1999). Nevertheless, to obtain quantitative information from the results, extension to qPCR is necessary. Therefore, the first question of this work concerned the further development and replacement of an existing conventional PCR protocol with qPCR. Time can be saved through a first qualitative protocol by combining short enrichments with subsequent qPCR. This utilizes the advantages of a rapid method as well as the included confirmation reaction by probe hybridization in qPCR (see also introduction 3.2.).

In general, scientific questions that arise during the implementation of qPCR for food pathogen detection are diverse and result from the particular underlying principles of the enzymatic assay; the qPCR (Rossmanith and Wagner 2010a). Heterogeneous food samples and various possibly interfering chemical substances comprised in the selective enrichment media composition have to be observed. Interference of these various components with the underlying fluorescence based detection mechanism of qPCR must be taken into consideration. In this context DNA isolation/purification methods play a key role and must be tested thoroughly for application in food pathogen detection.

The resulting main question is the necessary basic design of reliable molecular biological food pathogen detection. If additional sample preparation is the prime solution to ensure the perfect environment for the enzymatic polymerase-chain-reaction then an analytical chain

consisting of sample preparation, DNA isolation/purification and subsequent qPCR detection is the resulting structure (Stevens and Jaykus 2004; Brehm-Stecher et al., 2009). Consequently, reliable sample preparation methods have to be developed as a first step, which, in their widest context, cover the heterogeneous field of food samples.

2. Fragestellung

Die Fragestellungen, welche dieser Arbeit zugrundeliegen, sind mit der steigenden Wichtigkeit von schnellen und zuverlässigen Nachweismethoden für lebensmittelpathogene Bakterien verknüpft. Diese Methoden müssen auch die Möglichkeit zum quantitativen Nachweis inkludieren. Dies ist wegen entsprechender gesetzlichen Vorschriften notwendig. Ein vielversprechender Ansatz ist die Ergänzung mikrobiologischer Verfahren durch molekularbiologische Methoden, vor allem PCR. Es wurden in der Vergangenheit einige Arbeiten veröffentlicht, welche konventionelle PCR für diese Aufgabe nutzen (Border et al., 1990; Bubert et al., 1999). Um jedoch quantitative Information zu erhalten ist die Weiterentwicklung hin zur qPCR notwendig.

Die erste Aufgabe dieser Arbeit war daher die Weiterentwicklung eines bestehenden konventionellen PCR-Assays zur Verwendung als qPCR-Assay. Zeitersparnis als notwendige Fragestellung kann in einem ersten Ansatz beantwortet werden, indem man den qPCR-Assay mit einer kurzen Anreicherung kombiniert. Dies nutzt außerdem den Vorteil der inkludierten Bestätigungsreaktion der qPCR-Methode, da diese den Hybridisierungsschritt mit einer zusätzlichen Bestätigungssequenz durch die mittels Fluoreszenz-Farbstoff markierte Sonde in der Amplifikationsreaktion inkludiert.

Die wissenschaftlichen Fragestellungen, welche sich während der Entwicklung und Einführung von quantitativen molekularbiologischen Nachweisverfahren stellen, sind vielfältig und resultieren aus den Eigenschaften der verwendeten Methodik; dem Enzym-Assay qPCR (Rossmanith and Wagner 2010a). Die Verschiedenartigkeit der unterschiedlichen Lebensmittelmatrices muß ebenso berücksichtigt werden wie der Einfluss der unterschiedlichsten chemischen Substanzen welche in den Anreicherungsmedien enthalten sind. Die mögliche Beeinflussung der qPCR-Methode durch Eigenfluoreszenz der

selektiven Anreicherungsmedien muss ebenfalls untersucht werden. In diesem Zusammenhang spielen DNA Isolations/Aufreinigungsverfahren eine große Rolle und müssen sorgfältig getestet werden um den speziellen Anforderungen der molekularen Detektion lebensmittelpathogener Bakterien zu entsprechen.

Die resultierende endgültige Fragestellung ist das grundlegende Design zuverlässiger molekularbiologischer Methodik im Bereich der Lebensmitteldiagnostik. Wenn Probenvorbereitung unmittelbar notwendig ist um das entsprechende Umfeld für die Durchführung der sensiblen Detektionsmethodik, des enzymatischen qPCR-Assays zu gewährleisten, dann resultiert daraus eine analytische Kette bestehend aus Probenvorbereitung, DNA Isolation/Aufreinigung und anschließender qPCR Detektion (Stevens and Jaykus 2004a; Brehm-Stecher et al., 2009). Daraus folgt, daß in einem ersten Schritt eine zuverlässige Probenvorbereitungsmethode entwickelt werden muss, welche nach Möglichkeit das gesamte heterogene Feld der verschiedenen Lebensmittelmatrices abdeckt.

3. Introduction

3.1. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive rod shaped bacterium with increasing relevance since the early eighties of the last century (Fig.1). In particular, its occurrence in food and the associated distribution of the related illness, listeriosis, has made this pathogen an important threat to health. Listeriosis is a great risk to pregnant women, the elderly and the immuno-compromised. Infection with *L. monocytogenes* may result in non-invasive listeriosis with febrile gastroenteritis, or in invasive listeriosis accompanied by meningitis, septicemia, primary bacteremia, endocarditis, non-meningitic central nervous system infection, conjunctivitis, and influenza-like illness. It may lead to preterm birth, miscarriage or severe health problems for the newborn. The illness is frequently fatal when compared with other food-borne infections (Anonymous, 2005a).

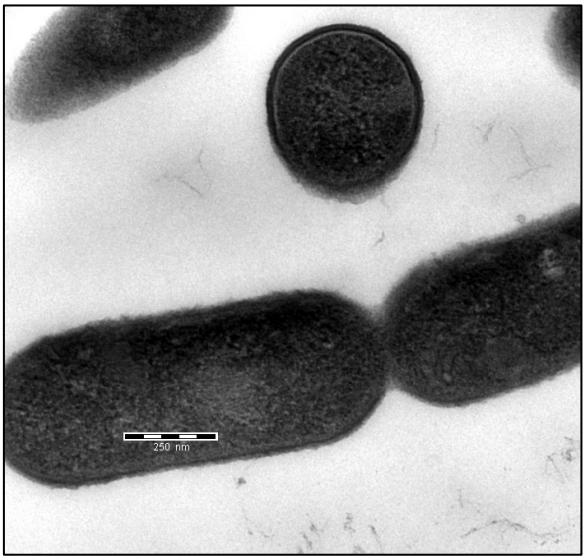

Figure 1. Transmission electron microscopic (TEM) picture of an overnight culture of *L. monocytogenes*.

In Austria the incidence of listeriosis in 2004 was 0.24 cases per 100 000 inhabitants and the mortality rate was 21%. (Anonymous 2005b). *L. monocytogenes* is a Gram-positive bacterium

with a robust cell wall. The size of its genome is approximately 3.0 Mbp, including the highly conserved *prfA* locus which regulates pathogenicity.

3.2. Food pathogen detection methods

Detection of food borne pathogens relies traditionally on microbiological methods. This also applies to *L. monocytogenes* and the associated official method protocols for European standards are collected in the ISO 11290-1 for qualitative investigation, and in ISO 11290-2 for quantitative purposes (Anonymous, 1996; Anonymous, 1998). These methods are based on selective enrichment with subsequent plating techniques and are thoroughly validated and reliable. The underlying selective Fraser broth is well established. However, the drawbacks of these microbiological methods are that they are time consuming in that several subsequent enrichment steps are included and that direct quantification of the pathogens is thereby difficult. Therefore rapid quantitative methods are required to meet the needs of modern food production and distribution.

Molecular biological methods, such as the polymerase chain reaction (PCR), theoretically offer the option of fast quantitative detection of bacterial pathogens from foodstuffs. PCR was the invention in molecular biology that made the greatest impact on methodology in the eighties and nineties of the last century. This now well-known method is based on the cyclic amplification of a given DNA sequence, the template, by use of the 5` to 3` polymerisation of a heat resistant DNA-dependent polymerase. Thus a microscopic amount of DNA is amplified so that it can be verified by a macroscopic detector. For conventional PCR this is usually performed by staining and separating the amplificate on an agarose gel. A further development of PCR, the so called real-time PCR or quantitative PCR (qPCR), utilises an additional feature, a fluorescence labelled probe which attaches to a sequence within the template and releases the fluorescent dye when a successful amplification occurs. This

fluorescent signal can be detected cycle by cycle during the PCR run and therefore it qualifies as a real-time procedure. Based on this cycle by cycle evaluation of the increasing fluorescent signal, the initial amount of template DNA can be determined by comparison with a given calibration function. Furthermore, qPCR is more sensitive than conventional PCR due to its shorter execution time, by omitting the so called elongation step which facilitates the safe polymerization of longer fragments in conventional PCR. The required shorter amplificates (< 400bp) in qPCR are reliably polymerized during the annealing step of the protocol. However, the main advantage of qPCR, which is especially useful in food pathogen detection, is the fact that a probe that binds within the target DNA sequence simultaneously includes the necessary confirmation reaction as demanded in diagnostics. In contrast, the conventional PCR approach additionally requires subsequent blotting and hybridisation steps. In summary, qPCR is a promising technique to improve food pathogen detection by means of reliable, time saving and quantitative methods.

3.3. Prerequisites for the implementation of molecular biological detection methods

The implementation of this task is nevertheless challenging as some prerequisites have to be met. Molecular biological methods are essentially derived from basic research efforts, where these methods have had a long history of applicability. Unfortunately the scientific questions addressed in that field do not extend to the requirements of food pathogen detection. For example, in basic research bacteria target numbers are invariably controllable by the researcher, which are simply dependent on the size of his overnight culture. In food pathogen detection the actual contamination of the foodstuff is not predictable, and in most cases the detection limit of the method has to be matched with “zero tolerance.”

Most of the molecular biological methods used are in *vitro* methods, which work perfectly in a standardised environment, which is practically under perfect laboratory conditions. Clearly

the requirements of these "borrowed" methods, developed for historical reasons, have to be re-adapted for applied use with food matrices, under far from perfect laboratory conditions. Thus the heterogeneous micro-environment of the food sample has to be matched to the requirements of highly sophisticated enzymatic assays, such as qPCR.

3.3.1. Characteristics of the food matrix: The major obstacle to overcome

The environment of food borne pathogens "the food" is a highly heterogeneous group from a chemical point of view. This topic is covered in various texts on food chemistry, technology and processing (Berlitz et al., 2009). Nevertheless, the classification of foodstuffs into food class, chemical composition and properties, structure and physical properties and grade of processing helps to appreciate the task of separating bacterial or viral targets from food matrices. It also offers good insight into the complexity of this challenge and how far removed it has become from basic in *vitro* applications.

- Traditionally food is classified as follows: dairy products, eggs, meat, fish and seafood, edible fat and oil, cereals and cereal products, pulses, vegetables, fruit, sugar and alcohol and honey, spices and coffee, vinegar and salt and water (Berlitz et al., 2009). This classification gives a good overview about the dimension of the field.
- The chemical composition and properties of food can be classified as follows: Water content; amino acids, peptides and proteins; lipids; carbohydrates; food additives and the pH.

  The variety of different chemical compounds and molecules in foodstuffs is comparable to that offered by organic chemistry itself. Fortunately most foodstuffs can be classified as above and these classes categorise foodstuffs according to their chemical content. The influence of the chemical composition of the food on the

performance of the following methods has to be taken into account. These could as well be biased due to remaining food additives or aberrant pH values.

- The structure and physical properties of food which are of importance are: Cell walls; tissue cohesion; solubility; viscosity; the state of aggregation and food can be existent as emulsion, solution and solid mixture.
- Physical properties of the food samples also have a great influence on detection methods. Pre-sample preparation methods, such as enzymatic or chemical treatment for solubilisation of the foodstuff, help to circumvent this problem.
- Food can be processed as follows: Raw; cooked (heat treatment); matured (cheese); fermented; smoked; salted; dried; irradiation and freezing.

  Food processing confers chemical or physical changes to a given foodstuff at a secondary level. The type and grade of processing plays a major role, as these different treatments affect the structure of the food matrices as well as their chemical composition. Additional characteristics have an impact on the performance of detection methods, regardless of the original composition of the food matrix. The following similar foodstuffs can demonstrate some different characteristics depending on the type of processing.

Therefore the requirements of molecular methods have to be observed regarding to the new application in food analysis and the resulting protocols have to be matched to the whole system starting from the heterogeneous food samples: The influence of chemical ingredients of the several food matrices and components of the test system on the performance of the enzymatic core reaction by means of possible inhibitory effects have to be investigated (i). The qPCR assay itself has to be carefully designed and thoroughly tested (ii). Several DNA isolation methods have to be tested for their performance to optimize the sensitivity of the method (iii).

3.4. Combined approaches

An alternative and a first step towards direct quantification of food borne pathogens is the combination of conventional enrichment based methods and molecular biological methods. After short-term (≤ 16 h) enrichment of the food sample in an appropriate selective enrichment medium, a PCR is performed, whether including an additional DNA isolation/purification step or not. In most cases few contaminating bacterial cells are obtained and these can then be enriched and subsequently detected and classified with PCR. Although several studies have been published previously presenting newly developed qPCR assays combined protocols for practical application had somehow been neglected until the start of this work (Hein et al., 2001; Hein et al., 2005; Piknova et al., 2005). Nguyen et al. (2004) presented in their work the rapid detection of *Escherichia coli* O157:H7 and *L. monocytogenes* from raw ground beef and beef hot dogs respectively. *E. coli* O157:H7 was detected after a 4 h enrichment step in modified trypton soy broth (TSB) and *L. monocytogenes* after 30 h in Fraser broth. The detection limit of the qPCR assays were 10^3-10^4 CFU/ml for *E. coli* O157:H7 and 10^3-10^4 CFU/ml for *L. monocytogenes*.

It is useful to replace existing protocols based on conventional PCR assays with combined approaches based on qPCR detection due to the above mentioned advantages. The combination of microbiological enrichment and molecular pathogen detection is useful and links these two fields. The advantages of both approaches add up simultaneously partly eliminating the disadvantages. The detection limit of the whole method is improved after successful enrichment. Short enrichments if performed in an appropriate way are providing enough DNA for qPCR detection and subsequent confirmation and classification of the pathogen is simultaneously performed during the detection step. Missing quantification due to the enrichment prior to qPCR is a disadvantage of combined approaches. Therefore these methods do provide only qualitative results. Nevertheless, these fast and comparatively

simple methods are an important contribution to implementation of molecular methods to the field of food pathogen detection. They support traditional microbiological testing and increase the confidence of the user in molecular methods.

3.5. Direct isolation of target DNA from food samples

Direct isolation of target DNA is based on the more-or-less selective lysis of either the bacterial cell wall, the core shell of the spore or viral envelope, without prior separation of target cells from the food matrix. Subsequent purification of the DNA from cell debris and food remnants is usually performed via affinity binding on silica gel or nitrocellulose. Overall, methods that do not separate the target cells from the rest of the sample protocols should be distinguished from methods that employ a series of steps from cell separation to DNA purification. Direct isolation of DNA is either limited by sample size or associated with impairment of subsequent PCR detection due to an enormous amount of background DNA that is usually present. Several isolation methods for DNA directly from foodstuffs using commercial DNA isolation kits are published. Pinzani *et al.*, (2004) extracted *Oenococcus oeni* DNA directly from 0.5 ml wine samples using the NucleoSpin® food kit and observed corresponding results of copy number and CFU as investigated with qPCR. Rudi *et al.*, (2005) used the DNAeasy® tissue kit, DNA DIRECT®, and the "Bugs and beads" kit to isolate DNA from 100µl volumes of Gouda cheese to detect directly *L. monocytogenes* with qPCR. They obtained a detection limit of 3.2×10^2 CFU per gram. Consequently, direct isolation of DNA is limited since the detection limit of these protocols is usually far from the contamination levels that can be expected in naturally contaminated samples. While pure DNA can be obtained, the method is cost intensive. In summary, this approach is restricted for basic research and should preferably not be used in routine diagnostics.

3.6. The analytical chain

The combined approaches (chapter 3.4.) and the direct application of qPCR (chapter 3.5.) to food pathogen detection both have serious disadvantages as discussed above. Subsequently it becomes clear that pathogen detection from foods must be a multi-step process: single-step applications, such as those offered by microbiological methods, e.g. enrichment of pathogens or plate counts, are impracticable if quantitative data are to be obtained. As discussed in chapter 3.3.1., most molecular methods were developed solely for *in vitro* applications, and their use for diagnostic purposes in food science, quasi *in vivo*, requires some adjustment. Core detection assays (qPCR) cannot be applied directly, but must be complimented with supporting methods. Therefore, qPCR, must be preceded by sample preparation steps, and followed by DNA isolation and purification methods, in order to establish a standard of purity suitable for reliable measurement. The entirety of these processes comprises a detection or analytical chain, which is defined by numerous elements connected in a logical sequence (Fig. 2.; from Rossmanith and Wagner, 2010a).

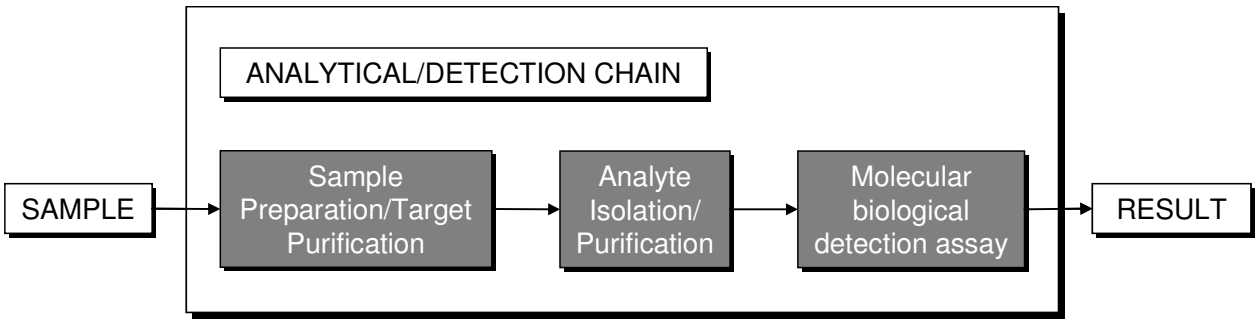

Figure 2. The analytical chain.

3.7. Sample preparation in food pathogen detection.

3.7.1. What should sample preparation be capable of?

In summary, sample preparation should meet the following essential requirements (Rossmanith and Wagner 2010b):

- Reduction of the sample size to volumes that can easily be handled in downstream steps, such as analyte isolation/purification from the target organism and molecular biological detection.
- Concentration of the target organism.
- First removal of possible inhibitory substances that affect subsequent molecular biological methods.
- Provide stable sample quality adequate for subsequent methods in the detection chain, independent of the original nature or composition of the respective food matrices.
- Provide maximum recovery (at least log scale accurate). Or more precisely, a maximum throughput rate with minimal loss of targets during the process.
- Provide linear recovery over the whole range of target concentrations down to low contamination levels, which is especially important for the subsequent use of quantitative methods.
- Provide a minimum detection limit. Or more precisely, no cut off against low concentrations.
- The integrity of the targets should be supported, by means of quantitative recovery of the analyte (e.g. DNA for qPCR). At best the viability of the targets should be conserved.
- Provide handling that supports reproducibility.
- Be cost effective, as this extra step results in some extra costs.
- Time-saving.

