

INNOVATIVE TOOLS FOR DETECTION OF CONTAMINANT MICRO-ORGANISMS IN THE POULTRY FOOD SUPPLY CHAIN

**Marmioli N., Palumbo G., Consigli C.,
Agrimonti C., Sanangelantoni A., Maestri E.**

Division of Genetics and
Environmental Biotechnology-
Department of Environmental Sciences- University of Parma

Abstract. This work describes the development of rapid tests based on Quantitative Real Time PCR to monitor the microbiological load in poultry samples. An interest in developing DNA based methods has been raised by control and surveillance bodies and companies dealing with poultry supply chain, as emerged from a survey conducted with written questionnaires and phone interviews. Among the questions: i) the bacterial species of greatest interest, ii) the method actually used for their detection, iii) the interest about the implementation of DNA rapid methods. Bacterial species of highest interest resulted *Campylobacter*, *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogenes*. Prompted by the results of these interviews we have developed Real Time PCR test to detect and enumerate bacterial species in commercial poultry samples. So far we have developed pairs of primers suitable for this kind of tests which are specific for *Salmonella enterica* and the other for *Listeria monocytogenes*. They have been used to construct a standard curve to quantify the DNA of these species in poultry samples. Primer identification of other species, either pathogen or spoilage microorganisms, is still in progress.

Keywords: poultry, supply chain, Real Time PCR, bacteria detection

Introduction

The results here reported have been obtained within the EC 6th Framework Programme Integrated Project CHILL-ON “*Developing and integrating novel technologies to improve safety, transparency and quality assurance of the chilled/frozen food supply chain. Test case fish and poultry*”. The objective of this project is to improve the chilled frozen supply chain to extend product shelf life, improving both low temperature technologies and microbiological controls.

A certain level of safety in food supply chains is also achieved by applying systems and mechanisms as Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and Hazard Analysis Critical Control Points (HACCP). In all these systems, tests for microbial

contamination are applied: standard detection tests, counting methods, typing and fingerprinting. Methods and targets for analysis have been prescribed by legislation: the European Union has a new and comprehensive legislation on the themes of food safety and on the application of HACCP, which includes: Regulations (EC) 852/04, 853/04, 854/04, 882/04, 183/05, 2073/2005 and Directive 2002/99. The Regulation 2073/2005 establishes in particular the microbiological criteria for foodstuffs. The main issues are related to the application of HACCP and of GHP and the microbiological parameters are instrumental to the validation and testing of HACCP procedures and in defining those values which are considered a limit for acceptability.

This Regulation has taken into account opinions and position papers issued on different micro-organisms by the Scientific Committee on Veterinary Measures relating to Public Health and by the Scientific Panel on Biological Hazards of EFSA, international guidelines, and especially the Codex Alimentarius guideline on "Principles for the establishment and application of microbiological criteria for foods". Novelty in this Regulation is the attribution to food operators of the responsibility in deciding the sampling and testing frequency as part of an autonomous HACCP procedure. It also establishes reference methods associated with each microbiological criterion, but it allows any food operator to choose alternative methods, even more rapid, as long as the results are reliable and the method was validated and authorised by competent authorities.

In a recent publication, EFSA (2006) has addressed the topic of food-borne zoonoses and identified the main causative agents: *Salmonella*, *Campylobacter*, *Listeria monocytogenes*, VTEC *Escherichia coli*, *Yersinia*, *Trichinella*, *Echinococcus*, *Toxoplasma* and *Cysticercus*. The first two organisms are the most commonly reported in case of infections in humans and it is recognised that their main occurrence is from poultry meat (Zhao et al., 2001; Wilson, 2002). According to EFSA (2005) poultry meat is the major source of sporadic

campylobacteriosis: the etiological agent, *Campylobacter*, is a thermophilic Gram negative bacterium. Infection occurs mainly because of contamination from poultry meat to ready-to-eat foods, hand to mouth contact and undercooked meat. Few hundred bacteria are considered sufficient to cause disease. *Campylobacter* spp. are relatively inactive from the biochemical point of view, and their speciation with classical biochemical tests is difficult (Logan et al., 2001). EFSA (2005) claims that there is a need for improved application of new typing methods for *Campylobacter*, and for more qualitative and quantitative data sources on its occurrence in food chains.