3.7.2. Sample preparation methods

Recently many different approaches to sample preparation for food pathogen detection have been described. A brief overview is given below based on the underlying physical and chemical processes. For more detailed information several reviews and book contributions have been published recently (Stevens and Jaykus, 2004; Brehm-Stecher et al., 2009, Rossmanith and Wagner 2010b, Rossmanith et al., 2010d).

3.7.2.1. Physical Separation Methods

- *Filtration and Dielectrophoresis/Ultrasound*

  The difference in particle size of target cells and food matrix is the basis for filtration as a separation method. Filtration often involves sieving, which is related to but still different from the physical separation method being rapid, simple and inexpensive. The recoveries of target cells demonstrated in recent publications using filtration for sample treatment vary from ten to one hundred percent (Stevens and Jaykus, 2004a). Still, solid foods are incompatible with this method as the filter easily clogs and therefore limits the amount of processable food samples (Bylund, 1995). Also, the method is likely to be influenced by bacterial targets absorbing to the filter or capturing of foodstuff particles. The major advantage of filtration is the near unlimited sample volume that can be processed.

- *Dielectrophoresis* separates targets from matrices by generating a high frequency electric field (0.1 – 10 MHz) in which targets move according to their charges. Given a minimum concentration of 10^7 CFU per millilitre 50% recovery from pure bacterial culture could be obtained. The question remains whether these targets are viable. Additionally, the method is not suitable for direct application to food due to numerous

chemical reactions in the samples themselves, e.g., oxidation and reductions at both electrodes (Hamann and Vielstich, 2005).

- *Sonication* of samples leads to concentration of particles in resonance nodes dependent on the wavelength and energy used. For this, 10^7 cells are the minimum amount necessary to achieve reasonable results and recoveries from 72% to 96% were reported for $>10^7$ cells (Limaye et al., 1998).
- *Centrifugation* is the method of choice for separating particles from liquid media. The underlying mechanism of sedimentation depends on particle density and diameter, viscosity of the liquid and the relative centrifugation force applied (Fliss et al., 1991). *Differential centrifugation* works by a stepwise increase of centrifugation speed. This rids the samples from heavier food particles at the beginning; the increased speed eventually leads to pelleting of the sample itself (Neiderhauser et al., 1992). Apart from the one-time costs for a suitable centrifuge the method is fast, easy and inexpensive. Also, detection limits of 10^3 to 10^4 CFU per millilitre were shown (Stevens and Jaykus, 2004a). Still, the adhesion of target cells to the food matrices and co-sedimentation of the targets with the food particles both lead to loss of targets and therefore false results.
- *Density gradient centrifugation* takes advantage of the targets seeking the equilibrate portion of the sample tube while continuously decreasing the density of the suspending solution during centrifugation. The constant change in the system as well as some food components, e.g., high fat contents, makes this method difficult to standardise (Stevens and Jaykus, 2004a). Therefore this method is currently limited to liquid samples (Lindqvist et al., 1997; Wolffs et al., 2004; Fukushima et al., 2007). Recovery rates varied between 11 and 45% with detection limits between 10^3 and 10^4 cells (Stevens and Jaykus, 2004a).

- *Adsorption in Liquid Systems and onto Solid Phases*

  Different adsorption effects like van der Waal's forces, electrostatic interactions, hydrophobic interactions and hydrogen bonding mediate nonspecific adsorptions to the surface of solid materials: metal hydroxides, ion exchange resins and lectins as well as targets are forced to matching liquid phases. These reversible interactions are also valid in immunological binding of epitope and antibody or viral binding respectively.

  A representative for liquid systems is the *aqueous two-phase system*. Recovery rates from 1% to 50% with a detection limit of 10^4 CFU per millilitre were reported (Lantz et al., 1994; Pedersen et al., 1998). Recoveries in both phases change with varying pH-values and polymers. Two-phase systems are also sensitive to temperature and chemical composition of samples; partitioning may be impaired by the presence of food components. The resulting difficulties in standardisation as well as high detection limits and heterogeneous results are major disadvantages of this method. This can only be partially compensated by the ability to process up to 4 g of sample volumes.

  Several nonspecific *solid phase adsorption* methods such as metal hydroxide coatings and ion exchange resins were shown to separate bacterial cells from sample matrices with varying recoveries of 9.5% to 99% (Berry and Siragusa, 1997; Lucore et al., 2000; Stevens and Jaykus, 2004a).

  Another rapid and simple method involves lectins, proteins binding the N-acetyl glucosamine residue of the bacterial cell wall. Lectins in turn can be bound to agarose beads and used in affinity columns or in conjunction with magnetic vehicles such as paramagnetic beads. Recoveries from 23% to 50% could be obtained (Payne et al., 1992; Stevens and Jaykus, 2004a), but the requirements of pre-sample treatment,

the lack of efficient release of bound cells and high costs make this method unfavourable.

3.7.2.2. Biochemical and Biological Separation Methods

The high affinity of antibodies and viral binding proteins for their targets can be exploited to separate bacterial target cells from food matrices. This brings the benefit of a pre-selection step, which concentrates the targets and separates them from their matrices at the same time. Other recently introduced methods make use of aptamers and antimicrobial peptides, both related to lectins in their nonspecific binding capacity. All in all, publications regarding affinity bindings and beads have risen over the past few years. Antibodies, viral binding proteins and antimicrobial peptides (AMP) from higher plants, such as magainin I, all bind to the surface of bacteria. They have been used in combination with magnetic beads by Amagliani et al. (2006), Nogva et al. (2000), Jung et al. (2003), Kretzer et al. (2007), Loessner et al. (2002), Hallier-Soulier and Guillot (1999), and Niederhauser et al. (1994), with silanised glass slides (Kulagina et al., 2005) and direct colony blot (Belyi et al., 1995). For enhanced recovery, nonspecific magnetic beads were combined with qPCR for specific target identification (Nogva et al., 2000).

- *Antibodies*

  Skjerve et al. (1990) were the first to report the separation of *L. monocytogenes* from culture media and heterogeneous suspensions by means of monoclonal antibodies. Jung et al. (2003) combined an immunogenetic separation method with flow cytometry and obtained recovery rates from 7% to 23% per ml sample of culture medium. In general, the detection limits of imunnoassay separation methods are 10^2 to 10^3 CFU per millilitre (Belyi et al., 1995; Nogva et al., 2000; Stevens and Jaykus, 2004a).

- *Viral binding proteins*

  Viral binding proteins in combination with paramagnetic beads were used for the separation of bacterial cells by Kretzer et al. (2007), but this was performed from culture media only. The detection limit of this approach is reported to be lower than for antibody-covered beads (< 10 CFU). Promising results for *L. monocytogenes* were achieved with listeriolysin O coated beads and the following phage endolysin-derived cell wall binding domain (CBD) based magnetic separation (Amagliani et al., 2006; Kretzer et al., 2007). Recovery rates were not determined but a detection limit of less than 10 CFU per millilitre for all artificially contaminated foods was established.

- *Aptamers and antimicrobial peptides (AMP)*

  So far these methods have been limited to pure cultures of bacteria or liquid matrices such as blood, skimmed milk or water and their application to solid foods remains problematic (Stevens and Jaykus, 2004a). Heterogeneous detection limits and recoveries, insufficient processed volumes and overall high costs are the common disadvantages of this category of methods.

3.7.2.3. Chemical and enzymatic digestion of the food matrix - The Latest Approach: Pre-Separation Methods for Sample Preparation

These methods are defined as pre-separation methods because in all cases digestion of the food matrix is followed by a physical separation method such as centrifugation.

- *Enzymatic Digestion of the Food Sample Matrix*

  Foods can be degraded by enzymatic digestion through specific cleavage of macromolecules. Only a few investigators have worked on enzymatic digestion methods for dairy products using pronase or an enzymatic system combining pronase with lysozyme and proteinase K (Wegmuller et al., 1993; Allmann et al., 1995). In one

case a detection limit of 50 CFU per gram following conventional PCR was published. Enzymatic digestion is a widespread molecular biological method but quickly reaches its limits in terms of sample numbers and volumes due to high costs of the enzymes. Only a few foodstuffs can be processed in this way as each class of chemical compound needs a matching enzyme to cut it. Moreover, most of the applications are time-consuming, as over-night incubation is usually necessary to achieve acceptable recoveries. But these approaches mark the first attempt of easing the separation process by removal of the food matrix.

3.7.2.3.1. Chemical Digestion of the Food Sample Matrix

Chemical digestion or solubilisation of the food matrices is executed by chemical cleavage and solubilisation of the foodstuffs in aqueous solutions. The reaction conditions have to be selected specifically, utilising the cell wall or other target-specific characteristics. To set up a protocol for such a heterogeneous group as foods is difficult as the reactions are influenced by pH-value, temperature, salt concentration, viscosity, or the main component of each foodstuff such as fat, protein or carbohydrates. Therefore, the food composition is more important for this sample preparation approach than in comparison to separation techniques which are not based on digestion of the sample matrix. Mechanisms which can be used to bring the foodstuff into aqueous solution are: Micelle formation with integration of water insoluble components within the micelle, chaotropic effects, change of pH, salting in, salting out, and heat.

- For the use of micelle formation detergents such as SDS can be used. The formation of micelles is based on the amphiphilic properties of the detergent molecules with lipophilic and hydrophobic components. The lipophilic compounds of the food matrix, such as fat, are then enclosed in the micelle, whereas the hydrophilic parts of the

molecules build a surface on the outside of the vesicle, which is in contact with the aqueous solution via hydrogen bonds. Due to their molecular structure detergents are the most relevant compounds if used in chemical solubilisation, in terms of negative effects on the integrity and viability of the target cells. The molecular structure of detergents is highly similar to the structure of the cell membrane of bacterial cells, therefore structurally affecting this membrane, which is crucial for the condition of the cell.

- Chaotropic substances such as urea change the surface tension of aqueous solutions, thus they also facilitate the solubilisation of various food compounds, but mainly the protein fraction.
- The role of the change of pH-value is not fully understood as yet, but again, proteins are mainly affected. The folding of the proteins is changed according to their iso-electric point and therefore hydrophilic properties of the molecule are brought to the surface, which then facilitate the solubilisation of these proteins.
- The refolding of proteins is also the mechanism of action of salting in/out. Salting in/out also helps to disrupt tissue coherence, such as releasing myofibrils from the muscle fibers.
- Heat helps to accelerate every chemical reaction and brings fat into a liquid state, which then accumulates in a liquid phase independent of the aqueous phase. This phase settles on top of the sample after centrifugation and can easily be discarded.

Few studies have been published using chemical reactions for reduction of sample size:

- First approaches of *chemical based sample preparation.*

  Choi and Hong (2003) developed a guanidine thiocyanate/phenol/chloroform extraction method for direct preparation of DNA from *L. monocytogenes, Y.*

enterocolitica and *Salmonella* subsp. *enterica* serovar Enteritidis. They were able to detect up to 10^3 CFU of *L. monocytogenes* per millilitre after conventional PCR (Choi and Hong, 2003). Another group used a diethylether/chloroform extraction method and performed an incubation step in 2 M urea and 1%SDS (Ramesh et al., 2002). Stevens and Jaykus (2004b) could reduce 11 g of plain non-fat yoghurt or cheddar cheese to a pellet of 500 µl by combining sodium citrate in a protocol with centrifugation and the DNAzol®BD reagent (Fig.2.). The resulting pellet size from the first step in sodium citrate was 1 to 5 g. Subsequently, the cheese was blended, filtered and the yoghurt strained through sterile cheesecloth. In this way a major amount of food matrix could be removed. Following centrifugation the bacterial recoveries varied from 53% to 143%. This data was obtained by directly plating the pellets of *L. monocytogenes* and *S.* Enteritidis from both foodstuffs. Depending on the food matrix and the bacterial species analysed, detection limits from 10^0 and 10^3 CFU per gram were established by conventional PCR and following Southern hybridisation.

3.7.2.3.2. Matrix Lysis.

Following from the previous statements one can differentiate two major approaches in sample preparation: separating the targets from the more or less unchanged food matrix or by removing the foodstuff by solubilisation with subsequent harvesting of the remaining targets. The impressive benefit of the latter approach is the simplification of the separation process, which can be easily performed by centrifugation. Centrifugation is the most promising method when it comes to recovery. If performed accurately in a liquid of low viscosity the theoretical recovery of 100% in a reasonable time is almost achievable in practice. Once the problem of accurate lysis of the foodstuff is solved, the resulting advantages of the food-solubilisation approach are obvious: simple handling steps, low costs, a maximum recovery,

unlimited sample volume, a maximum rate of target-concentration/volume-reduction, a minimum of interaction with the food matrix during separation, unspecific application for every target, which has a higher specific weight than the buffer solution and a broad range of applicable foodstuffs. Therefore this approach was chosen for the development of a new sample preparation method in this work.

4. Manuscripts

4.1. Rossmanith et al., 2006; Research in Microbiology, 157, 763-771.

Rossmanith, P., Krassnig, M., Wagner, M. and Hein, I.

Detection of *L. monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA* gene.

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Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA* gene

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Abstract

A combined enrichment/real-time PCR method for the detection of *Listeria monocytogenes* is presented. The method is based on a conventional PCR assay targeting the *prfA* gene, which has been validated and suggested as an international standard PCR method for identifying *L. monocytogenes* in food. This real-time PCR assay includes an internal amplification control. Inclusivity and exclusivity were 100% each when testing 100 *L. monocytogenes* isolates, 30 *Listeria* spp. isolates other than *L. monocytogenes*, and 29 non-*Listeria* isolates. The theoretical detection limit was one copy of the target gene per PCR reaction and the practical detection limit was about 5 copies per PCR. Using the combined enrichment/real-time PCR method, 7.5 CFU/25 ml of artificially contaminated raw milk, and 9, 1, and 1 CFU/15 g of artificially contaminated salmon, pâté, and green-veined cheese, respectively, were detected. When analyzing 76 naturally contaminated food samples of various types and comparing the results with the ISO 11290-1 standard method, the relative accuracy was 96%, the relative specificity 100%, and the relative sensitivity, 76.9%.

Keywords: *Listeria monocytogenes*; Real-time PCR; Food; Enrichment

1. Introduction

Facultative anaerobic Gram-positive *Listeria monocytogenes* is a food-borne pathogen widely distributed in nature. It is commonly found in decaying vegetation, soil and water. Various dairy products, meat products, and types of seafood have been reported to be contaminated with this pathogen and are implicated in sporadic as well as epidemic cases of listeriosis [5,11,15,16,24,33]. Individuals at greatest risk for listeriosis are pregnant women, the elderly and immunocompromised persons. Infection with *L. monocytogenes* may result in non-invasive listeriosis with febrile gastroenteritis, or invasive listeriosis with meningitis, septicemia, primary bacteremia, endocarditis, non-meningitic central nervous system infection, conjunctivitis, and influenza-like illness. In pregnant women, listeriosis may lead to preterm birth, miscarriage, or severe health problems for the newborn. The mortality rate is rather high when compared with other food-borne pathogens [7]. In Austria the incidence of listeriosis in 2004 was 0.24 cases per 100 000 inhabitants and the mortality rate, 21% [8].

As is the case for other bacterial food-borne pathogens, traditional methods for the detection of *L. monocytogenes* in food are time-consuming. The methods include enrichment in selective media, subsequent plating on agar plates, and various tests for species identification [2,6]. Rapid detection methods are required for microbiological quality control programs, which have to be applied throughout the food production chain. Various tests have been developed for this purpose. Many of these employ DNA-based PCR methods to detect the organisms of interest [28]. Recently, in an EU-funded project (FOOD-PCR I; http://www.pcr.dk; [25]) efforts were made to standardize the application of post-enrichment PCR in the field of food hygiene. One of the outcomes of this project was the proposition of well defined conventional PCR assays as standard methods for the detection of various food-borne pathogens, including *L. monocytogenes*. Compared to conventional PCR, real-time PCR involves a lower risk of cross-contamination because the presence of the target in the sample is indicated by an increase in fluorescence, and no post-PCR processing of the samples is

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required [17]. In addition, the fluorescent signal is proportional to the amount of target present in the sample and the method has the potential for automation.

The aims of the present investigation were as follows: (a) to adapt the above-mentioned validated conventional *L. monocytogenes*-specific PCR assay for real-time PCR, (b) to evaluate the performance of this assay (inclusivity, exclusivity, and detection limit) in view of subsequent validation according to ISO 16140 [4], and (c) to apply this assay to the detection of *L. monocytogenes* in artificially and naturally contaminated food of various types, and compare it with traditional microbiological methods.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used for inclusivity and exclusivity testing included 130 *Listeria* isolates (100 *L. monocytogenes*, 13 *L. innocua*, 3 *L. ivanovii*, 5 *L. seeligeri*, 6 *L. welshimeri*, 3 *L. grayi*) and 29 non-*Listeria* isolates (Table 1). *L. monocytogenes* isolates with the internal numbers 3220, 2729, 2782, and 2305 were used to artificially contaminate green-veined gorgonzola-like cheese, salmon, liver pâté (referred to as "pâté") and raw milk samples, respectively, and were originally isolated from these types of food. *L. monocytogenes* EGDe (internal number, 2964) was used to optimize real-time PCR and as the DNA standard. The bacteria were maintained at −80 °C using the MicroBank™ technology (Pro-Lab Diagnostics, Richmont Hill, Canada) and were part of the collection of bacterial strains at the Department of Veterinary Public Health and Food Science, University of Veterinary Medicine, Vienna, Austria. *Listeria* isolates were grown in tryptone soy broth with 0.6% yeast (TSB-Y; Oxoid, Hampshire, UK) at 37 °C overnight. All other bacteria were grown according to their individual requirements for 24 h prior to DNA isolation [32].

2.2. Artificially and naturally contaminated food samples

Food samples (salmon, pâté, and green-veined cheese) for artificial contamination were purchased in local supermarkets. Raw milk was taken from a dairy farm at the University of Veterinary Medicine, Vienna, Austria.

For comparison of different DNA isolation methods, 25 ml of raw milk was added to 225 ml half-Fraser medium (Oxoid) and inoculated with 2.5 ml of a 10-fold dilution series in Ringer's solution (Oxoid) of a pure culture of *L. monocytogenes*. The dilution series was prepared to contain 1–10, 10–100, and 100–1000 CFU/ml. The number of CFU for each step of the dilution series was obtained by the plate count method using tryptone soy agar with 0.6% yeast (TSA-Y; Oxoid). The inoculated samples were incubated for 24 h at 30 °C, and 1 ml of the enrichment was subjected to DNA isolation.

The best-performing DNA isolation method was then applied to artificially contaminated salmon, pâté, and green-veined cheese. In this case, 15 g of each sample was added to 135 ml half-Fraser medium (Oxoid) and homogenized in a Stomacher 400 laboratory blender (Seward, London, UK) for 2 min. One hundred microliters of a pure culture of *L. monocytogenes* was added to 9.9 ml of the homogenate. Preparation of the dilution series, calculation of the number of CFU in the dilution series, and incubation of the inoculated samples were performed as described above. *L. monocytogenes* in the enrichment were counted using the plate-count method and Palcam agar plates (Biokar, Beauvais, France). One milliliter of the enrichment was subjected to DNA isolation.

Food samples for artificial contamination were tested according to ISO 11290-1 to ensure they were *L. monocytogenes*-negative prior to inoculation [2,6]. In addition, real-time PCR targeting of the *prfA* gene of *L. monocytogenes* was performed as described below.

Naturally contaminated samples of different types of food, including fish, meat, meat products, and dairy products were obtained from a local laboratory for food analysis and stored at −80 °C for up to three months prior to analysis. In addition, some samples were purchased at local supermarkets and freshly processed. Nine cheese samples were obtained from the material used for routine diagnostic procedures performed at the Institute of Milk Hygiene, Milk Technology, and Food Science, University of Veterinary Medicine, Vienna, Austria. These samples were stored at 4 °C for one week prior to analysis. The naturally contaminated samples were subjected to microbiological analysis according to ISO 11290-1 [2,6] and real-time PCR targeting of the *prfA* gene of *L. monocytogenes*, as described below. Due to the limited quantity of available samples, the analyzed sample volume was reduced to 10 g. Therefore, 90 ml half-Fraser broth was added. For plating, Palcam (Biokar), Oxford (Biokar), and ALOA (Biolife, Milan, Italy) agar plates were used in parallel.

2.3. PCR for confirmation of suspected colonies on agar plates

For confirmation of *Listeria* species-suspected colonies on Palcam, Oxford or ALOA agar, all colonies were subjected to PCR after a short Chelex-based DNA isolation method as described below. Two PCR reactions were performed in a Perkin–Elmer 2400 thermocycler (Applied Biosystems, Foster City, CA, USA): one targeting the 16S rRNA gene specific for all *Listeria* spp. and the *hly* gene specific for *L. monocytogenes* [10], and the other targeting the *iap* gene of *Listeria* spp. yielding fragments typical of each species or group of species [12]. The 25-µl volume for the first PCR reaction contained 20 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1.1 µM of each primer L11, U1, LM1, and LM2, 200 µM (each) of dATP, dTTP, dGTP, and dCTP, 1.25 U of Platinum© *Taq* DNA polymerase (Invitrogen, Lofer, Austria), and 2 µl isolated DNA. Amplification following initial denaturation at 94 °C for 2 min was performed in 30 cycles, at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. A final extension was performed for 5 min at 72 °C. The 25 µl volume for the second PCR reaction contained 20 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 128 nM of each primer Siwi 2, Ino 2, Mono A, Murga I, and Lis 1B, 200 µM (each) of dATP, dTTP, dGTP, and dCTP, 1.5 U of Platinum© *Taq* DNA

Table 1
Listeria and non-*Listeria* isolates used for inclusivity and exclusivity testing of the *prfA*-specific real-time PCR assay

Genus and species	Serotyp	Origin	Internal number	Genus and species	Serotyp	Origin	Internal number
L. monocytogenes	1/2a	Cheese	6	*L. monocytogenes*	4b	Cheese production	180
L. monocytogenes	1/2a	Cheese	11	*L. monocytogenes*	4b	Cheese production	183
L. monocytogenes	1/2a	Cheese	20	*L. monocytogenes*	4b	Cheese production	191
L. monocytogenes	1/2a	Cheese	28	*L. monocytogenes*	4b	Cheese production	646
L. monocytogenes	1/2a	Cheese	49	*L. monocytogenes*	4b	Cheese production	704
L. monocytogenes	1/2a	Cheese	267	*L. monocytogenes*	4b	Cheese production	3517
L. monocytogenes	1/2a	Cheese	304	*L. monocytogenes*	4b	Paté	2129
L. monocytogenes	1/2a	Cheese	3296	*L. monocytogenes*	4b	Clinical	1515
L. monocytogenes	1/2a	Cheese production	208	*L. monocytogenes*	4b	Liquor (human)	1517
L. monocytogenes	1/2a	Cheese production	3299	*L. monocytogenes*	4b	Blood (human)	580
L. monocytogenes	1/2a	Cheese production	284	*L. monocytogenes*	4b	French outbreak	3078
L. monocytogenes	1/2a	Cheese production	316	*L. monocytogenes*	4c	ATCC 5579	2940
L. monocytogenes	1/2a	Sheep abortus	142	*L. monocytogenes*	4d	NCTC 10888	2939
L. monocytogenes	1/2a	Fish	715	*L. monocytogenes*	4d	Cheese production	3301
L. monocytogenes	1/2a	Turkey	3303	*L. monocytogenes*	4d	Sausage salad	3316
L. monocytogenes	1/2a	Meat	3317	*L. monocytogenes*	4d	u.k.	1447
L. monocytogenes	1/2a	Sausage	3344	*L. monocytogenes*	4e	ATCC 19118	2941
L. monocytogenes	1/2b	ATCC 7644	1468	*L. monocytogenes*	4e	Salad	260
L. monocytogenes	1/2b	Cheese	45	*L. monocytogenes*	7	NCTC 10890	2935
L. monocytogenes	1/2b	Cheese	47	*L. innocua*	4ab	NCTC 10528	2943
L. monocytogenes	1/2b	Cheese	79	*L. innocua*	6a	DSM 20649	2951
L. monocytogenes	1/2b	Cheese	103	*L. innocua*	6a	Cheese	18
L. monocytogenes	1/2b	Cheese	200	*L. innocua*	6a	Cheese	199
L. monocytogenes	1/2b	Cheese	202	*L. innocua*	6a	Cheese production	58
L. monocytogenes	1/2b	Cheese	222	*L. innocua*	6a	Cheese production	83
L. monocytogenes	1/2b	Cheese	297	*L. innocua*	6a	Cheese production	122
L. monocytogenes	1/2b	Cheese	308	*L. innocua*	6b	CIP 8012	TS86
L. monocytogenes	1/2b	Cheese	3298	*L. innocua*	6b	Cheese	2
L. monocytogenes	1/2b	Soft cheese	761	*L. innocua*	6b	Cheese	40
L. monocytogenes	1/2b	Cheese production	69	*L. innocua*	6b	Cheese	80
L. monocytogenes	1/2b	Cheese production	91	*L. innocua*	6b	Cheese	104
L. monocytogenes	1/2b	Cheese production	188	*L. innocua*	6b	Cheese	136
L. monocytogenes	1/2b	Cheese production	213	*L. ivanovii* subsp. *Londoniensis*		CCM 4698	TS122
L. monocytogenes	1/2b	Cheese production	229	*L. ivanovii*	–	Cheese	520
L. monocytogenes	1/2b	Cheese production	242	*L. ivanovii*	–	Cheese production	874
L. monocytogenes	1/2b	Cheese production	246	*L. seeligeri*	1/2b	DSM 20751	2953
L. monocytogenes	1/2b	Cheese production	274	*L. seeligeri*	6b	CIP 79.46	TS82
L. monocytogenes	1/2b	Cheese production	326	*L. seeligeri*	–	Cheese production	175
L. monocytogenes	1/2b	Cheese production	349	*L. seeligeri*	–	Cheese	784
L. monocytogenes	1/2b	Cheese production	353	*L. seeligeri*	–	Salad	1644
L. monocytogenes	1/2b	Cheese production	356	*L. welshimeri*	1/2b	DSM 20650	2950
L. monocytogenes	1/2b	Raw milk	2305	*L. welshimeri*		CIP 81.48	TS90
L. monocytogenes	1/2b	Fish	738	*L. welshimeri*	–	Cheese	382
L. monocytogenes	1/2b	Fish	748	*L. welshimeri*	–	Cheese	683
L. monocytogenes	1/2b	Fish	1424	*L. welshimeri*	–	Meat	3305
L. monocytogenes	1/2b	Fish	1603	*L. welshimeri*	–	Sausage	3334
L. monocytogenes	1/2b	Fish	3376	*L. grayi*	–	DSM 20596	1653
L. monocytogenes	1/2b	Fish	3378	*L. grayi*		DSM 20601	1654
L. monocytogenes	1/2b	Poultry	1715	*L. grayi*	–	Raw milk	783
L. monocytogenes	1/2b	Meat	3326	*Bacillus subtilis*	–	NCTC 10400	TS72
L. monocytogenes	1/2b	Meat	3360	*B. cereus*	–	NCTC 7464	TS355
L. monocytogenes	1/2b	Sausage	3347	*B. cereus*	–	NCTC 10460	TS67
L. monocytogenes	1/2b	Sausage	3374	*B. licheniformis*	–	CCM 2145	TS109
L. monocytogenes	1/2b	Salad	264	*B. pumilus*	–	CCM 2144	TS128
L. monocytogenes	1/2b	Sausage salad	3380	*B. lentus*	–	CCM 2214	TS123
L. monocytogenes	1/2b	Shrimps	171	*Brochotrix thermosphacta*	–	CCM 4769	TS333
L. monocytogenes	1/2b	Sheep abortus	141	*B. thermosphacta*	–	DSM 20171	TS465
L. monocytogenes	1/2b	Lymph node human	1742	*Citrobacter freundii*	–	CCM 4475	TS125
L. monocytogenes	1/2b	Blood human	570	*Enterococcus faecalis*	–	NCTC 775	TS62
L. monocytogenes	1/2c	ATCC 5577	TS199	*E. faecalis*	–	CCM 1875	TS108
L. monocytogenes	1/2c	CIP 58.33	TS96	*E. faecalis*	–	CCM 4224	TS120
L. monocytogenes	1/2c	NCTC 5348	470	*Escherichia coli*	–	NCTC 12900	TS54
L. monocytogenes	1/2c	Cheese	96	*Klebsiella pneumoniae*	–	ATCC 13883	TS388
L. monocytogenes	1/2c	Cheese production	65	*K. oxytoca*	–	DSM 5175	TS87
L. monocytogenes	1/2c	Cheese production	67	*Kurthia zopfii*	–	DSM 20580	TS103
L. monocytogenes	3a	NCTC 5105	2938	*K. gibsoni*	–	CCM 3321	TS331
L. monocytogenes	3a	Cheese	10	*Lactobacillus casei*	–	NCTC 10302	TS47
L. monocytogenes	3b	SLCC 2540	2937	*Paenibacillus polymyxa*	–	CCM 1459	TS130
L. monocytogenes	3b	Emmentaler cheese rind	301	*Proteus mirabilis*	–	NCTC 10975	TS5
L. monocytogenes	3c	CCM 5881	2936	*Rhodococcus equi*	–	NCTC 1621	TS9
L. monocytogenes	3c	Meat	3383	*Salmonella typhimurium*	–	DSM 554	TS43
L. monocytogenes	4a	NCTC 5214	472	*Shigella flexneri*	–	ATCC 12022	TS414
L. monocytogenes	4a	u.k.	1503	*Staphylococcus aureus*	–	NCTC 6571	TS10
L. monocytogenes	4b	NCTC 10527	2942	*S. warneri*	–	CCM 2730	TS111
L. monocytogenes	4b	NCTC 11994	TS203	*Streptococcus thermophilus*	–	DSM 20617	TS101
L. monocytogenes	4b	Cheese	94	*S. thermophilus*	–	CCM 4757	TS117
L. monocytogenes	4b	Cheese	197	*S. agalactiae*	–	ATCT7221	TS423
L. monocytogenes	4b	Cheese	221	*Yersinia enterocolitica*	–	NCTC 10460	TS67
L. monocytogenes	4b	Cheese	647				

u.k.: unknown.

polymerase (Invitrogen), and 2 µl isolated DNA. Amplification following initial denaturation at 94 °C for 2 min was performed in 30 cycles, at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. A final extension was performed for 5 min at 72 °C. PCR products were separated in 1.5% agarose gels at 90 V for 25 min and stained with 0.5 µg/ml ethidium bromide (Sigma-Aldrich GmbH, Steinheim, Germany). GeneRuler 100 bp (MBI Fermentas, St. Leon-Rot, Germany) was used as a standard.

2.4. DNA isolation

For inclusivity and exclusivity testing, one milliliter of a pure culture was pelleted at 5000 *g* for 5 min. The pellet was resuspended in 100 µl 0.01 M Tris–HCl (pH 7.0) and 400 µl lysis solution (0.25 mM Tris–HCl, pH 7.0; 2.5%, w/v, Chelex 100 resin (BioRad, Hercules, CA, USA)), and incubated for 10 min at 100 °C. The debris was pelleted at 14 000 *g* for 5 s. The DNA concentration in the supernatant was measured fluorimetrically using a Hoefer DyNA Quant200 apparatus (Pharmacia Biotech, San Francisco, CA, USA) and the DNA was diluted to 1 ng/µl in ddH_2O.

For confirmation of suspected colonies on agar plates, the colonies were suspended in 1 ml 0.01 M Tris–HCl and subjected to the same Chelex-based DNA isolation method as described above.

For DNA isolation from enriched food, the NucleoSpin© tissue kit and the support protocol for Gram-positive bacteria (Machery-Nagel, Düren, Germany; method A), the Nexttec® kit for genomic DNA from bacteria (Nexttec GmbH Biotechnologie, Leverkusen, Germany; method B), and previously published DNA isolation protocols were tested.

The DNA isolation protocol published by Allmann et al. [1] was applied with modifications [18] (method C). One milliliter of the enrichment broth was incubated with 130 µl digestion buffer (100 mM Tris–HCl, 100 mM EDTA, 0.5% sodium dodecyl sulfate, pH 8.0) and 200 µl Pronase solution (10 mg/ml ddH_2O; Roche Diagnostics GmbH, Mannheim, Germany) at 40 °C for 3 h. The sample was centrifuged for 15 min at 5700 *g* and 4 °C. The pellet was washed three times for 5 min at 5700 *g* and 4 °C with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5), followed by a final wash with PCR buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.4). Cell pellets were resuspended in 50 µl PCR buffer, and 5 µl lysozyme solution (10 mg/ml ddH_2O; Sigma-Aldrich GmbH) was added. The samples were incubated for 15 min at room temperature. Two µl of a proteinase K solution (10 mg/ml ddH_2O; Roche) was added and the samples were incubated for 1 h at 60 °C. The samples were then boiled for 15 min. After a final centrifugation step at 13 000 *g* for 5 min, 5 µl of the supernatant was used for real-time PCR reactions as template.

Using the DNA isolation method described by Longhi et al. [23] (method D), cells were collected at 6000 *g* for 5 min and washed twice in 1 ml 1× PBS (155.7 mM NaCl, 2.8 mM KCl, 2.97 mM Na_2HPO_4, and 1.06 mM KH_2PO_4, pH 7.4) for 5 min at 2000 *g*. Next, the pellets were resolved in 200 µl 1× PBS containing 0.05% Tween-20 (Sigma-Aldrich GmbH) and boiled for 12 min. After centrifugation at 14 000 *g* for 5 min, 5 µl of the supernatant was used for real-time PCR. Additionally, one modified protocol of this method was tested, including an ethanol precipitation step [31] after boiling (method E).

2.5. Targets and oligonucleotides for real-time PCR

The forward primer (LIP1: 5′-GATACAGAAACATCGGTTGGC-3′) and the reverse primer (LIP2: 5′-GTGTAATCTTGATGCCATCAGG-3′) amplify a 274 bp fragment of the *prfA* gene [14]. This conventional PCR assay was modified by the addition of a TaqMan™ probe in order to be used in a real-time PCR format. The probe was designed according to general guidelines [3]. Two different TaqMan™ probe formats with increased melting temperature were used. The LIP probe 2 (5′-FAM-CAGGATTAAAAGTTGACCGCA-MGB-3′) used an MGB modification while the LIP probe 3 used LNA modifications (5′-FAM-CAGGATTAAAAGTTGACCGCA-BHQ1-3′; LNA-modified bases are underlined). The LNA-modified probe was used on the Mx3000p thermocycler only (Stratagene, La Jolla, CA, USA).

An internal amplification control (IAC) was introduced using an artificial single-stranded DNA target based on the sequence of the pGL2-Basic Vector (Promega, Mannheim, Germany) with modifications in order to adjust the GC content of the fragment, and with the primer sequences LIP1 and LIP2 at the 5′ and 3′ ends, respectively (IAC: 5′-GAT ACA GAA ACA TCG GTT GGC GTA TTC GAA ATG TCC GTT CGG TTG GCG CTA TGA AGA GAT ACG CGG TGG AAC CTG GAA CCT GAT GGC ATC AAG ATT ACA C-3′). A probe for the IAC of the assay (Pluclm 4: 5′-HEX or TET-TTCGAAATGTCCGTTCGGTTGGC-BHQ1-3′) was designed according to the general guidelines mentioned above. Matching the FAM-labeled *prfA* specific probe, the probe for the internal control was HEX labeled for using the Stratagene Mx3000p real-time PCR thermocycler, and TET labeled for the application of the assay on the ABI PRISM™ 7900 Sequence Detection System real-time PCR thermocycler (Applied Biosystems, Foster City, CA, USA). The artificial 100 bp target for the IAC was synthesized by VBC Genomics (Vienna, Austria). Primers and probe Pluclm 4 were purchased at MWG Biotech (Ebersberg, Germany). The MGB-modified probe was purchased at Applied Biosystems and the LNA-modified probe at Proligo (Boulder, CO, USA).

2.6. Real-time PCR

Real-time PCR was performed in an Mx3000p real-time PCR thermocycler (Stratagene) and an ABI PRISM 7900 SDS real-time PCR thermocycler (Applied Biosystems). If not indicated otherwise in the results section, the Mx3000p and the LNA-modified probe were used for all experiments. The 25 µl volume contained 20 mM Tris–HCl, 50 mM KCl, 3.5 mM $MgCl_2$, 500 nM of each primer, 250 nM of each probe, 200 µM (each) of dATP, dTTP, dGTP, and dCTP, 1.5 U of Platinum© *Taq* DNA polymerase (Invitrogen, Lofer, Austria), 25 copies of the IAC and 5 µl isolated DNA. Amplification following initial

denaturation at 94 °C for 2 min was performed in 45 cycles, at 94 °C for 15 s, and 64 °C for 1 min.

To check for non-specific amplification during optimization of the assay, the PCR products were separated in 1.5% agarose gels as described above.

2.7. DNA standard for real-time PCR quantification

One milliliter of a pure culture of *L. monocytogenes* strain EGDe was subjected to DNA isolation using the NucleoSpin© tissue kit and the support protocol for Gram-positive bacteria. The DNA concentration was measured fluorimetrically using a Hoefer DyNA Quant200 device (Pharmacia Biotech). The copy number of the *prfA* gene was determined by assuming that, based on the molecular weight of the genome of *L. monocytogenes,* 1 ng of DNA equals 3.1×10^5 copies of the entire genome, and that the *prfA* gene is a single-copy gene [26]. The slope (s) of the standard curve was used for calculation of the PCR efficiency (E) with the following equation: $E = 10^{-1/s} - 1$ [21].

2.8. Statistical analysis

In order to determine the detection limit of the real-time PCR assay, the number of positive and negative results expected according to Poisson distribution assuming the ability to detect a single copy of the target molecule were compared to the observed numbers using the Chi-square test and the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). The expected percentage of negative results (P^0) as a function of the concentration of the target gene (C) was calculated according to the calculation of Wang and Spadoro (1998) [35]: $P^0 = 1/e^C$.

2.9. Definitions

The terms "inclusivity", "exclusivity", "relative accuray", "relative sensitivity" and "relative specificity" are used according to the ISO 16140 method for validation of alternative methods [4]. The term "detection limit" describes the lowest target copy number yielding a positive result if present in the real-time PCR sample tube, and the "practical detection limit" is calculated by taking into consideration the distribution probabilities of the target copies when present in low numbers [9,35].

3. Results

3.1. Design, optimization, and characterization of the L. monocytogenes-specific real-time PCR assay

A 274 bp fragment of the *prfA* gene was chosen as a target for detection of *L. monocytogenes* by real-time PCR. This gene encodes a regulator of virulence gene expression [30] and therefore represents an optimal target for the detection of *L. monocytogenes*. The primers amplifying this fragment of the gene were already evaluated, performed well and were suggested for inclusion in an international standard for the detection of *L. monocytogenes* using PCR [14]. Thus it was decided that this assay should be modified in order to be used in a real-time PCR format by adding a TaqMan® probe and including an internal amplification control suitable for duplex real-time PCR. The IAC consisted of an artificial 100 bp target, which was amplified with the same primers as the *prfA* target.