Poultry meat is also a major source of salmonellosis, caused by *Salmonella* spp. The highest numbers of outbreaks rest from *Salmonella enteridis* and many others depends on *S. typhimurium*. The ISO methods for the detection of these micro-organisms in food samples requires pre-enrichment and selective enrichment followed by isolation and confirmation. More than seven days are normally required for this entire procedure.

Other bacteria, such as *Listeria* and *Escherichia coli*, represent important source of concern during the production and distribution of poultry meat and during preparation.

The general objective of our work is to develop appropriate tools to monitor the presence or absence of micro-organisms of interest for human health and for product quality in the chilled/frozen poultry processing chain. The final goal is to provide methods and materials along with the information to start an efficient HACCP procedure and for quality certification at national and international (ISO) level. The work will include analysis of poultry samples along supply chain and involve the comparison between existing officially prescribed methods based on microbial culture and those based on DNA extraction and PCR analysis which are still in development and have not been validated (Jaykus, 2003).

The work has been divided in three parts: i) a survey among the producers, retailers and control agencies for establishing a list of poultry and bacteria species relevant for industries;

ii) the evaluation of the efficiency of existing methods and the optimization of standard methods for an efficient retrieval of contaminant bacteria from food; iii) the development of multiplex and quantitative PCR (qPCR) methods for the detection and enumeration of the bacteria of interest. At this purpose specific primers have been designed on sequences of bacterial genes and applied to qualitative and quantitative PCR analysis. Parameters considered for assessing the efficiency of these new test will be the limits of quantification (LOQ) and the limit of detection (LOD).

Material and methods

An international survey among poultry producers

The tools utilised were written questionnaires and phone interviews. Five questions were delivered: 1. Which bacteria are currently analysed in your company when checking contamination of poultry material and/or live animals; 2. Which bacteria are routinely analysed in order to comply with the requirements of the specific legislations of your country; 3. Which analytical methods are currently used in your company for detection and/or typing of the bacteria in the poultry food chain; 4. Which species of groups of bacteria could be of additional interest for your company, besides those you already analyse; 5. Would the company be interested in new analytical methods based on molecular analysis (DNA proteins); 6. Which would be the requirements in terms of speed of obtaining of the results; 7. How much will you be willing to pay for a test of microbial contamination.

For the distribution of the written version of the questionnaire, e-mail was exclusively employed. More than 50 companies of European and Extra-European countries were contacted (Table 1).

Table 1. List of companies and countries contacted during survey

Country	N° of companies
Brazil	2
Chile	2
China	2
Germany	12
Greece	5
Iceland	3
Israel	1
Italy	18
Netherlands	8
United Kingdom	2

Primer design for RealTime Quantitative PCR (qPCR)

Primers were designed to amplify candidate genes of *Campylobacter jejuni*, *Salmonella enterica*, *Listeria monocytogenes* and *Staphylococcus aureus*.

Candidate genes for primer design were selected on the basis of information available in literature. In general regions showing no or low similarity with other bacteria genomes were selected, after a BLAST (Altschul et al., 1997) search against the microbial DNA sequences available at NCBI.

Different softwares (Oligo analyzer 1.2, Fast PCR and Primer 3) were used to design primers in species-specific regions of each candidate gene. The selected oligonucleotides were analyzed for: i) the annealing temperature 58-60 °C; ii) the amplicon size 70-80°C; iii) the primer size 18-22 bp and; iv) the loss of secondary structures.

DNA extraction

Genomic DNA isolated from certified ATCC bacterial strains was purchased from LGC Promochem (Teddington, UK). DNA was also extracted from 1 gr of chicken meat using kit Puregene (Gentra- Minneapolis, USA) and from 1 gr of soil using Fast DNA Spin Kit (Q-Bio Gene Irvine, CA)

Quantitative Real Time PCR

Reference standard curves for quantification were made with the selected primers using serial dilutions of DNA of each bacteria corresponding to 100000, 10000, 1000, 100 and 1 genome copies. PCR reactions were carried out in ABI PRISM[®] 7000 (Applied Biosystems, Foster City, CA-USA) using SYBR[®] GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA), following the supplier recommendations. Three replicates for each dilution were carried out.