All experiments to optimize the performance of the assay on the DNA level were carried out using a 10-fold dilution series of genomic DNA in ddH_2O of *L. monocytogenes* from 1.55×10^1 to 1.55×10^5 copies of the genome/PCR as a standard. Concentrations of primers, probes, and $MgCl_2$ were optimized to the amounts cited in Section 2. Annealing time and temperature proved to be crucial parameters, as the assay tended to yield non-specific PCR products when used at temperatures below 61 °C and annealing/extension times above 60 s. The best performance was achieved using an annealing/extension step at 64 °C for 1 min. The performance of the assay was compared on the Mx3000p and ABI 7900 real-time PCR thermocycler using LIP probe 2. PCR efficiencies were similar on both real-time PCR thermocyclers (Mx3000p: 96.2%; ABI 7900: 94.2%), and all steps of the dilution series of the DNA could be detected. On the Mx3000p, both the LIP probe 2 and the LIP probe 3 were tested, and yielded comparable results: the PCR efficiency of the assay was 95.7% when using the LNA-modified probe, compared to 96.2% when using the MGB-labeled probe within one amplification batch. The optimal copy number of the artificial target of the internal amplification control per PCR reaction was determined to be 25, as this quantity permitted amplification of the IAC while avoiding interference with the main reaction (Fig. 1).

Poisson analysis was used to investigate the detection limit of the assay [35]. Poisson statistics can be used to calculate the number of negative results when performing repeated analysis of samples containing small numbers of target. According to the Poisson distribution, 0.04, 5, and 37% of replicate measurements should contain no target molecule if the average concentration of the target molecules per PCR is 10, 3, and 1, respectively. Thirty replicates containing an average of each 10, 3, and 1 target molecules/PCR were analyzed, and 0, 1, and 9.9 negative results were expected. The expected and the achieved results yielded no statistically significant differences, regardless of whether the ABI 7900 thermocycler (assay without IAC: 0, 1, and 11 negative results ($P = 0.991$)) or the Mx3000p [assay without IAC: 0, 3, and 9 negative results ($P = 0.957$); assay including IAC: 0, 1, and 7 negative results ($P = 0.93$)) was used.

Inclusivity and exclusivity of the primers LIP1 and LIP2 have been validated recently [14]. As the TaqMan® probe had been introduced additionally, the assay was tested again using 100 *L. monocytogenes* strains, 30 other *Listeria* isolates (*L. grayi*, *L. welshimeri*, *L. innocua*, *L. ivanovii*, and *L. seeligeri*) and 29 non-*Listeria* isolates (Table 1). Both inclusivity and exclusivity were 100%. Ct values of *L. monocytogenes* isolates averaged 19.23 (standard deviation: 0.88), indicating similar real-time PCR amplification and detection of all isolates tested.

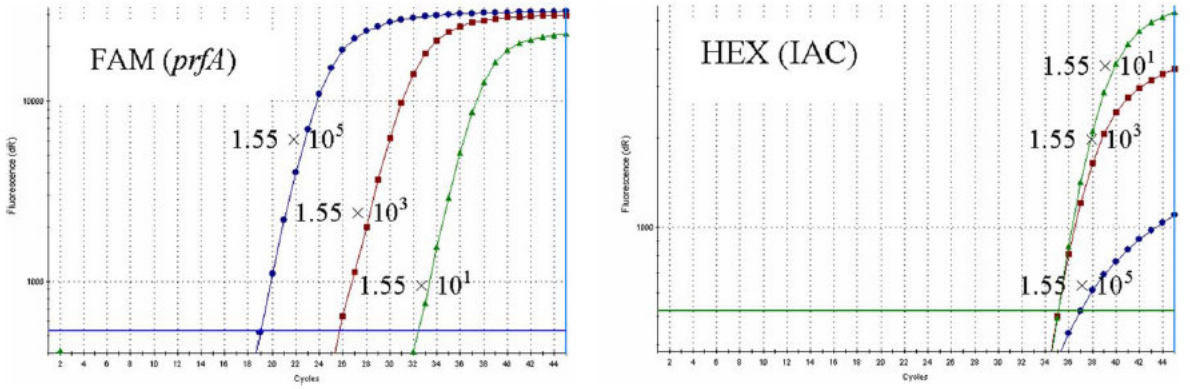

Fig. 1. Real-time PCR amplification of the *prfA* target and the IAC within one reaction. Each reaction contained 25 molecules of IAC target plus 1.55×10^5 (◆), 1.55×10^3 (■), or 1.55×10^1 (▲) copies of the *prfA* target.

Table 2
Comparison of different DNA isolation methods for real-time PCR detection of *L. monocytogenes* in raw milk after enrichment in half-Fraser broth

Pre-enrichment (CFU/25 ml raw milk)	Replicate[a]	Copies of the *prfA* gene/PCR (average percentage of recovery[g])									
		Method A[b]		Method B[c]		Method C[d]		Method D[e]		Method E[f]	
7.5×10^0	A	5.9×10^2	(100.0%)	3.5×10^2	(78.8%)	Negative	(0.0%)	2.8×10^1	(7.4%)	2.6×10^1	(5.4%)
7.5×10^0	B	3.0×10^2		3.4×10^2		Negative		3.8×10^1		2.2×10^1	
4.3×10^1	A	9.2×10^3	(100.0%)	5.7×10^3	(93.5%)	5.0×10^0	(0.0%)	7.4×10^2	(8.1%)	2.8×10^1	(15.5%)
4.3×10^1	B	5.6×10^3		8.2×10^3		1.0×10^0		4.8×10^2		2.3×10^3	
5.9×10^2	A	8.0×10^4	(48.8%)	1.9×10^5	(100.0%)	2.0×10^2	(0.1%)	4.2×10^4	(21.8%)	3.5×10^2	(0.3%)
5.9×10^2	B	6.0×10^4		8.3×10^4		1.1×10^2		1.8×10^4		Negative	

[a] Enrichment was performed in duplicates.
[b] NucleoSpin© Tissue kit.
[c] Nexttec© kit for genomic DNA from bacteria.
[d] Boiling after treatment with pronase, lysozyme, and proteinase K [1].
[e] Boiling of the bacterial pellet in 0.05% Tween 20 and 1× PBS [23].
[f] Method D, including ethanol precipitation.
[g] Average percentage of recovery was calculated from results of replicate A and B.

3.2. *Performance of different DNA isolation methods from enriched food*

Five different DNA isolation methods were compared for their ability to produce amplifiable template DNA for *L. monocytogenes*-specific real-time PCR: two commercially available DNA isolation kits, the NucleoSpin© Tissue kit (method A) and the Nexttec© kit for genomic DNA from bacteria (method B), two previously published DNA isolation methods according to Allmann et al. [1] (boiling after treatment with pronase, lysozyme, and proteinase K; method C) and Longhi et al. [23] (boiling of the bacterial pellet in 0.05% Tween 20 and 1× PBS; method D), and one modified isolation method according to Longhi et al. [23], including ethanol precipitation to remove PCR inhibitors (method E).

Artificially contaminated raw milk was enriched in half-Fraser medium. One milliliter was subjected to DNA isolation and analyzed by real-time PCR. Recovery rates were calculated in relation to the method performing best (Table 2). Method A showed the best performance at low concentrations, whereas method B performed best at high concentrations. The recovery rate of method D allowed reliable detection of all contamination levels, although yielding approximately one tenth the amount of DNA compared to methods A and B. Methods C and E did not perform well. The IAC was positive in all negative samples, indicating the absence of PCR inhibitors in the samples. PCR reactions were repeated using a tenfold dilution of the DNA isolations as a template to check for inhibitory effects in positive samples. The detected copy numbers of these PCR reactions were in accordance with those using undiluted DNA, indicating no inhibitory effects within the PCR reactions (data not shown).

The influence of food matrices other than raw milk on DNA isolation method D was examined using artificially contaminated samples of salmon, pâté, and green-veined cheese. After enrichment in half-Fraser broth, real-time PCR detection of *L. monocytogenes* was possible at all levels of contamination and in all food matrices tested (Table 3). The effect of different growth rates of *L. monocytogenes* assigned to the varying food matrices was evident. Salmon-derived enrichment cultures showed growth up to $>10^7$ CFU/ml after incubation, even at the lowest inoculation level, whereas inoculation of one CFU

Table 3
Real-time PCR detection of *L. monocytogenes* in enriched salmon, pâté, and gorgonzola-style cheese after DNA isolation using method D

Food type	Pre-enrichment (CFU/15 g)	Post-enrichment (CFU/ml)	Real-time PCR
Salmon	9.0×10^0	4.0×10^8	+
	1.1×10^2	5.0×10^7	+
	8.0×10^2	4.8×10^7	+
Pâté	1.0×10^0	5.6×10^4	+
	5.2×10^1	1.9×10^5	+
	6.2×10^2	1.3×10^6	+
Green-veined Cheese	1.0×10^0	1.8×10^3	+
	5.8×10^1	6.5×10^4	+
	5.2×10^2	3.2×10^5	+

into pâté and green-veined cheese generated cultures of $<10^5$ and $<10^4$ CFU/ml enrichment, respectively. However, it was sufficient for real-time PCR detection.

Isolation method D was subsequently used to analyze naturally contaminated samples, as it permitted detection at all contamination levels tested and was fast, easy to perform, and economical.

3.3. Analysis of naturally contaminated samples

Seventy-six food samples of different types were tested for the presence of *L. monocytogenes* using the combined enrichment/real-time PCR method and microbiological analysis according to ISO 11290-1 (Table 4). Comparison of the results of real-time PCR and the microbiological method showed concordant results for the samples stored at 4 °C and the fresh samples, whereas three of 42 frozen samples yielded positive results when processed according to ISO 11290-1 and negative results with real-time PCR. Thus, the relative accuracy of the combined enrichment/real-time PCR method was 96.0%, the relative specificity was 100.0%, and the relative sensitivity was 76.9%.

4. Discussion

Recently, an EU-funded project (FOOD-PCR I) to standardize the detection of foodborne pathogens with PCR-based methods, as faster alternatives to traditional microbiological methods, was completed (http://www.pcr.dk; [25]). One of the outcomes of this project was the proposition of well defined conventional PCR assays as standard methods for the detection of various food borne pathogens like *L. monocytogenes*. Given the advantages of real-time PCR, such as less time for analysis, the reduced risk of contamination, and the possibility to quantify the target and operate the procedure in an automatic mode, it was decided to adapt the previously evaluated conventional *L. monocytogenes*-specific PCR assay for real-time PCR.

Due to the AT-rich consensus sequence of the amplicon, both minor-groove-binder (MGB) labeled and locked-nucleic-acid (LNA) modified TaqMan™ probes were used. Otherwise the

Table 4
Detection of *L. monocytogenes* in naturally contaminated samples according to ISO 11290-1 and with the combined enrichment/real-time PCR method

Food type	Internal number	Storage	Microbiological analysis	Real-time PCR
Salmon	1	−80 °C	–	–
Semolina dumpling	2	−80 °C	–	–
Salmon	3	−80 °C	–	–
Salmon	4	−80 °C	+	
Salmon	5	−80 °C	+	+
Salmon	6	−80 °C	–	–
Noodle salad	7	−80 °C	–	–
Semolina dumpling	8	−80 °C	–	–
Salmon	9	−80 °C	–	–
Salmon	10	−80 °C	–	–
Raw sausage	11	−80 °C	–	–
Prawn	12	−80 °C	–	–
Liver loaf	13	−80 °C	–	–
Ham	14	−80 °C	–	–
Salmon	15	−80 °C	–	–
Salmon	16	−80 °C	–	–
Cooked ham	17	−80 °C	+	+
Strudel	18	−80 °C	–	–
Bacon	19	−80 °C	+	+
Salmon	20	−80 °C	–	–
Salmon	21	−80 °C	+	–
Salmon	22	−80 °C	–	–
Salmon	23	−80 °C	–	–
Minced meat	24	−80 °C	–	–
Poultry	25	−80 °C	–	–
Poultry	26	−80 °C	+	+
Poultry	27	−80 °C	–	–
Pâté	28	−80 °C	–	–
Salmon	29	−80 °C	+	–
Fish	30	−80 °C	+	+
Pizza dough	31	−80 °C	–	–
Salmon	32	−80 °C	–	–
Fish	33	−80 °C	–	–
Sausage	34	−80 °C	–	–
Frying sausage	35	−80 °C	+	+
Frying sausage	36	−80 °C	–	–
Cooked ham	37	−80 °C	–	–
Salad	38	−80 °C	–	–
Salmon	39	−80 °C	–	–
Salmon	40	−80 °C	–	–
Salmon	41	−80 °C	–	–
Salmon	42	−80 °C	–	–
Hard cheese	43	4 °C	+	+
Hard cheese	44	4 °C	+	+
Hard cheese	45	4 °C	+	+
Hard cheese	46	4 °C	+	+
Hard cheese	47	4 °C	–	–
Hard cheese	48	4 °C	–	–
Hard cheese	49	4 °C	–	–
Hard cheese	50	4 °C	–	–
Hard cheese	51	4 °C	–	–
Salmon	52	No storage	–	–
Salmon	53	No storage	–	–
Salmon	54	No storage	–	–
Blue-veined cheese (raw milk)	55	No storage	–	–
Brie	56	No storage	–	–
Brie	57	No storage	–	–
Brie	58	No storage	–	–
Soft cheese	59	No storage	–	–
Soft cheese	60	No storage	–	–
Blue-veined cheese	61	No storage	–	–
Pâté	62	No storage	–	–
Pâté	63	No storage	–	–
Pâté	64	No storage	–	–
Pâté	65	No storage	–	–
Salmon	66	No storage	–	–
Blue-veined cheese	67	No storage	–	–
Pâté	68	No storage	–	–
Pâté	69	No storage	–	–
Fish	70	No storage	–	–
Salmon	71	No storage	–	–
Pâté	72	No storage	–	–
Pâté	73	No storage	–	–
Blue-veined cheese	74	No storage	–	–
Blue-veined cheese (raw milk)	75	No storage	–	–
Blue-veined cheese	76	No storage	–	–

probe would have been too long which would have reduced the efficiency of the real-time PCR assay [3]. The MGB-labeled probe performed equally well on both real-time PCR platforms tested. Moreover, there was no difference between the performances of the two probes on the Mx3000p. These results confirm those of other authors who compared the two types of TaqMan probes [22]. As the LNA-modified probe was the cheaper alternative, it was used for the experiments with artificially and naturally contaminated samples. Inclusivity and exclusivity of the real-time PCR assay were 100%, thus proving that the addition of the probe did not alter these values [14]. Applying Poisson statistics, a theoretical detection limit of one copy of the target gene per PCR reaction was observed, irrespective of the real-time PCR thermocycler used and whether there was IAC present in the PCR reaction or not. Thus, the practical detection limit is about 5 copies of the target gene per PCR reaction, depending on the number of replicate analyses performed and the likelihood of missing a positive result tolerated. In this case, 0.67% of the analyzed replicate sample volumes would contain no target [35]. The detection limit analyzed in the present work using purified genomic DNA cannot be directly compared to that achieved recently with the conventional version of the PCR assay. In the latter setting, a defined number of cells were directly added to the PCR reaction, combining the effects of the efficiency of DNA release from the cells during PCR, and PCR efficiency. Three to 10 cells were required to achieve a positive result [14].

As the assay performed satisfactorily on the genomic DNA level, it was applied to artificially and naturally contaminated food. First, different DNA isolation methods were compared in order to find an appropriate pre-PCR processing strategy of the samples using raw milk as a model food matrix. Both commercial kits tested included a column-based purification step of the DNA and yielded the largest quantities of DNA for PCR amplification. The DNA isolation method according to Allmann et al. [1] employing boiling after treatment with pronase, lysozyme, and proteinase K did not work well, although the method was suitable for direct isolation of bacterial DNA from raw milk and cheese for PCR and real-time PCR, as shown previously [1,18,19]. The best non-commercial method includes boiling of the bacterial pellet in the presence of Tween-20. This method is fast and easy to perform, and was therefore chosen for further analysis. It was expected to yield sufficient quantities of DNA for post-enrichment PCR detection. The addition of an ethanol-based DNA precipitation step did not improve the method, probably due to loss of DNA during this step. The method worked equally well for all food matrices tested, and allowed the detection of 7.5 CFU/25 ml artificially contaminated raw milk, and 9, 1, and 1 CFU/15 g artificially contaminated salmon, pâté, and green-veined cheese, respectively. Other authors have reported post-enrichment-based real-time PCR detection of 5 CFU of *L. monocytogenes* per 25 g or ml of dairy products, 3 CFU/g of sausage, 1 CFU/10 ml of milk and water, and 1.2–6 CFU/g beef hotdogs using different targets and/or enrichment broths compared to those used in the present investigation [13,20,27,34]. Thus, the real-time PCR method described here performs equally as well as recently published methods while offering the following additional benefits: (a) it permits the use of primers that have been suggested for use in an international standard PCR detection method for *L. monocytogenes*, and (b) the type of enrichment broth is the same as that used for ISO 11290-1, thus allowing further analysis of PCR-positive samples according to this standard.

As the method might perform differently when analyzing artificially and naturally contaminated samples [29], 76 field samples of various types of food were investigated in parallel according to ISO 11290-1 and with the combined enrichment/real-time PCR method. The relative accuracy was 96% and the relative specificity, 100%. The relative sensitivity was 76.9% due to 3 false-negative PCR results. As the IAC was positive in these samples, the presence of PCR inhibitors was not the reason for these results. Probably the enrichment was not sufficient to produce the number of *L. monocytogenes* cells required to yield a positive real-time PCR result for these samples. In comparison, no artificially contaminated sample contained less than 10^3 CFU/ml enrichment. DNA isolation using the more efficient commercial kits might improve the relative sensitivity of the method. Other authors used the conventional version of this PCR assay with a relative specificity of 81.8% in artificially contaminated raw milk; these data were collected in the course of a collaborative trial. The relative sensitivity depended on the initial contamination of the samples and ranged from 42.4% for low-level to 93.9% for high-level contamination [14]. As we did not perform quantitative analysis of the samples, no direct comparison of the results is possible. However, the fact that we encountered no false-positive results confirmed the reduced risk of cross-contamination when using real-time PCR. Cox et al. [13] examined different dairy products with a combined enrichment/real-time PCR method using another target gene, a different enrichment broth, and artificially contaminated samples, registering a relative accuracy of 96.2%, a relative specificity of 96.7%, and a relative sensitivity of 95.2%. In comparison, the number of positive field samples in the present study was limited, which could lead to a bias in the reported relative sensitivity.

In summary, the combined enrichment/real-time PCR method described above, allowing fast and sensitive detection of *L. monocytogenes* in various food matrices, could serve as a rapid screening method and has the potential for operation in an automatic mode. The procedure employs the same enrichment medium as that used for ISO 11290-1, thus facilitating its integration in routine laboratory diagnostics. The real-time PCR assay is based on a previously evaluated conventional PCR assay suggested as a standard PCR detection method. Further work should focus on performance testing in a collaborative trial setting, including replicate analysis of samples and statistical analysis according to the ISO 16140 method for validation of alternative methods [4].

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The Fluorescence Characteristics of Enrichment Media in the Wavelength Range of Real-Time PCR Thermocycler Optical Path Assignments

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Abstract The aim of this study was to investigate the fluorescence characteristics of common enrichment media with regard to disturbance of fluorescence readings of real-time polymerase chain reaction (PCR) reactions. Confirmation of effective amplification of target DNA during the real-time PCR process depends on measurement of the fluorescence emitted from the probe fluorophore used. Possible background fluorescence emitted from enrichment media, when applied directly to the sample, could lead to interference with this measurement. This inhibition of the detection process leads to false negative results, even although amplification of the target DNA was successful. Among the 11 enrichment media investigated, half-Fraser broth displayed significant fluorescent emission if excited in the wavelengths 492, 517 and 535 nm, according to the FAM, TET and HEX filter sets of the real-time PCR thermocycler. A shift to the excitation wavelengths of 585 (ROX) and 635 nm (CY-5) leads to no measureable emission caused by half-Fraser broth. A further investigation revealed that Fraser supplements containing acriflavine and nalidixic acid show strong emission if exited with the tested wavelengths. The related molecular structures of acriflavine and fluorescein, the basic molecular structures of FAM, HEX and TET, implicate similarity of the fluorescent properties of these molecules. In summary, consideration of the fluorescence characteristics of the applied enrichment media is important if subsequent direct quantification of food borne pathogens with real-time PCR from enrichment media is anticipated. This requirement was demonstrated as Fraser supplements contained in half-Fraser broth did raise the background fluorescence substantially.

Keywords Enrichment Media · Fluorescence · Real-Time PCR · Direct Detection · Inhibition

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Introduction

The application of polymerase chain reaction (PCR) as a qualitative diagnostic tool for detection of microorganisms in complex biological, environmental and food samples is now definitely established. When it comes to routine application of real-time PCR as a quantitative tool in analysis of samples containing a complex matrix, some problems may occur (Stevens and Jaykus 2004; Rådström et al. 2004). Direct detection of target DNA from such samples requires pre-PCR sample preparation steps (Stevens and Jaykus 2004; Rådström et al. 2004). These steps are often laborious and expensive (Rådström et al. 2004). The combination of enrichment procedures by culture followed by real-time PCR provides some advantages as recently published (Fricker et al. 2007; O'Grady et al. 2009). The use of enrichment media with subsequent PCR detection without purification of the target DNA was, amongst others, reported for *Salmonella* spp. from animal feed samples (Löfström et al. 2004) and for *Salmonella enterica* from poultry (Kanki et al. 2009). However, some disadvantages are also associated with direct application of enrichment media in real-time PCR

samples (Knutsson et al. 2002). In particular, inhibitory effects of the enrichment media or its ingredients that interfere with PCR have been reported (Rossen et al. 1992; Wilson 1997). These factors have been related to chemical or physical properties of the amplification reaction.

In carrying out real-time PCR, confirmation of a positive reaction is performed by the measurement of increasing fluorescence of a reporter dye released proportionally to the amplification of the target DNA. Real-time PCR thermocyclers offer a broad range of wavelengths for detection measurement. These wavelengths are linked to the characteristics of the dyes which are used and which are selected for operational reasons to achieve optimal performance in terms of real-time PCR.

Proposed fluorescent qualities of enrichment media could serve as a possible source of inhibitory effects, interfering with the fluorescence measurement of real-time PCR thermocyclers (Table 1). The aim of this work was to investigate the fluorescent characteristics of nine enrichment media with regard to their use in direct quantification with real-time PCR (Table 1).

Materials and Methods

Enrichment Media

Fraser (FR) broth base for half-Fraser (HFR) broth, Bolton broth, buffered peptone water and Rappaport–Vassiliadis broth were purchased from Merck (Darmstadt, Germany). Müller–Kaufmann tetrathionate broth, brilliant green bile lactose broth, tryptone soya broth with yeast, Luria-Bertani broth and brain heart infusion broth were purchased from Oxoid (Hampshire, UK). The Fraser broth supplements were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) and Merck. Preston broth and the supplements for Bolton broth, including horse blood, were purchased from Oxoid. All enrichment media were prepared according to the manufacturers' instructions and stored at 4 °C.

Spectroscopic Data

The spectroscopic characteristics of the enrichment broths investigated were analysed using a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan). The respective excitation and emission wavelengths of the fluorescent dyes FAM, HEX, TET, CY5 and ROX (see Table 2 for abbreviations), which are mainly used in real-time PCR, were applied to a tenfold dilution of the enrichment broths (Table 1). The appropriate wavelengths of excitation were chosen according to the Stratagene Mx-3000 thermocycler (La Jolla, CA, USA). The measurement in the Hitachi F-4500 fluorescence spectrophotometer was performed in 1-ml quartz cuvettes (Hellma, Mühlheim, Germany). The scan data were as follows: scan speed, 1,200 nm/s; photomultiplier tube voltage, 950 V; excitation and emission slit, 5 nm and emission was measured from 200 to 800 nm in 0.2-nm steps.

Additionally, a plate read was performed using the Stratagene Mx-3000 thermocycler. The fluorescence values of the enrichment media were measured with the respective filter sets of the thermocycler for FAM, HEX, TET, CY-5 and ROX (Table 2). Samples for quantitative plate reads with the Mx-3000 thermocycler were performed in tripli-

Table 1 Enrichment media and contained fluorescing substances

Enrichment media	Fluorescing substances in the composition	Abbreviation	Reference
Preston broth	Rifampicin, cycloheximid, trimethoprim (TP)	PR	Bolton and Robertson (1982)
Bolton broth	Cefoperazone, TP, vancomycin, amphotericin B	BO	NACMCF National Advisory Committee on Microbiological Criteria for Foods (1994)
Fraser broth base	Aesculin	FR-	Fraser and Sperber (1988)
Fraser broth supplements	Nalidixic acid, acriflavine	F-supp.	Fraser and Sperber (1988)
Rappaport–Vassiliadis broth	Malachite green, novobiocin	RV	Vassiliadis (1983)
Buffered peptone water	–	BPW	Anon. (2002)
Müller–Kaufmann tetrathionate broth	Brilliant green	MK	Jeffries (1959)
Brilliant green bile lactose broth	Brilliant green	BGGL	Mackenzie et al. (1948)
Tryptone soy broth with yeast	–	TSB-Y	Roberts et al. (2003)
Luria-Bertani broth	–	LB	Bertani (1951)
Brain heart infusion broth	–	BHI	Eley et al. (1985)

Table 2 Excitation and emission maxima of the respective dyes as used in this study

Dyes and respective filter sets in brackets	Excitation (nm)	Emission (nm)
6-carboxyfluorescein (FAM)	492	516
6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein (HEX)	535	555
5-carboxy-X-rhodamine (ROX)	585	610
Indodicarbocyanine (CY-5)	635	665
6-carboxy-2′,4,7,7′-tetrachlorofluorescein (TET)	517	538

cate and included 5 µl of enrichment broth in ddH_2O and a total volume of 25 µl. The concentration of the respective enrichment media for plate read measurements was chosen to reflect the actual circumstances, thus 5 µl of undiluted media in a total volume of 25 µl. The concentration of the enrichment media as applied to the spectroscopic analysis resulted from the useful measurement range of the Hitachi F-4500 fluorescence spectrophotometer.

Results and Discussion

The data obtained by quantitative plate read are illustrated in Fig. 1. The fluorescence values obtained by quantitative plate read measurements of the enrichment media, which showed no significant fluorescence emission in the Mx-3000 thermocycler ranged from Δ118 (6.7% relative standard deviation (RSD)) fluorescence units (fu_{Mx}) using the TET filter set in comparison with ddH_2O. The respective values for the FAM, HEX, ROX and CY-5 filter sets obtained by quantitative plate read were Δ687 fu_{Mx} (4.2% RSD), Δ−7 fu_{Mx} (4.9% RSD), Δ−49 fu_{Mx} (4.3% RSD) and Δ−67 fu_{Mx} (7.3% RSD) compared with ddH_2O (Fig. 1).

The measurement of HFR-broth resulted in Δ28,094 fu_{Mx} using the TET filter set in comparison with ddH_2O, and the values for the FAM, HEX, ROX and CY-5 filter sets were Δ48,917, Δ36,627, Δ718 and Δ−242 fu_{Mx} compared with ddH_2O (Fig. 1). These results indicate a strong fluorescence of HFR-broth in the ranges of the FAM, HEX and TET filter sets. The other enrichment media that were tested showed no significant emission if excited at the respective wavelengths of all five filter sets, such as HFR-broth in the wavelength ranges of the CY-5 and ROX filter sets. FR-broth base also showed no significant emission when excited at the wavelength of all filter sets. The supplements of FR-broth emitted in comparison with ddH_2O Δ8,834, Δ54,376, Δ7,930, Δ34 and Δ−270 fu_{Mx} in the respective wavelengths of the TET, FAM, HEX, ROX and CY-5 filter sets (Fig. 1).

The results obtained with the Mx-3000 thermocycler are supported by the spectral analysis of the enrichment media. A strong fluorescent band from approximately 460 to approximately 650 nm was emitted by HFR-broth if excited with 492 nm (FAM), and a strong emission from approximately 500 to approximately 650 nm was measured when HFR-broth was excited with 535 nm (HEX). A strong emission from approximately 480 to approximately 650 nm was obtained (Fig. 2) if it was excited at the respective wavelength of the TET filter set.

The FR-supplements exhibited fluorescence from approximately 450 to approximately 630 nm when excited with 492, 517, 535 and 585 nm (FAM, TET, HEX and ROX). An excitation of the FR-supplements with 350 nm, representing the maximum excitation wavelength of nalidixic acid and aesculin, resulted in a strong emission band from approximately 450 to approximately 630 nm (Fig. 3).

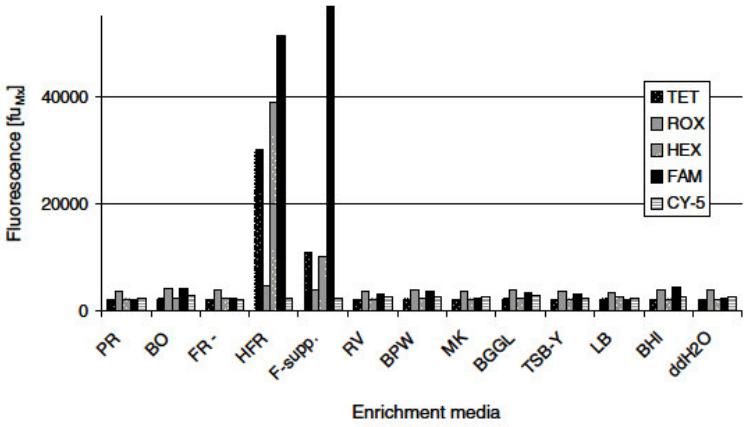

Fig. 1 The emission measured via quantitative plate read with the Mx-3000 thermocycler of the examined enrichment media after excitation with the respective wavelengths for the FAM, TET, HEX, CY-5 and ROX filter sets of the Mx-3000 thermocycler. The mean relative standard deviation of the measurement was 1.96% for all triplicates

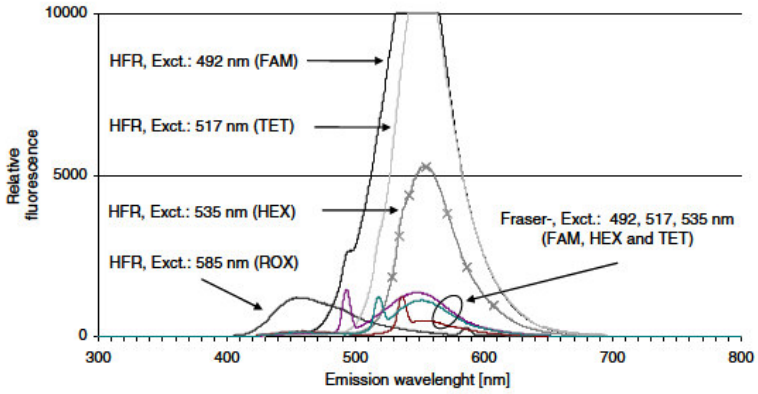

Fig. 2 The emission spectra of half-Fraser broth with supplements (*HFR*) and Fraser broth base (*Fraser-*) obtained after excitation with the respective wavelengths for the FAM, TET, HEX and ROX filter sets of the Mx-3000 thermocycler. These *graphs* illustrate the influence of the supplements on the spectral properties of HFR-broth. The spectra obtained by excitation with the respective wavelengths of FAM, TET and HEX show strong emission thus demonstrating possible disturbance of fluorescence readings in the respective channels of the real-time polymerase chain reaction thermocycler. The spectrum obtained by excitation with 585 and 635 nm according to ROX and CY-5 show no significant emission in the wavelengths of the respective channels of the thermocycler (610 and 656 nm). The spectrum of CY-5 is not labelled in the illustration since the detected values are too low to be presented in the scale of the illustration

FR-broth base showed strong emission from approximately 400 to approximately 600 nm when excited with 350 nm (Fig. 3).

The majority of the tested enrichment media exhibited no fluorescence emission at the respective wavelengths used for measurement in the real-time PCR cycler. This observation is surprising considering the various fluorescent properties of the substances included in these compositions (presented in Table 1). Nevertheless, these substances do not emit fluorescence in the range of the analysed wavelengths. Analysis of the composition of HFR-broth and the supplements thereof resulted in three fluorescent substances, which possibly emitted the high values of fluorescence, which were measured: acriflavine, aesculin and nalidixic acid (Fig. 4). The molecular structure of aesculin includes the fluorescent group coumarin. Nalidixic acid contains a two-ring hetero-conjugate as a strong fluorophore. The low fluorescence of FR-broth without supplements, in contrast to the strong signal emitted by the supplements, suggests that aesculin, which is exclusively contained in the broth, is not the interfering fluorescent substance. Furthermore, aesculin and nalidixic acid emit a maximum fluorescence of approximately 450 and approximately 500 nm, respectively, when excited with approximately 350 nm (Fig. 3). The respective excitation wavelengths of the optical path assignment of the real-time

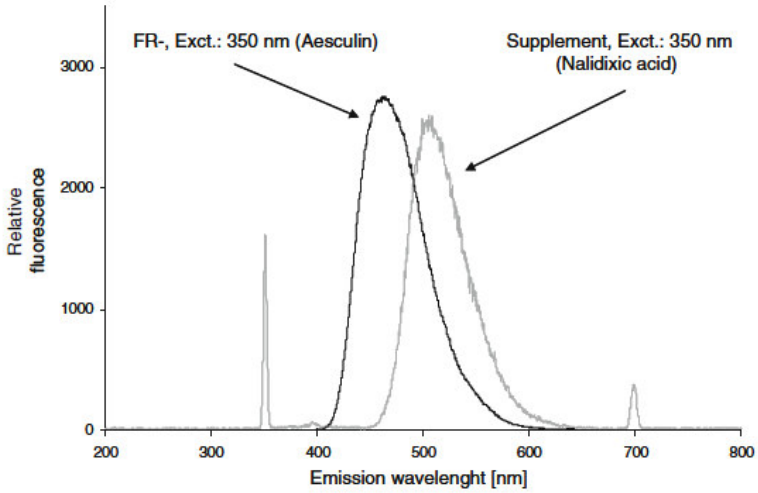

Fig. 3 The emission spectra of Fraser supplements and Fraser broth base (*FR-*) obtained after excitation with 350 nm using the Hitachi F-4500 fluorescence spectrophotometer. The approximately 60-nm shift of the peak emission wavelength of the fluorescent properties of nalidixic acid and aesculin is clearly demonstrated

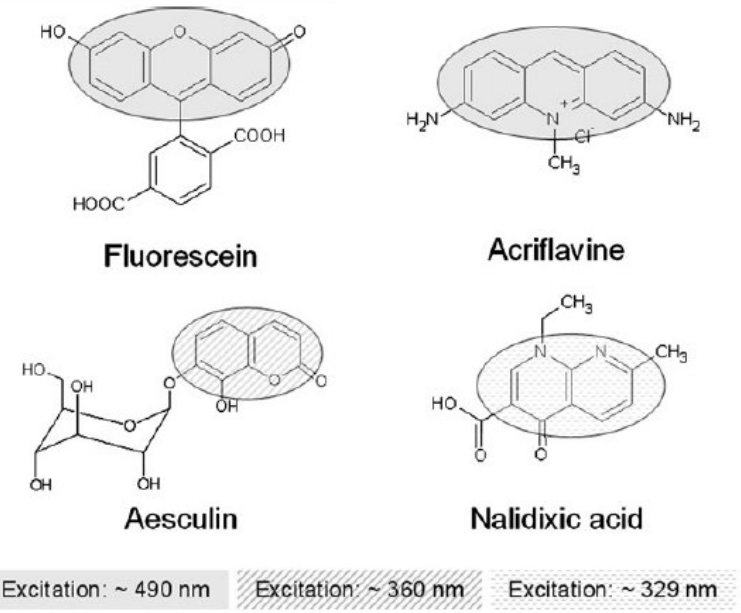

Fig. 4 The molecular structure of fluorescein, acriflavine, aesculin and nalidixic acid. The structural analogy of fluorescein and acriflavine illustrates the corresponding fluorescence characteristics of these substances. In contrast, the excitation wavelengths of the fluorescent properties of aesculin and nalidixic acid are reflected in the analogy of their structure, which differs from the basic fluorescent properties of fluorescein and acriflavine

PCR thermocycler do not include this wavelength range, so aesculin and nalidixic acid are not the origin of the strong emission in the wavelength of the FAM, TET and HEX filter sets. Data obtained by excitation of FR-broth base and the FR-supplements with 350 nm support this conclusion, as two distinct spectra with a wavelength shift of approximately 60 nm have been measured as was expected, according to the literature (Fig. 3; Khalfan et al. 1986; Wang et al. 2002).

Acriflavine includes a hetero-conjugated three-ring system, which is a structural analogue of fluorescein, the fluorescent basic structure of the real-time PCR reporter dyes FAM, TET and HEX (Fig. 4). These hetero-conjugates emit a maximum fluorescence at approximately 510–550 nm when excited with approximately 490–535 nm (Du et al. 1998). The structural similarities of fluorescein and acriflavine suggest the latter to cause the strong fluorescence emission observed. This is because the spectral data demonstrate that acriflavine emits a strong signal from 468 to 650 nm, and the respective detection wavelengths of the real-time PCR thermocycler of 516 (FAM), 555 (HEX) and 538 nm (TET) range within these values.

Inhibitory effects of foodstuffs, clinical specimens, chemicals and enrichment media on PCR have been previously reported (Rossen et al. 1992; Wilson 1997; Al-Soud and Rådström 2000). The effects of these substances on real-time PCR results have to be rated similarly, as fluorescence measurement adds some possible points of action for inhibitory effects rather than preventing inhibition. Previous publications basically highlight the interference of inhibitory components with the polymerisation process of the PCR amplification (Rossen et al. 1992; Knutsson et al. 2002). These compounds may chemically or physically interfere with the availability or activity of essential reaction compounds within the sample (Wilson 1997). The detection process, via measurement of the fluorescence of the released reporter dye, as a prerequisite of real-time PCR detection of a DNA target, was not taken into consideration. This study shows that at least one of the examined enrichment media contains fluorescent ingredients, which possibly inhibit the detection process in real-time PCR, even if a positive amplification occurred. The fluorescence values of HFR-broth and the supplements therein correlate to the range of the measurable amount in the optical path assignment in the FAM, TET and HEX filter sets of the thermocycler. The Stratagene Mx-3000 thermocycler has a maximum value of 65,535 fu_{Mx}. The measured value for FR-supplements using the FAM filter set was 56,826 fu_{Mx}. This value covers nearly the full range of the measurable fluorescence, though overlaying the detection process. A dilution of the enrichment broth by one tenth before application to the PCR sample should be sufficient to enable detection, nevertheless influencing the signal-to-noise ratio of the detection unit of the thermocycler and impairing the detection limit of the method. The choice of a reporter dye for probe design, which covers an emission/excitation wavelength not impaired by the enrichment media, should be taken into consideration if direct detection of target DNA from cultural enrichment of foodstuffs or other complex sample matrices with real-time PCR is desired. As demonstrated in this study for

HFR-broth, ROX and CY-5 would be alternative probe dyes with excitation wavelengths of 585 and 635 nm, respectively, and emission wavelengths of 610 and 656 nm. This would circumvent interference of the fluorescence of the FR-supplements with the probe dyes FAM, HEX and TET otherwise leading to disturbance of fluorescence readings in the respective channels of the real-time PCR thermocycler. Nevertheless, in general, the application of real-time PCR directly from enrichment media is unproblematic even though most of the investigated enrichment media contain strong fluorescent compounds (Table 1). These compounds do not interfere with the detection process, as their fluorescent properties do not cover the wavelength range of real-time PCR thermocycler optical path assignments.