Results and discussion

Survey among poultry food chain producers

All Institutions contacted replied to the first question of the survey, which contributed to the identification of the micro-organisms of highest interest: *Salmonella* spp., *Listeria/Listeria monocytogenes*, *Campylobacter*, *Escherichia coli* and *Staphylococcus aureus* for routine analyses, while interest for sporadic analyses concerned *Pseudomonas*, *Enterococci* and other micro-organisms, such as moulds and yeasts (Figure 1). Some companies raised the issues of flock control in live animals for detection of pathogenic bacteria before slaughter.

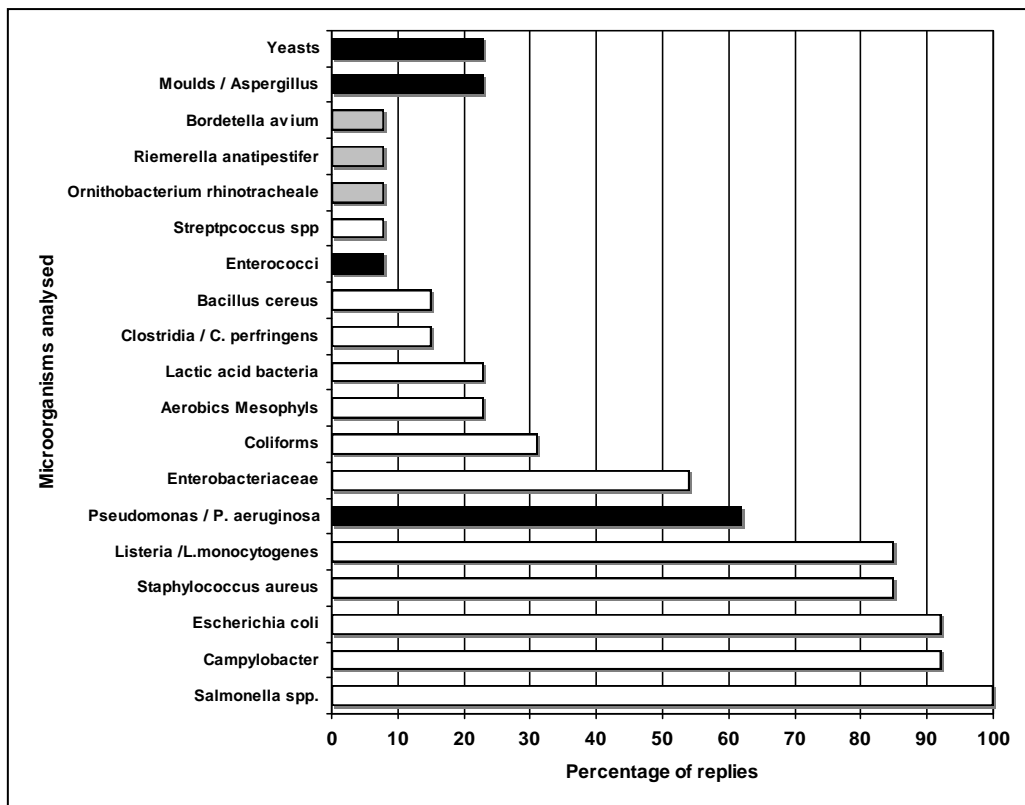


Figure 1. Micro-organisms of highest interest to poultry companies, as resulted from the analysis of the questionnaire. White bars represent routine analysis, and black bars sporadic analyses. The grey bars indicate organisms which are monitored in living animals for diagnostic purposes.

All companies, even those outside Europe, were referring to the EU legislation, in particular Regulation (EC) 2073/2005. The legislation requires the assessment, in samples of neck skin, of total bacteria at 30°C, *Escherichia coli*, *Salmonella* spp., and *Listeria monocytogenes*. *Salmonella* and *Escherichia coli* are searched also in prepared foods.

Approximately 25% of companies make also use of tests based on DNA, but the smallest companies pointed out they do not have this technical and economical possibility. According to the survey, DNA based tests are currently performed mainly with the aid of analytical laboratories outdoor or, in big companies, within their own laboratories. In general, laboratories perform DNA analysis without using commercial kits. It is felt that commercial

kits, which do not disclose the target sequences, do not give enough confidence. Preference is given to tests adapted from scientific literature and EC consortia for testing and validation.

Regarding future needs, most companies have identified the detection and typing of *Campylobacter* in skin samples and quantification of bacteria and/or virulence factors through Real Time PCR as an important issue. Other issues concerned *E. coli* VTEC, *Listeria* and spoilage bacteria (especially *Lactobacillus* and *Pseudomonas* spp.). Some companies pointed out that they have to deal also with pathogens infecting birds in slaughterhouses, even when they are not responsible for zoonoses.

Companies expressed a interest in PCR-based methods to detect *Salmonella*, *Campylobacter*, *Listeria* and *Staphylococcus*. A general requirement was for a rapid and simple test to give results in 2-4 hours, or at least within the same day of sampling. Though all companies perform the controls required by legislation exclusively using official methods prescribed by legislation, additional controls are carried out, referring to National requirements or to voluntary monitoring plans. In this case, official methods are always used but there is occasion also for tests based on protein or DNA analysis.

Companies are also willing for additional tests for bacteria they are not able to analyse. The highest number of requests concerned typing and quantification of *Campylobacter*, which is seen as a possible target for future legislation (recent publications of EFSA, 2005, 2006). In this case, PCR-based methods are the only practical option.

All companies have underlined that the innovative tests should be cost-effective, rapid and simple; which may favour the DNA based analysis.

Development of qPCR

On the basis of results of the survey, the research was aimed to develop qPCR assays to detect and quantify *Salmonella*, *Campylobacter*, *Listeria*, and *Staphylococcus*.

Instead of traditional qPCR based on TaqMan probes (Afonina et al., 1997; Kutuyavin et al., 2000) that are rather expensive, we tried to develop a quantification method based on SYBR Green (De Medici et al., 2003).

Firstly a literature search was performed to find sequences which can be used for drawing specific primers for each of the species mentioned above. We tried the specificity of these primers with BLAST algorithm against micro-organism sequences available at NCBI. Finally we obtained a number of primers varying from 3, for *Listeria monocytogenes*, to 12 for *Campylobacter jejuni*

Afterwards we tested all these primers in Real Time PCR with SYBR Green to assess: *i*) the absence of multiple amplicons; *ii*) the intensity of amplicon fluorescence and *iii*) the absence of primer dimers. Finally the best primers resulted JEJ1 and JEJ3 for *Campylobacter jejuni*, L3 for *Listeria monocytogenes*, SA4 for *Salmonella enterica*, and AU3 for *Staphylococcus aureus*. Standard curves were constructed with these primers based on serial dilutions of purified bacterial DNA. The standard curves will be used to quantify bacterial DNA in poultry samples. The quality of standard curve was evaluated considering the parameters recommended for Real Time PCR such as: *i*) the limit of detection (LOD): 1 copy; *ii*) the limit of quantification (LOQ): 10 copies; *iii*) the correlation between quantity of DNA and threshold cycles (R^2)= 0.99; *iv*) the distance between serial dilutions: 3 cycles.

The standard curves obtained fitted with these parameters when using primers SA4 (*Salmonella enterica*) and L3 (*Listeria monocytogenes*). As evidenced in Figure 2, qPCR can detect down to one copy of bacterial genome.