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Development of matrix lysis for concentration of gram positive bacteria from food and blood

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Abstract

The development of a fast, reliable and inexpensive protocol for the concentration of bacteria from food by the removal of fat, carbohydrates and proteins that is compatible with downstream alternative DNA-based quantification methods is described. The protocol was used for dairy products, cooked and smoked fish and meat, carbohydrate-rich cooked products, ready-to-eat sauces, egg and blood. Lysis resulted in pellets of reasonable size for further processing. Starch, plant materials, fungi, tissues such as sinew, and chalaza could not be dissolved. Using *L. monocytogenes*, *S. aureus* and *B. cereus* as model organisms, microscopic analysis of the remaining bacterial pellets revealed that the recovered bacteria remained physically intact, albeit that the viability of the cells was compromised. Using real-time PCR, 7.3 CFU of *L. monocytogenes* were detected in artificially contaminated ultra-high temperature treated (UHT) milk and raw milk.

Keywords: Listeria; Direct quantification; Foodstuffs; Real time PCR; Food pathogens

1. Introduction

Real-time PCR allows reliable detection and quantification down to one single nucleic acid target per PCR sample, but requires highly purified template DNA (Radstrom et al., 2004; Rossmanith et al., 2006). However many foodstuffs hinder PCR (Radstrom et al., 2004; Wilson, 1997). Reliable and efficient recovery of the target organisms from the food sample is another requirement which is difficult to meet, given the relatively large sample volumes prescribed for analysis by international standard methods (Anonymous, 2006). Alternative techniques have to be evaluated in comparison to these methods (Hallier-Soulier and Guillot, 1999; Kulagina et al., 2005; Loessner et al., 2002; Nogva et al., 2000; Pedersen et al., 1998; Radstrom et al., 2004; Stevens and Jaykus, 2004a,b). One commonly used approach when applying real-time PCR as a diagnostic tool for pathogenic bacteria in food is to combine the method with a prior enrichment step, thus increasing the target concentration but losing the ability of quantification (Hein et al., 2006; Rossmanith et al., 2006; Yang et al., 2002).

The vast number of procedures used for this purpose and articles published focusing on this topic indicate that these problems are still far from being solved. Most methods have drawbacks such as the insufficient size of the processed sample volume (Jung et al., 2003; Lantz et al., 1994; Lindqvist et al., 1997; Wolffs et al., 2004). and the application of these methods has been largely restricted to a small number of food matrices (Allmann et al., 1995; Wegmuller et al., 1993). Based on the requirements for direct quantification of bacteria in food, which include (i) a large sample volume, (ii) a reproducible recovery rate over a wide range of target concentrations, and (iii) removal of inhibitors to aid alternative molecular methods for downstream analysis, we developed a protocol for the separation of gram positive organisms from various food matrices and blood. Our approach was focused on the elimination of the food matrix.

2. Material and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes EGDe (internal number, 2964) was used as a DNA quantification standard for real-time PCR and for

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artificial contamination of food samples. *S. aureus* (NCTC 1803) and *B. cereus* (NCTC 7464) were used to analyse the effects of the matrix lysis protocol on the growth and cellular appearance on gram positive bacteria other than *L. monocytogenes*. The bacteria were maintained at −80 °C using the MicroBank™ technology (Pro-Lab Diagnostics, Richmont Hill, Canada) and were part of the collection of bacterial strains at the Department of Veterinary Public Health and Food Science, University of Veterinary Medicine, Vienna, Austria. All bacterial strains were grown overnight in tryptone soy broth with 0.6% (w/v) yeast (TSB-Y; Oxoid, Hampshire, United Kingdom) at 37 °C. For artificial contamination of food, 1 ml of the overnight culture was transferred to 1 ml of fresh medium and incubated at 37 °C for 3 h, and 100 µl of appropriate dilutions in 1×PBS were added to the samples. The plate count method and tryptone soy agar plates supplemented with 0.6% (w/v) yeast extract (TSA-Y; Oxoid, Hampshire, United Kingdom) were used for quantification of *L. monocytogenes*. The agar plates were incubated at 37 °C for 24 h.

For assessment of the survival rate of *L. monocytogenes* treated with different chemical compounds of the matrix lysis buffer system, 100 µl of an overnight culture of *L. monocytogenes* was pelleted at 8000×g for 5 min, re-suspended in 1 ml of the chemical compound of choice, and incubated for 2 h at 45 °C and 300 rpm on an Eppendorf shaker (Eppendorf, Hamburg, Germany). The bacterial cells were then washed twice in 1×PBS at 5000×g and resuspended in 120 µl 1×PBS. One-hundred µl of the suspension was plated onto TSA-Y agar plates (Oxoid). Incubation was performed for 48 h at 37 °C.

2.2. Food samples

All food samples except raw milk were purchased at local supermarkets (Table 1). Raw milk was taken from a dairy farm at the University of Veterinary Medicine, Vienna, Austria. All samples used for artificial contamination were tested to be *L. monocytogenes* negative using the protocol as described below.

2.3. Microscopic investigation

Viability staining was performed using the Live/Dead® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Willow Creek, OR, USA) as prescribed by the manufacturer. Ten fields per filter were analysed, and two filters were prepared for each sample.

2.4. Optimisation of the matrix lysis protocol and sample treatment

Combinations of five different detergents (Tween 20®, Tween 80®, Triton X-100®; SDS, CHAPS), four different buffer systems (1×PBS, pH 8; 1×TE; 1×TBS, pH 8; 1×Tris, pH 8), three hydrophilic solvents (8, 6, and 4 M urea; 8 M guanidine; 1 M NaOH) and two lipophilic solvents ($CHCl_3$; *n*-Hexane) were compared with respect of their ability to dissolve food matrices such as salmon, hard cheese and milk, which were taken as model foodstuffs to develop the matrix lysis protocol. All chemicals used [with the exception of SDS, Sigma-Aldrich, Steinheim, Germany] were purchased at Merck (Darmstadt, Germany). In addition, different centrifugal forces for optimal recovery of *L. monocytogenes* and different conditions of incubation (time,

Table 1
Application of the optimized matrix lysis protocol to various food matrices and blood

Matrix	Amount processed	Pellet weight (wet weight)		Pellet size (weight volume)		General comments
		1st lysis	2nd lysis	1st lysis	2nd lysis	
UHT milk	12.5 ml	n.d. [a]	n.p. [b]	<2 µl	n.p.	
Raw milk	12.5 ml	n.d.	n.p.	<2 µl	n.p.	
Yoghurt, 3.6% fat in dry matter	12.5 g	0.07 g	0.07 g	70 µl	70 µl	Pellet mainly bacteria
Cottage cheese, 2.2% fat in dry mat	12.5 g	0.04 g	0.038 g	40 µl	40 µl	
Mozzarella cheese and brine	12.5 g	0.07 g	0.06 g	70 µl	70 µl	Pellet mainly bacteria
Camembert	6.25 g	2.4 g	1.4 g	n.d.	n.d.	Pellet consist mainly of penicillum roqueforti
Green-veined cheese	6.25 g	2.0 g	0.9 g	n.d.	n.d.	Pellet consist mainly of penicillum roqueforti
Hard cheese	6.25 g	0.15 g/0.03 g [c]	n.p.	150 µl/<10 µl [c]	n.p.	Good results with sedimentation for 5–10 min
Ice cream (vanilla)	12.5 g	0.08 g	0.08 g	75 µl	75 µl	
Ice cream (strawberry)	12.5 g	0.34 g	0.34 g	350 µl	350 µl	Pellet due to vegetable remnants
Cooked perch	6.25 g	n.d.	0.42 g	n.d.	420 µl	Total lysis after 4–5 lysis steps
Smoked salmon	6.25 g	n.d.	1.6 g	n.d.	n.d.	Total lysis after 4–5 lysis steps
Cooked minced meat	6.25 g	n.d.	3 g	n.d.	n.d.	Lysis problematic due to heterogenous composition
Cooked noodles	6.25 g	no lysis	no lysis	n.d.	n.d.	No lysis
Cooked rice	6.25 g	no lysis	no lysis	n.d.	n.d.	No lysis
Cooked dill sauce	12.5 g	0.28 g	0.28 g	n.d.	n.d.	Good lysis of sauce, no lysis of vegetable portion
Cooked tomato sauce	12.5 g	5 g	5.5 g	n.d.	n.d.	No lysis of vegetable portion
Egg	12.5 g	0.25 g/<0.01 g	0.25 g/<0.01 g	300 µl/<2 µl	n.d.	Physical removal of chalaza necessary
Blood	12.5 ml	n.d.	n.p.	<2 µl	n.p.	

[a] n.d. indicates that this parameter was not determined.
[b] n.p. indicates that this analysis was not performed.
[c] Weight and volume are given for the pellet of the supernatant, which was removed after sedimentation for 5 min.

temperature, and pH) with the matrix lysis buffer and the washing buffer chosen were tested. The optimised matrix lysis protocol is described below.

For experiments using artificially contaminated samples, 100 µl of a pure culture of *L. monocytogenes* was added to 12.5 ml raw milk or ultra-high temperature treated (UHT) milk in a 50-ml polypropylene tube (Corning, NY, USA). Matrix lysis buffer (8 M urea, 1% SDS, and 1×PBS) was added to a final volume of 40 ml. The samples were incubated in a water bath at 45 °C and constant shaking at 200 rpm for 30 min in horizontal position. The samples were then centrifuged at 3220×g for 30 min at room temperature. The supernatant was discarded gently, leaving 1 ml in the tubes. The pellet was re-suspended in 40 ml washing buffer [1×PBS, 1% Exact industrial detergent (containing anionic detergents and various solvents, pH 7; E. Mayr; Vösendorf; Austria)] and incubated in a water bath at 45 °C and constant shaking at 200 rpm for 30 min in horizontal position. Afterwards the samples were centrifuged at 3220×g for 30 min at room temperature and the supernatant was gently discarded, leaving 500 µl in the tubes. The pellet was re-suspended in 500 µl 1×PBS, transferred to a 1.5-ml plastic tube (Eppendorf, Hamburg, Germany), and washed twice in 1 ml 1×PBS with additional centrifugation for 5 min at 5,000×g.

Hard cheese was added to 12 ml matrix lysis buffer in 6.25-g portions and homogenised twice in the laboratory blender Stomacher 400 (Seward, London, UK) for 3 min each. The homogenate was transferred to 50-ml polypropylene tubes (Corning, NY, USA), and matrix lysis buffer was added to a volume of 40 ml. Lysis was performed as described for raw milk and UHT milk. The re-suspended pellet was transferred to a 2-ml plastic tube (Eppendorf, Hamburg, Germany) because of the size of the pellet. Two washing steps, each consisting of 1.5 ml 1×PBS at 5000×g were performed at room temperature. After re-suspending the pellet in 1.5 ml 1×PBS, 5 minutes were allowed to elapse for sedimentation of the calcium phosphate remnants. The supernatant was transferred to a fresh tube and centrifuged at 8000×g for 5 min in order to collect the bacteria.

Food matrices other than milk and hard cheese were processed in 6.25-g or 12.5-g portions as indicated in Table 1, by adding 12 ml of matrix lysis buffer before homogenisation and following the protocol described for hard cheese, except for the fact that the re-suspended pellets were transferred to 1.5-ml plastic tubes and no sedimentation step was required.

DNA isolation of the remaining bacterial pellet after matrix lysis was performed using the NucleoSpin® tissue kit (Machery-Nagel, Düren, Germany) and the support protocol for Gram positive bacteria.

2.5. DNA standard for real-time PCR quantification

One millilitre of a pure culture of *L. monocytogenes* was subjected to DNA isolation using the NucleoSpin® tissue kit (Macherey- Nagel) and the support protocol for Gram positive bacteria. The DNA concentration was measured fluorimetrically using a Hoefer DyNA Quant200 apparatus (Pharmacia Biotech, San Francisco, CA, USA). The copy number of the *prfA* gene was determined by assuming that, based on the molecular weight of the genome of *L. monocytogenes*, 1 ng of DNA equals 3.1×10^5 copies of the entire genome, and that the *prfA* gene is a single-copy gene (Nelson et al., 2004).

2.6. Real-time PCR

Real-time PCR was carried out as published previously by targeting a 274 bp fragment of the *prfA* gene of *L. monocytogenes* (D'Agostino et al., 2004; Rossmanith et al., 2006). The real-time PCR result was expressed as bacterial cell equivalents (BCE).

For determination of the inhibitory effects of the food matrices or the chemical compounds used in the matrix lysis protocol, either 5 µl of a dilution of raw milk or hard cheese in ddH_2O ranging from 10^{-1} to 10^{-3}; or 0.8% to 0.005% (w/v) SDS; or 0.05 M, 0.1 M, and 0.2 M urea was added directly to the PCR mix. In addition, an aliquot of the diluted food matrices was boiled at 100 °C for 15 min prior to supplementation to the PCR. The PCR mix either contained 15.5 or 1550 copies of the target gene/PCR. The real-time PCR signal in terms of Ct values of these PCR samples was compared to the signal of PCR samples containing no food matrix or special chemical compound, in duplicate.

3. Results

3.1. Ability of different detergents, buffer systems, solvents, and additives to dissolve food matrices in aqueous solution

The specific composition of the lysis buffer used for matrix lysis – 1×PBS, 8 M urea, and 1% SDS – was chosen after testing a large number of chemicals (Table 2). Following lysis of the food matrices, a washing step in 1% industrial detergent Exact® in 1×PBS (washing buffer) was introduced to dissolve fat remnants and release *L. monocytogenes* cells bound to the 50-ml polypropylene tube.

3.2. Optimisation of the centrifugation steps, pH of the matrix lysis buffer and the washing buffer, and the incubation times and temperatures used

The optimal centrifugal force required for maximum recovery of *L. monocytogenes* was tested using a pure culture of these bacteria suspended in 1×PBS. Recovery was determined semiquantitatively by visually assessing the pellet size. A centrifugation step of 30 min at 3220×g was optimal to collect the bacteria. With respect to the degradation of the food matrix, lysis of the food matrix with the matrix lysis buffer worked well when performed in a pH range of 7.0 to 9.5, resulting in a reduction of the pellet size. A pH less than 7.0 yielded no sufficient reduction of the pellet size. Experiments with temperatures ranging from 25 °C to 56 °C for incubation of different food matrices with the matrix lysis buffer and the washing buffer showed 45 °C to be the optimal incubation temperature. The optimal duration of incubation with the matrix lysis buffer and the washing buffer was 30 min.

Table 2
Performance of various combinations of buffers, solvents, detergents, and additives used in the development of the matrix lysis protocol

	8 M Urea	6 M Urea	4 M Urea	8 M Guanidin	1 M NaOH	1×TBS pH 8 [a]	1×TE [a]	1×PBS pH 8	1×TRIS pH 8 [a]	1% SDS	Tween 20(2%)	Tween 80(2%)	Triton X-100(1%)	CHAPS	EDTA [a]	CHC13 [b]	N-HEXAN [b]
8 M Urea	n.l. [c]	–	–	–	n.l.	–	–	+	–	++	–	–	–	++/ ex.	–	–	–
6 M Urea	– [d]	n.l.	–	–	–	–	–	n.l.	–	–	–	–	–	–	–	–	–
4M Urea	–	–	n.l.	–	–	–	–	n.l.	–	–	–	–	–	–	–	–	–
8 M Guanidin	–	–	–	–	–	–	–	n.l. /ex	–	++	–	–	–	++/ ex.	–	–	–
1 M NaOH	n.l.	–	–	–	n.l.	n.l.	–	n.l.	–	n.l.	–	–	–	–	–	–	–
1×PBS pH 8	+ [e]	n.l.	n.l.	+/ex. [f]	n.l.	–	–	n.l.	–	n.l.	n.l.	n.l.	n.l.	n.l./ex.	–	n.l/lp. [g]	n.l.
1×TE [a]	–	–	–	–	–	–	–	–	–	n.l.	–	–	–	–	–	–	–
1×TBS pH 8 [a]	–	–	–	–	–	–	–	–	–	n.l.	–	–	–	–	–	–	–
1×TRIS pH 8 [a]	–	–	–	–	–	–	–	–	–	n.l.	–	–	–	–	–	–	–
1% SDS	++	–	–	++	n.l.	n.l.	n.l.	n.l.	n.l.	n.l.	n.l.	n.l.	n.l.	–	–	n.l/lp.	n.l.
Tween 20 (2%)	–	–	–	–	–	–	–	n.l.	–	n.l.	n.l.	n.l.	n.l.	–	–	–	n.l.
Tween 80 (2%)	–	–	–	–	–	–	–	n.l.	–	n.l.	n.l.	n.l.	n.l.	–	–	–	n.l.
Triton X-100 (1%)	–	–	–	–	–	–	–	n.l.	–	n.l.	n.l.	n.l.	n.l.	–	–	–	n.l.
CHAPS	++/ ex.	–	–	++/ex.	–	–	–	n.l./ex	–	–	–	–	–	n.l./ex.	–	–	ex/n.l.
CHI [b]	–	–	–	–	–	–	–	n.l./lp.	–	n.l/l.p.	–	–	–	–	–	n.l/lp.	–
n-HEXAN [b]	–	–	–	–	–	–	–	n.l./lp.	–	+	n.l.	n.l.	n.l.	–	–	–	n.l.
1×PBS, 8 M Urea	–	–	–	–	–	–	–	–	–	++ [h]	n.l.	n.l.	n.l.	–	–	–	–
1×TBS, 8 M Urea [a]	–	–	–	–	–	–	–	–	–	++	n.l.	n.l.	n.l.	–	–	–	–
1×TRIS, 8 M Urea [a]	–	–	–	–	–	–	–	–	–	++	n.l.	n.l.	n.l.	–	–	–	–
1×PBS, 6 M Urea	–	–	–	–	–	–	–	–	–	+	n.l.	n.l.	n.l.	–	–	–	–
1×PBS, 4 M Urea	–	–	–	–	–	–	–	–	–	+	n.l.	n.l.	n.l.	–	–	–	–
1×PBS, 8 M Guanadine	–	–	–	–	–	–	–	–	–	++/ex.	n.l.	n.l.	n.l.	–	–	–	–
1×TE, 8 M Urea [a]	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–	–	–
1×PBS ,8 M Urea, 1% SDS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+
1×TBS ,8 M Urea 1% SDS [a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–
1×TRIS ,8 M Urea 1% SDS [a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–

[a] Tested to avoid the formation of calcium phosphate [$Ca_3(PO_4)_2$] remnants in hard cheese.
[b] Formed a viscous gel in combination with 8 M urea and 1% SDS, thus hindering proper centrifugation.
[c] n.l. indicates no lysis.
[d] – indicates that this combination was not performed.
[e] + indicates that the resulting pellet hindered subsequent handling (due to pellet size and/or consistency).
[f] ex. indicates that this reagent was considered too expensive and therefore excluded from the protocol.
[g] lp. indicates a lethal procedure for *Listeria*.
[h] ++indicates that the resulting pellet permitted good subsequent handling.