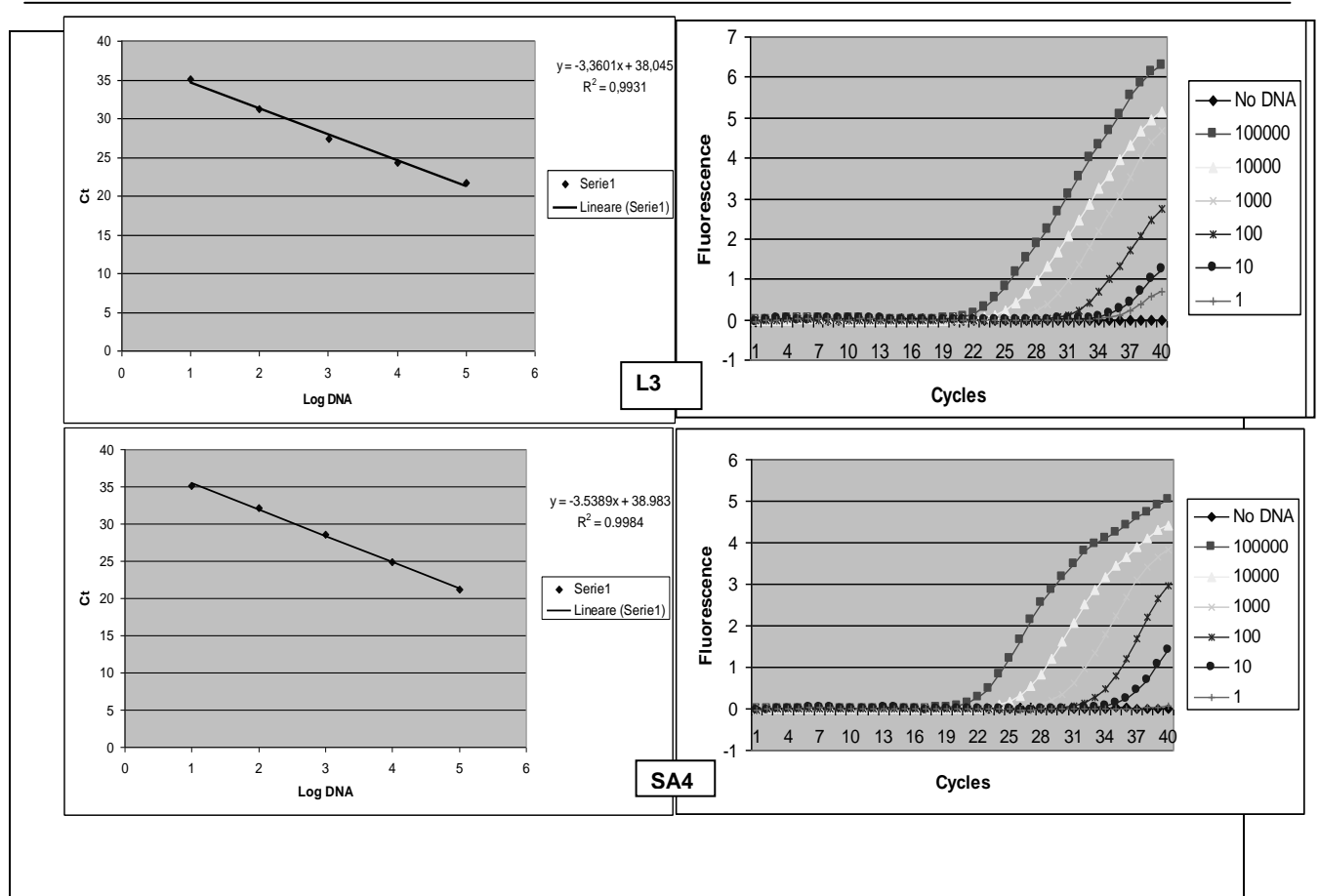


Figure 2. Left: reference curve for Real Time obtained from serial dilutions of DNA of *Listeria monocytogenes* (L3) and *Salmonella enterica* (SA4). On the right the original plots are represented.

Primers for *Campylobacter* and *Staphylococcus* were also tested, but the standard curves obtained need further improvements.

To check that primers L3 and SA4 do not cross amplify with other bacterial species we conducted PCR on DNA extracted from soil samples, that contain an high number of bacterial species. No cross amplifications were observed (data not shown) confirming that these primers are sufficiently specific to recognize *Salmonella* and *Listeria* in food samples. The same primers were also tested on DNA extracted from chicken meat, to assess that no cross-amplifications occurs. Since no amplifications were observed we concluded that these primers

can be considered suitable to assess the presence of *Salmonella* and *Listeria* in chicken samples.

Conclusions

A survey conducted in companies and institutions dealing with the poultry food chain evidenced an interest for PCR-based methods to detect *Salmonella*, *Campylobacter*, *Listeria* and *Staphylococcus*. A general requirement was for rapid tests which may give results in 2-4 hours or at least within the same day of sampling. We have developed a method based on Real Time quantitative PCR to quantify the DNA of the bacterial species mentioned above. At this purpose a considerable number of primers designed on bacterial sequences was tested. So far, primers L3 for *Listeria monocytogenes* and SA4 for *Salmonella enterica* have been used to construct a standard curve which quantifies the DNA of these bacterial species in poultry samples. Tests of primers for *Campylobacter jejuni* and *Staphylococcus aureus* are in progress.

Acknowledgements

This study has been carried out within the Integrated Project FP6-016333-2 "Developing and integrating novel technologies to improve safety, transparency and quality assurance of the chilled/frozen food supply chain-test case fish and poultry"(CHILL-ON) and supported by European Commission Sixth Framework Programme.

This work was also supported financially by Emilia-Romagna (IT) Regional Project SIQUAL within the research framework PRRIITT, Misura 3.4., and by EU Project "Implementation of GNSS tracking and tracing Technologies for EU regulated domains" (MENTORE)

References

- Afonina I., Zivarts M., Kutuyavin I., Lukhtanov E., Gamper H., Meyer R.B. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res.* 1997, 25, 2657 – 2660.
- Altschul S.F., Madden T.L., Schaffer A.A., Zhang J.H et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* 1997, 25, 3389-3402.

- De Medici D., Croci L., Delibato E., Pasquale S., et al., Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time PCR to detect *Salmonella enterica* serotype *enteridis* in poultry. *Appl. Environ. Microb.* 2003, 69, 3456-3461.
- EFSA Scientific Report of the Scientific Panel on Biological Hazards on the request from the Commission related to *Campylobacter* in animal and foodstuffs. Annex to The EFSA Journal 2005, 173, 1-105.
- EFSA Opinion of the Scientific Panel on Biological Hazards and of Scientific Panel on Animal Health and Welfare on "Review of the Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Antimicrobial Resistance in the European Union in 2004". The EFSA Journal 2006, 403, 1-62.
- Logan J.M.J., Edwards K.J., Saunders N.A., Stanley J. Rapid identification of *Campylobacter* spp., by melting peak analysis of bioprobes in real time PCR. *J. Clin. Microbiol.* 2001, 39, 2227-2232.
- Kutyavin I.V., Afonina I.A., Mills A., Gorn V.V., Lukhtanov E.A., Belousov, E.S., Singer, M.J., Walburger D.K., Lokhov S.G., Gall A.A., Dempcy R., Reed M.W., Meyer R.B., Hedgpeth J. 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res.* 2000, **28**, 655-661.
- Jaykus, L.A. Challenges to developing real-time methods to detect pathogens in food. *ASM News* 2003, 69, 341-347.
- Zhao C., Ge B., De Villena J., Sudler R., et al. Prevalence of *Campylobacter* spp., *Escherichia coli* and *Salmonella* serovars in retail chicken, turkey, pork and beef from the Greater Washington, D.C., area. *Appl. Environ. Microb.* 2001, 67, 5431-5436.
- Wilson I.G. *Salmonella* and *Campylobacter* contamination of raw retail chickens from different producers: a six year survey. *Epidemiol. Infect.* 2002, 129, 635-645.
- Wolffs P., Knutsson R., Norling B., Rådström P. Rapid quantification of *Yersinia enterocolitica* in pork samples by a novel sample preparation method, flotation, prior to real-time PCR. *J. Clin. Microbiol.* 2004, 42, 1042-1047.