3.3. Application of the optimised matrix lysis protocol to various food matrices

Five groups of foodstuffs (dairy products, cooked and smoked fish and meat, carbohydrate-rich cooked products, ready-to-eat sauces, and egg) were examined (Table 1). In addition, the matrix lysis protocol was applied to blood. Within the group of dairy products, the lysis of UHT milk, raw milk, yoghurt, mozzarella, and cottage cheese resulted in a pellet of reasonable size for further processing steps. Lysis of 6.25 g of various types of hard cheeses yielded pellet sizes of approximately 150 µl, regardless of the degree of cheese ripening. Microscopic analysis showed that the pellet mainly consisted of round crystalline structures which remained insoluble in water, 1 M NaOH, and 1 N HCl, at both 25 °C and 100 °C. The main part of the remaining pellet consisted of $Ca_3(PO_4)_2$ (analysis not shown). The introduction of a final sedimentation step for 5 min and subsequent centrifugation of the supernatant decreased the pellet size to approximately 10 µl. Ice cream was reduced to 75 µl during matrix lysis if 12.5 g was applied, except when the ice cream contained bits of fruit or nut. Cooked perch and smoked salmon were lysed completely in five consecutive lysis steps. Cooked minced meat could not be lysed satisfactorily, yielding pellet sizes of up to 3 ml even after 5 consecutive lysis steps. The carbohydrate-rich foodstuffs tested (rice and noodles) remained insoluble when processed with the matrix lysis buffer. Tomato sauce and dill sauce were reduced to their vegetable ingredients, which remained insoluble. Egg was completely lysed after two consecutive lysis steps, except the chalaza of the egg, which was removed after the second lysis step prior to centrifugation. Blood was lysed, leading to a pellet size <2 µl after one lysis step.

3.4. Effects of the matrix lysis protocol on the growth and cellular appearance of L. monocytogenes, S. aureus and B. cereus

The chemical compounds used in the matrix lysis protocol (8 M urea, 1% SDS, 1×PBS, industrial detergent Exact®) were tested, alone and in combination, for their impact on the growth and cellular appearance of *L. monocytogenes*, *S. aureus* and *B. cereus*. The bacteria were able to grow when treated with 8 M urea or 1×PBS. Complete inhibition of growth was observed for 1% SDS and for the combination of 8 M urea, 1% SDS, and 1×PBS. The industrial detergent Exact® also completely inhibited growth. Nevertheless, microscopic analysis revealed that all treatments inactivated the cells but left them physically intact. The cell count could not be accurately determined because of the poor signal-to-noise ratio of weakly stained bacterial cells and the background.

3.5. Inhibitory effects of food matrices and detergents on real-time PCR

Real-time PCR samples containing hard cheese or raw milk diluted from 10^{-1} to 10^{-3} yielded Ct values of 33.3 (RSD: 0.92%) when they contained 15.5 copies of the target gene/PCR, and 27.0 (RSD: 1.3%) when they contained 1550 copies of the target gene/PCR. The results were similar when the diluted food samples were boiled before addition to the real-time PCR sample, yielding Ct values of 33.2 (RSD: 0.26%) and 26.9 (RSD: 0.59%), respectively. For comparison, respective real-time PCR samples without food matrix yielded Ct values of 32.9 (RSD: 0.57%) and 26.7 (RSD: 0.19%). These results indicate the absence of inhibitory effects of the food matrices tested on real-time PCR. Analogous experiments with urea and SDS showed inhibitory effects for SDS in a range of 0.8% to 0.005%, with no amplification of the targets at any of the tested concentrations (15.5 and 1550 copies/PCR). The samples containing 1550 copies of target DNA and urea concentrations of 0.2 M, 0.1 M, and 0.05 M achieved Ct values of 26.1 (RSD: 0.94%.) in comparison to 26.7 (RSD: 0.19%) for samples containing no urea. Thus, we decided to use a column-based DNA purification step prior to real-time PCR analysis of the bacterial pellet after matrix lysis.

3.6. Real-time PCR and microscopic analysis of the recovery of L. monocytogenes from milk after matrix lysis

Artificial contaminated raw milk and UHT milk containing a decimal dilution series of *L. monocytogenes* in 4 steps starting at 1.1×10^5 CFU per 12.5 ml of sample was subjected to DNA isolation and real-time PCR after application of the matrix lysis protocol. The number of BCE per sample obtained by real-time PCR from raw milk was 2.0×10^4 (RSD: 68.3%), 1.9×10^3 (RSD: 24.4%), 2.2×10^2 (RSD: 74.8%), and 9.0×10^1 (RSD: 85.7%). From UHT milk, 3.2×10^4 (RSD: 30.8%), 4.7×10^3 (RSD: 30.2%), 2.3×10^2 (RSD: 49.0%), and 6.9×10^1 (RSD: 51.0%) BCE per sample were obtained. Based on these results, which indicate a similar recovery rate at all *L. monocytogenes* concentrations tested, a more detailed investigation was performed by comparing real-time PCR data, total cell counts as determined by microscopy, and CFU before and after the

Table 3
Determination of the recovery rate of *L. monocytogenes* from milk after matrix lysis as determined by real-time PCR

Recovery related to	Real time PCR			Microscopy[a]	Plate count method
	Control[b] BCE/ml (RSD)	Raw milk BCE/ml (RSD)	UHT milk BCE/ml (RSD)	Cells/ml (RSD)	CFU/ml (RSD)
	9.3×10^4 (64.0%)	2.1×10^4 (68.4%)	3.2×10^4 (30.8%)	2.2×10^5 (20.1%)	1.1×10^5 (46.9%)
Microscopy[a]	42.0%	93%	14.0%	100%	
Plate count method	86.9%	19.1%	29.9%	–	100%
Real time PCR	100%	22.0%	34.5%	–	

[a] Inoculation level of milk prior to matrix lysis.
[b] Bacterial culture directly processed with the NucleoSpin® tissue kit, without matrix lysis.

application of the matrix lysis protocol (Table 3). The recovery rate of *L. monocytogenes* specific real-time PCR from raw milk was 9.3% when compared to the total cell count, and 19.1% when compared to the number of CFU. A yield of 22.0% was achieved when compared to a control sample (real-time PCR analysis of the culture before addition to milk). For UHT milk, the recovery rate of *L. monocytogenes* was 14.0% and 29.9% when compared to the total cell count and number of CFU, respectively, and 34.5% in comparison to the control sample.

4. Discussion

4.1. Optimisation of the matrix lysis protocol

The fundamental idea was to remove fat, carbohydrates and proteins and to disintegrate eukaryotic cells in the food matrices. Special care was taken to develop an optimal procedure for the removal of fat from the food matrices. Initial attempts to separate fat consisted of freezing the samples and the use of lipophilic solvents such as *n*-hexane or chloroform. The separation of the fat from frozen samples proved difficult, resulting in incomplete separation. Both tested lipophilic solvents formed viscous gels with the proteins present in the food samples, thus preventing the separation of bacterial cells by means of centrifugation. Further experiments proved that the ability of the matrix lysis buffer containing 1×PBS, 8 M urea and 1% SDS to resolve fat was sufficient when performed at 45 °C. A post-lysis washing step performed in 1% industrial detergent Exact® in 1×PBS increased the recovery of *L. monocytogenes* cells from the food matrix (data not shown), as fat remnants on the surface of the 50-ml tubes were removed and the adsorption of bacterial cells on the surface of the tubes was prevented (Harden and Harris, 1953).

To develop a useful hydrophilic solvent system, a wide range of solvents, detergents, buffer systems and incubation conditions were tested. Based on protocols used in chemical analysis of food, NaOH was tested as a solvent at concentrations that have been reported to exert no effect on the integrity of *L. monocytogenes* cells (≤1 M). However, the ability of NaOH to dissolve food matrices was inadequate, regardless of whether it was used in combination with detergents (Lou and Yousef, 1999). Urea and guanidine/HCl showed good performance in lysing food matrices when used alone, and even better performance when combined with detergents. Nevertheless, urea was incorporated into the matrix lysis protocol because it is cheaper, which is of concern in routine diagnosis.

Members of three groups of detergents were tested. The non-ionic detergents Tween 20, Tween 80 and Triton X 100® did not support lysis of the food matrices. The anionic detergent SDS, which is known to bring membrane-associated proteins in solution, worked well in combination with 8 M urea or 8 M guanidine. CHAPS, a zwitterionic detergent, also worked well in combination with 8 M urea or 8 M guanidine, but was deemed too expensive for diagnostic purposes.

Proteins could be dissolved with good results because the protocol is primarily based on protein biochemistry. Thus, milk, dairy products, other protein-rich food matrices and whole blood were lysed well. The relatively large size of the remaining pellet of Camembert and green-veined cheese is a result of the high content of *Penicillum roqueforti* in both kinds of cheese, which could not be removed under the lysis conditions used.

For food matrices with a low water content such as hard cheese (Table 1), the processed volume had to be reduced to 6.25 g because of the resulting viscosity of the food/buffer mixture. Direct detection or quantification of bacteria in food using alternative DNA-based methods has been mainly reported for samples sizes up to 2 g or ml (Jung et al., 2003; Stevens and Jaykus, 2004b; Wegmuller et al., 1993). Direct processing of 11 g of plain non-fat yoghurt and cheddar cheese; 25 ml, 100 ml, and 40 ml of raw milk; and 4 g of cheese or sausage has been reported (Allmann et al., 1995; Herman et al., 1995a,b; Pedersen et al., 1998; Stevens and Jaykus, 2004a,b). Increasing the sample size exerts a positive effect on the detection limit.

All four buffer systems employed to obtain constant conditions at a pH of approximately 8 in order to preserve the bacteria during the matrix lysis protocol were feasible (Lou and Yousef, 1999). In addition, the physical conditions for the matrix lysis protocol such as temperature and centrifugation forces were adjusted for optimal lysis of the food matrices within the reported range of survival of *L. monocytogenes* (Lou and Yousef, 1999). Gram staining of the remaining bacterial pellet after matrix lysis showed that the cell walls of *L. monocytogenes*, *S. aureus*, and *B. cereus* remained intact. This was confirmed by fluorescent staining of the chromosomal DNA, which showed that the DNA remained within the bacterial cells. However, the cells showed a red and occasionally orange colour, indicating cell inactivation.

4.2. L. monocytogenes specific real-time PCR analysis of artificially contaminated milk after matrix lysis

The matrix lysis protocol was tested in combination with real-time PCR to show the ability for direct quantification of *L. monocytogenes* in raw milk and UHT milk. Good reproducibility of the recovery rate of the matrix lysis protocol was shown, as the decimal dilution of the artificial contaminated samples was reflected in the real-time PCR results. The recovery rates of 19.9% and 29.9% of *L. monocytogenes* added to raw milk and UHT milk, respectively, cannot be compared with published data because direct plating of the bacterial pellet could not be performed, as the cells were compromised during the matrix lysis protocol. DNA isolation might not have been fully efficient, and may have led to an underestimation of the recovery rate of the matrix lysis protocol as determined by the real-time PCR analysis. Recovery rates ranging from 71 to 161% have been reported for the concentration of *L. monocytogenes* from reconstituted non-fat dry milk, whole milk, and ice cream when using metal hydroxide immobilization in combination with centrifugation (Lucore et al., 2000). Using other food matrices and different methods, recovery rates of 5 to 128% have been reported (Fluit et al., 1993; Lantz et al., 1994; Pedersen et al., 1998; Stevens and Jaykus, 2004a,b). However, these recovery rates were all obtained by direct plating.

The detection limit of the matrix lysis protocol in combination with real-time PCR analysis when examining artificially contaminated UHT milk and raw milk of 7.3 CFU per ml compares favourably with other PCR-based methods. By the use of real-time PCR it was possible to detect 10 to 100 CFU/ml UHT milk, 40 to 200 CFU/ml raw milk and skim milk, and 10^3 CFU/ml reconstituted non-fat dry milk (Hein et al., 2001; Koo and Jaykus, 2003; Nogva et al., 2000). Other authors used conventional PCR methods with variable results (Choi and Hong, 2003; Herman et al., 1995a). The detection limit achieved in the present study is close to what would be theoretically possible: as 5 µl of the DNA solution was added to the PCR sample and the DNA of 12.5 ml of milk was concentrated in a volume of 100 µl after DNA isolation, the theoretical detection limit equals 1.6 CFU/ml milk. Concentration of the DNA solution to a smaller volume, which could be added completely to one PCR sample, might achieve a further reduction of the detection limit.

In summary, the matrix lysis protocol presented here allows the reduction of 6.25-g to 12.5-g volumes of various food matrices into small pellets that can be easily used for further processing with DNA-based methods. The method is easy to perform, inexpensive fast and can be used for on-line hygiene monitoring in food production. The method is a valuable tool for clinical diagnosis as well, as shown by its application with whole blood.

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The use of chromogenic bacteria as coloured substitutes for pathogens: A simple strategy during design and development of a new method for sample pre-treatment.

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NOTE TO THE EDITOR

The use of chromogenic bacteria as coloured substitutes for pathogens: A simple strategy during design and development of a new method for sample pretreatment

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Abstract

Aims: In the present study, chromogenic (red) bacteria were used to simulate actual target bacteria during set-up and optimization of an isolation process of bacteria, designed for food samples. Isolation of bacteria from food in the context of molecular biological detection of food pathogens is a multistep process. Development of such a separation method requires continuous monitoring of the location of the presumable targets in the sample tubes. Therefore, red-coloured pigmented bacteria were used as substitutes for the actual target bacteria, during the establishment of a new sample preparation technique.
Methods and Results: The chromogenic bacteria *Micrococcus roseus* and *Serratia marcescens* were confirmed to withstand the physical (e.g. centrifugal forces) and chemical (e.g. lysis buffer composition) conditions required during establishment of the new technique. Furthermore, the suitability of these model bacteria to substitute for the actual target pathogens (*Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Listeria monocytogenes*) was assured by testing the physical properties of the model bacteria with respect to the proposed separation methods.
Conclusion: Visibility of the pigmented bacteria within the complex sample matrices served to allocate bacterial content during the various steps necessary for finalization of the method protocol. The presumptive bacterial targets can be allocated simply by visualization of their bright red colour silhouetted against the background sample matrix.
Significance and Impact of Study: The use of pigmented bacteria as substitutes for actual colourless target bacteria during design and development of a bacterial isolation method is a simple and inexpensive application. It saves a huge amount of time and resources, as the proof of principle of new methods is possible in rapid succession.

Molecular methods for detecting and quantifying bacterial pathogens are growing in importance, as alternatives to conventional microbiology techniques. These methods offer precision and quantitative aspects that conventional microbiological methods lack (Stevens and Jaykus 2004). However, a precondition for their use is adequate sample preparation and reproducible isolation of the bacterial targets. This includes separation of the target bacteria from complex matrices and their digestion for DNA isolation. Recently, major efforts have been made to implement such sample preparation protocols but there is still need for reliable methods to be established (Rådström *et al.* 2004; Stevens and Jaykus 2004). Methods are required that overcome preparation difficulties, yet permit subsequent broad application of molecular methods in quantitative routine diagnostics of food-borne and clinical pathogens.

The design and development stages of new sample preparation methods are elaborate and expensive to perform. There is a need to establish the physical and chemical parameters of the new method, such as buffer systems, centrifugation times and temperatures, which require a vast number of individual experiments, all of which must preserve the target organisms and achieve maximum separation and recovery. From the outset, the key question in such a process is where to allocate the presumptive target organisms within the samples during the various handling steps. Common pathogen target organisms in food and clinical diagnostics are colourless and invisible and hence not easy to differentiate from sample matrices. Consequently, it is necessary to simulate the bacterial targets at the beginning of the development of a new method protocol to establish the essential physical and chemical parameter.

This prerequisite may now lend itself to the use of chromogenic bacteria. The *Micrococcus* species, particularly *M. luteus* and *M. roseus*, and the *Serratia* species, particularly *S. marcescens*, exhibit intense chromogenic properties (Greenberg 1978). *Micrococcus roseus* produces red carotenoid pigments (Jagannadham *et al.* 1991), which are very stable under diverse conditions (Fig. 1e). *Serratia marcescens* produces prodigiosin, a particularly intense red pigment (Bennett and Bentley 2000). From an operational safety perspective, *M. roseus* and *S. marcescens* are low hazard organisms when used under standard laboratory conditions (Hogue *et al.* 2007). Until now, there have been few reports in the literature regarding application of their chromogenic properties (Hoerr *et al.* 2004).

The present work resulted from the exemplary application of *M. roseus* and *S. marcescens* as substitutes for *Listeria monocytogenes* and serovar Typhimurium during the design and development of a sample preparation method, which was described by Rossmanith *et al.* (2007) and Mayrl *et al.* (2009). The method, called 'matrix lysis', involves solubilization (lysis) of a sample food matrix and subsequent separation of the target cells via centrifugation. This allows quantitative detection of bacteria with real-time PCR after isolation of the bacterial DNA.

The main steps of the 'matrix lysis' protocol, recommended by those authors, involve chemical solubilization of a 6·25–12·5 g ml^{-1} foodstuff sample, physical separation of the bacterial targets by centrifugation in two subsequent repeats and two wash steps (Rossmanith *et al.* 2007; Mayrl *et al.* 2009). The central principle of this separation method is a buffer system capable of solvation of the sample matrix, so releasing the enclosed bacteria. Solvation of the protein fraction is performed by chaotropic agents and detergents; the water-soluble carbohydrate fraction is solubilized by the hydrophilic nature of the buffer, and separation of the fat content is achieved by temperature-dependent liquefaction and centrifugation. Adjustment of chemical and physical factors during the first steps of the protocol led to difficulty in allocating presumable bacterial targets to the various sample fractions after solubilization and centrifugation (Rossmanith *et al.* 2007; Mayrl *et al.* 2009). It was not clear whether the bacteria were attached to the tube, trapped in the fat content or included in the water-soluble fraction. Alternatively, separate investigation of these fractions after each experimental step using microbiological or molecular biological methods would have been resource-intensive.

To circumvent this effort, *M. roseus* and *S. marcescens* were added whilst testing the necessary parameters of the

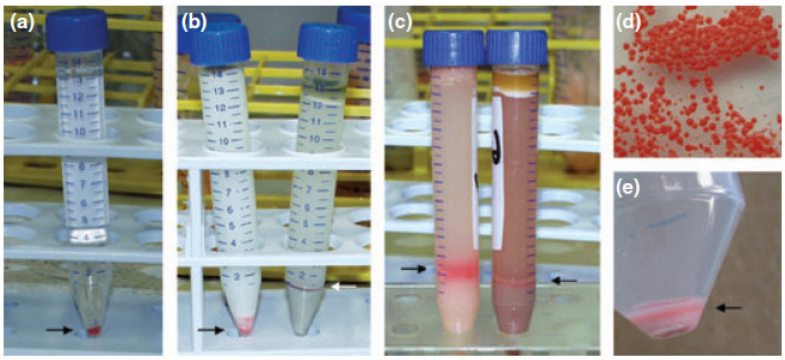

Figure 1 The use of *Micrococcus roseus* during several stages of matrix lysis method development. The arrows indicate the location of *M. roseus* within the samples. (a) A sample centrifuged to the bacterial pellet stage to determine the necessary centrifugation parameters (*M. roseus* in 8 mol l^{-1} urea, 1% SDS and 1 × PBS). (b) Raw milk after incubation and centrifugation in 1% Tween 20 before lysis of the food matrix (left) and Österkron cheese after a similar treatment (right). *Micrococcus roseus* can be seen as a thin band above the black residues of *Penicillum roqueforti*. (c) Smoked salmon (left) after incubation in 1% Triton-X 100 and centrifugation showing a broad band of *M. roseus* because of the viscosity of the semi-lysed food matrix, and liver pate following similar treatment (right). (d) *M. roseus* on a TSA-Y plate after 48 h of incubation at 25°C. (e) *M. roseus* after complete lysis of the foodstuff in 8 mol l^{-1} urea, 1% SDS and 1 × PBS (6·25 g of Gouda cheese).

protocol. *Micrococcus roseus* (Strain R4) and *S. marcescens* (Strain R54) were part of the collection of E. Schopf (Department of Veterinary Public Health and Food Science, VUW, Vienna, Austria). *Micrococcus roseus* and *S. marcescens* were grown separately for 48 h on tryptone soya agar with yeast (TSA-Y; Oxoid, Hampshire, UK) plates at 25°C to develop their characteristic red pigments. Samples of different food products [Milk (UHT, raw), yoghurt, cottage cheese, mozzarella cheese and brine, hard cheese, blue-veined cheese, ice cream, pate, beef, chicken, minced meat, smoked salmon, cooked salmon, eggs, strawberries, blood, noodles, rice and chives] blended in the chemical compounds, which were tested for applicability as buffer ingredients (for a complete list see supporting information and Rossmanith *et al.* 2007) were spiked with approximately 1×10^{10} cells for a sample size of 15–50 ml. The cells were transferred with a sterile spatula directly from the agar plates, or 5-ml bacterial cultures were centrifuged at 5000 ***g*** for 5 min and the resulting pellet transferred to the samples after resuspending in 100-μl double distilled H_2O. The samples were examined visually after each test during set-up of the essential parameters of the method protocol, taking advantage of the colour contrast of the pigmented bacteria silhouetted against the sample fractions (Fig. 1a–d). Dairy products including milk and most sorts of ice cream, eggs, meat, fish, noodles and rice clearly supported chromogenic contrasting because of the background colour of the foodstuff. Salmon and strawberries required inoculation with more than 1×10^{10} cells to allow for adequate visualization (Fig. 1c).

Prior to this application of *M. roseus* and *S. marcescens* in the developmental stage of the new method protocol, preliminary tests were performed to verify the comparability of the pigmented model bacteria to the actual targets (*L. monocytogenes* and serovar Typhimurium). This was done (i) regarding physical characteristics, as required by the proposed separation by centrifugation and (ii) chemical resistance of the bacterial pigment regarding the intended buffer composition.

To ensure that the velocity of descent of the chromogenic bacteria *M. roseus* and *S. marcescens* is equal to velocity of descent of the pathogenic target strains *L. monocytogenes* and serovar Typhimurium, centrifugation tests were performed (Koch and Blumberg 1976). *Listeria monocytogenes* EGDe (internal number 2964) and serovar Typhimurium (NCTC 12023) were grown overnight in tryptone soya broth with yeast (TSB-Y; Oxoid) at 37°C. *Micrococcus roseus* and *S. marcescens* were handled as described earlier in the text to obtain coloured bacterial cultures. One millilitre samples of the bacterial cultures were centrifuged at 5000 ***g*** for 5 min and the resulting pellet was resuspended in 1-ml double distilled H_2O for comparative velocity testing. Measurement of the optical density (OD_{600}) of the supernatant of the bacterial suspensions after 10-min centrifugation was performed in duplicates with an hp 8452 spectrophotometer (Hewlett Packard, Paolo Alto, CA, USA) to demonstrate comparability of the chromogenic strains with the actual targets. The used centrifugal forces were 4, 94 and 2348 ***g*** corresponding to 200, 1000 and 5000 rev min^{-1} in an Eppendorf 5424 centrifuge (Eppendorf, Hamburg, Germany) with a FA 45-24-11 rotor. The resulting data after measurement of OD_{600} showed correlation of the velocity of descent of *M. roseus* and *S. marcescens* cells in comparison to *L. monocytogenes* and serovar Typhimurium cells within 2·27% standard deviation for all tested centrifugation forces (Table S1).

Micrococcus roseus and *S. marcescens* pigment stability was demonstrated during the preliminary tests, covering the projected lysis buffer compounds. This was performed by incubation of a spatula-tipfull of the bacteria resuspended in one ml of the compounds at 30°C and 45°C. The samples were incubated on an Eppendorf Thermomixer 5437 at 700 rev min^{-1} for 45 min. Subsequent optical evaluation of the colour of the bacterial pellet was performed after a centrifugation at 6010 ***g*** for 5 min. *Micrococcus roseus* and *S. marcescens* were incubated in solutions of four groups of chemicals. The organic solvents n-hexan, iso-propanol, $CHCl_3$ and ethanol did not affect the red stain of the bacterial cells. Solutions containing 1–5% of the detergents SDS, Triton-X, Tween-20 and Lutensol AO-07 did also not affect the red stain of *M. roseus* and *S. marcescens*, as well as the chaotropic agents urea and guanidine/HCl at concentrations of 8 mol l^{-1}. An organic solution containing 1 mol l^{-1} HCl and 5 mol l^{-1} NaCl did not affect the red stain of *M. roseus* and *S. marcescens*, but 1 mol l^{-1} NaOH discoloured the bacteria resulting in white coloured pellets.

Overall, these preliminary findings show the suitability of *M. roseus* and *S. marcescens* to simulate *L. monocytogenes* and serovar Typhimurium during method development. Consequently, *M. roseus* and *S. marcescens* were incorporated into the large-scale development stages of the "matrix lysis" protocol, as mentioned earlier.

The number of tested parameters necessary for design and development of the "matrix lysis" protocol, based on possible combinations, totalled >400. To examine all these with conventional methods would have been prohibitive on cost and time resources. Moreover, molecular techniques, such as polymerase chain reaction analysis, were not practicable for investigating each phase of the sample (the lipid and solubilized food aqueous phases and the pellet would have had to be tested separately). If indeed separation of bacteria from the remains of the

sample was possible, laborious washing steps and a complex DNA isolation step would have complicated the procedure.

Overall, the use of chromogenic bacteria as indicators of bacteria distribution in complex matrices is time- and cost-effective. The final protocol and buffer composition of the 'matrix lysis' protocol, which reduced the food matrices to a minimal fraction, were established in a minimum of time. This protocol includes it 8 mol l^{-1} urea, 1% SDS or Lutensol AO-07 and 1 × PBS; 30 min incubation at 45°C with subsequent centrifugation at 3220 ***g*** as recently published by Rossmanith *et al.* (2007) and Mayrl *et al.* (2009). As internal controls, chromogenic bacteria can be matched to particular experimental circumstances and permit methodological flexibility. The broad range of colours represented within the various species of pigmented bacteria permits numerous experimental possibilities and the use of chromogenic organisms other than bacteria, e.g. chromogenic yeast species (Punkari and Henrici 1933), could widen the range of applications significantly.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Tests of various physical and chemical properties to demonstrate the suitability of *Micrococcus roseus* and *Serratia marcescens* as control organisms for matrix lysis method development.

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4.4.1. Supplement to Rossmanith et al., 2010c; Lett. Appl. Microbiol., 50, 230-3.

Table S1:

Table 1 Tests of various physical and chemical properties to demonstrate the suitability of *M. roseus* and *S. marcescens* as control organisms for matrix lysis method development.

	M. roseus	*L. monocytogenes*	*S. marcescens*	*S.* Typhimurium
OD_{600} *	1.305 (±0.001)	0.980 (±0.001)	1.456 (±0.002)	2.006 (±0.008)
Centrifugation† ***g*** / rpm	% of OD_{600} of bacterial samples after centrifugation			
4 / 200	93.2	96.0	98.2	99.2
94 / 1000	85.2	88.6	86.3	87.4
2348 / 5000	2.5	3.4	2.5	2.4
Chemical treatment‡	Bacterial cell colour following treatment			
1 mol l^{-1} NaOH	+§	-**	w††	-
1 mol l^{-1} HCl	+	-	+	-
n-Hexane	+	-	+	-
Isopropanol	+	-	+	-
Ethanol 96%	+	-	+	-
$CHCl_3$	+	-	+	-
5 mol l^{-1} NaCl	+	-	+	-
1 × PBS	+	-	+	-
Exact™ industrial detergent	+	-	+	-
Ringer´s sol.	+	-	+	-
1 - 5% SDS	+	-	+	-
1 - 5% AO-07, Lutensol	+	-	+	-
1 - 5% Triton-X	+	-	+	-
1 - 5% Tween 20	+	-	+	-
8 mol l^{-1} guanidine/HCl	+	-	+	-
8 mol l^{-1} urea	+	-	+	-

* OD_{600} of the bacterial samples before centrifugation: Measurement of the optical density (OD600) of the bacterial cultures was performed in duplicate with an hp 8452 spectrophotometer (Hewlett Packard, Paolo Alto, USA).

† Centrifugation: One millilitre of an overnight culture was centrifuged for 10 min in an Eppendorf 5424 using a FA 45-24-11 rotor, to provide corresponding physical characteristics in accordance with sample composition.

‡ 30°C and 45°C, 45 min. Performed to test the stability of the chromogenic stain under various conditions.

§ +: red colour of the bacterial cells after treatment with the respective chemical.

** -: Not determined.

†† w: white. *S. marcescens* pigmentation was not stable under these conditions. NaOH did not support solvation of the tested foodstuffs during matrix lysis and was therefore excluded during development of the protocol before coloured bacteria were introduced to the experimental workflow.

5. Discussion/Outlook

From a historical point of view this work reflects the progress of the scientific development in the field of molecular biological methods in food pathogen detection over the last few years: The successful advancement of conventional PCR to qPCR to obtain quantitative results; the investigation of various auxiliary factors such as inhibition of the enzymatic reaction, possibilities for enhancing the enzymatic reaction and the testing of various DNA isolation/purification methods; the development of time saving, but nevertheless qualitative combined enrichment/qPCR approaches, and the direct application of qPCR to food samples; and discussion of the necessary design of food pathogen detection methods by means of a sequence of successive methods leading to an analytical chain. This has led to extensive research in the topic of sample preparation. Given this analytical chain there are some prerequisites for reliable molecular biological food pathogen detection using qPCR, which can be summarized in an analytical trihedron (Fig. 3.). This analytical trihedron comprises three pillars providing the basis for optimal performance and unique determination of such an analytical chain: (i) reliable sample preparation, (ii) process controls for all crucial steps of the analytical chain, and (iii) thorough validation and specification of every step of the protocol, especially of the core detection method *per se*.

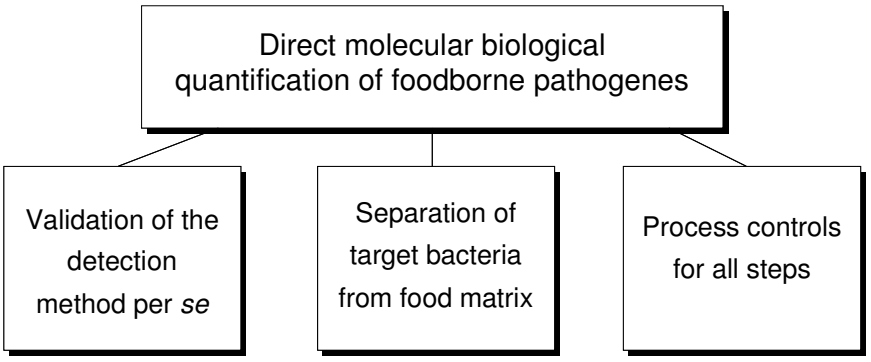

Figure 3. The analytical trihedron of food pathogen detection.

At this point recent scientific work has demonstrated that combined enrichment/qPCR and direct approaches are compromises, which meet the given requirements only to a certain degree. Combined approaches do successfully support traditional molecular biological methods, but nevertheless are not frequently used alone (Rossmanith et al., 2010a). Furthermore, DNA isolation/purification is necessary and sample preparation is the most promising approach to eliminate the influence of heterogeneous food matrices.

The qPCR assay presented in this work to detect the *prfA* locus was especially carefully validated to achieve a most reliable quantitative basis for all further developmental steps. The Poisson based testing of the assay at its limiting extreme was qualitative only, but nevertheless fulfilled a first step to validation (without comparison to a standard method) of this enzymatic reaction. This is routinely performed in analytical chemistry. A next step would be the extension of this test algorithm to include the quantitative purposes of qPCR as suggested by Rossmanith and Wagner (2010c and d). This corresponds to Boundary Limit Analysis, a test system regularly used in software engineering and electronics (Rossmanith and Wagner 2010a, c and d).

The influence of the fluorescence characteristics of enrichment broths on qPCR detection is a further reference to how important thorough testing and validation are, with respect to all factors possibly interfering with the underlying methodology of molecular biological food pathogen detection.

The presented sample preparation method was successfully demonstrated for *L. monocytogenes,* a Gram-positive bacterium. Gram-negative bacteria were not covered as the harsh chemistry employed lyses the bacterial cells during incubation of the food sample. A necessary further development is the modification of the buffer composition to extend the protocol to Gram-negative bacteria such as *Salmonella* (Mayrl et al., 2009). A further necessity is conservation of the viability status of the bacterial cells during the sample

preparation protocol. This is necessary as the question as to whether a contaminating bacterium is capable of proliferation is crucial to the assessment of the actual hazard. Therefore the buffer composition had to be developed further (Mester et al., 2010).

In summary, several of the tasks and questions that are fundamental to the successful broad implementation of qPCR into food pathogen diagnostics have been successfully executed during this work. The resulting analytical chain and the prerequisites summarized in the analytical trihedron together pose the scientific questions for future investigations.

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7. Curriculum and Publications

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11/06 –	Group Leader, Christian Doppler Laboratory for Molecular Food Analytics, University of Veterinary Medicine, Vienna. http://www.vetmeduni.ac.at/milchhygiene/forschung/cd-mofa/

PATENTS AND PATENT - APPLICATIONS

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STANDARDS

Validation of a national reference method for detection of *L. monocytogenes* of the §64, LFGB-Arbeitsgruppe "Molekularbiologische Methoden - Mikrobiologie" from Federal Office of Consumer Protection and Food Safety Berlin (BVL) in consideration of the method published in Res. Microbiol. 157, 763-771, Rossmanith et al., (2006).

APPLICATIONS

PCRFast® Listeria monocytogenes Realtime (IF/MR1002). *L. monocytogenes* Detection Kit. IFP Institute for Product Quality (Institut für Produktqualität GmbH, Berlin, Germany)

> Based on: Rossmanith, P., Krassnig, M., Wagner, M., Hein, I., 2006. Detection of *L. monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA* gene. Res. Microbiol. 157, 763-771, Rossmanith et al., (2006).

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- Rossmanith, P., Süß, B., Wagner, M., Hein, I.
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  19. DOSCH Symposion der Österr. Gesellschaft für Hygiene, Mikrobiologie und Präventivmedizin, 21.- 23.05.2007.

- Röder, B., Rossmanith, P., Hein, I., Wagner, M.
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- Mester, P., Röder, B., Mayrl, E., Huehn, S., Wagner, M., Rossmanith, P.
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  Enrichment /real-time PCR as a useful tool for the detection of food borne pathogens. *20th International ICFMH Symposium, Bologna, 29.08.-02.09.2006, p. 423.*

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  Development of a real-time PCR assay for direct detection and quantification of *Listeria monocytogenes* regarding to validation and standardization of online quantification during foodstuff production concerning food borne microorganisms.

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- Mayrl E., Mester P., Wagner, M., Rossmanith, P.
  Die Lebend/Tot – Problematik beim Nachweis bakterieller Zellen mittels molekularer Diagnostik: Implikation und Auswirkungen auf die Entwicklung von Matrix-Lysis als Probenvorbereitungsmethode
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  Cloning and characterization of a Δ-*prfA Listeria monocytogenes* strain containing a single copy genomic artificial internal amplification control (IAC) for use as internal sample process control. *IAFP 2010, 6th European Symposium on Food Safety, Dublin, Ireland, 9.6.-11.6.2010, p 55.*

- Rossmanith, P., Wagner, M.
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  Cloning and characterization of a Δ-*prfA Listeria monocytogenes* strain containing a single copy genomic artificial internal amplification control (IAC) for use as internal sample process control. *2nd ÖGMBT Annual Meeting 2010; Molecular and Applied Biosciences Austria 2010. SEP 27-29, Vienna, AUSTRIA, p99.*

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  Micrococcus roseus and *Serratia marcescens* as coloured bacterial indicators: A simple strategy during design and development of a new method for sample pre-treatment. *2nd ÖGMBT Annual Meeting 2010; Molecular and Applied Biosciences Austria 2010. SEP 27-29, Vienna, AUSTRIA, p99.*

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  Demonstration of the effective performance of a combined enrichment/real-time PCR method targeting the prfA gene of Listeria monocytogenes by testing fresh naturally contaminated acid curd cheese. *2nd ÖGMBT Annual Meeting 2010; Molecular and Applied Biosciences Austria 2010. SEP 27-29, Vienna, AUSTRIA, p98.*

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  Christian Doppler Laboratory for Molecularbiological Food Analytics: Vorstellung des Christian Doppler Labors *2nd ÖGMBT Annual Meeting 2010; Molecular and Applied Biosciences Austria 2010. SEP 27-29, Vienna, AUSTRIA.*

DIPLOMA THESIS

- Rossmanith, P.
  Nachweis von DNA–Sequenzen des Toxingens *apxIV* und des Oberflächenantigens *omlA* mittels Polymerasekettenreaktion zur Detektion von *Actinobacillus pleuropneumoniae* in Schweinebeständen.

PUBLICATIONS (OTHER)

- „Wie kommt das Bakterium aus dem Käse“
  News; Nr. 40/2008, S 79.

- Nominee Wiener Zukunftspreis
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- Broschüre der AWS (Austria Wirtschaftsservice) gemeinsam mit BMWFI und BMWF.
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  Beitrag Vetmeduni: CD-MOFA, Sichere Lebensmittel – Molekulare Lebensmittel-analytik: ein erfolgreicher Brückenschlag zwischen Grundlagenforschung und industrieller Anwendung.

- „Erreger von Ärgernis und Durchfall“
  Standard; 29.9.2010; Forschung Spezial; S11.

AWARDS

- „Hygienepreis 2007“ of the „Austrian Society for Hygiene, Microbiology and Preventive Medicine“ („Österreichischen Gesellschaft für Hygiene, Mikrobiologie und Präventivmedizin“, ÖGHMP) funded by Unilever.

- Nominee „Zukunftspreis der Stadt Wien 2008“.

- Overall winner: Poster award, IAFP 2009, 5th European Symposium on Food Safety, Berlin, Germany, 7.9.-9.9.2009: Rossmanith, P., Frühwirth, K., Süß, B., Schopf, E., Wagner, M. *Micrococcus roseus* and *Serratia marcescens* as coloured bacterial indicators: simple strategy during design and development of a new method for sample pre-treatment.