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Evaluation of real-time PCR vs automated ELISA and a conventional culture method using a semi-solid medium for detection of *Salmonella*

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ABSTRACT

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Aims: Evaluation of iQ-Check PCR *Salmonella* for *Salmonella* detection in artificially and naturally contaminated food and environmental field samples.

Methods and Results: Artificially contaminated samples (poultry meat and ground red meat) subjected to cold- and freeze-stress, and 120 naturally contaminated samples (swabs and meat) were tested for *Salmonella* using the diagnostic semi-solid *Salmonella* medium (DIASALM) method, the Vidas assay and the iQ-Check PCR assay after 24 h enrichment in buffered peptone water.

Conclusions: Both the iQ-Check PCR and the Vidas assay provide a rapid and user friendly screening method for detection of *Salmonella*. False negative samples were obtained for the inoculated samples using both the iQ-Check PCR assay and the Vidas method when *Salmonella* cells were severely stressed. In total 45 of 120 naturally contaminated field samples showed *Salmonella* positive using the DIASALM method. The agreement percentage with the DIASALM method was respectively 92% for the iQ-Check PCR and 95% for the Vidas method.

Significance and Impact of the Study: False-negative samples were obtained for the inoculated samples using both the iQ-Check PCR assay and the Vidas method when *Salmonella* cells were severely stressed, e.g. freezing at −18°C for 7 days. Of the 120 naturally contaminated field samples 45 showed *Salmonella* positive using the DIASALM method. The agreement percentage with the DIASALM method was 92% for the iQ-Check PCR and 95% for the Vidas method respectively.

Keywords: environmental swabs, naturally contaminated samples, pork, poultry.

INTRODUCTION

Detection of *Salmonella* is an important parameter in microbiological analysis to control food safety. Different methods have been developed in order to reduce the time required for the detection of the pathogen, as the standard culture method (ISO 6579) requires up to 5 days and is not suitable for routine testing of large numbers of samples. As a result of the low incidence of nonmotile *Salmonella* strains (Holbrook *et al.* 1989), the detection methods based on the motility of this organism are being used more often in routine laboratories. It was established that standard culture methods using semi-solid media such as modified semi-solid Rappaport-Vassiliadis (MSRV) or diagnostic semi-solid *Salmonella* medium (DIASALM) produce results that are equivalent to those obtained by ISO 6579 for the detection of *Salmonella* in foods of animal origin (Van der Zee *et al.* 2002). As an alternative to culture methods, immunological methods in different formats have been introduced for rapid detection of *Salmonella* (Patel 2000). Vidas is an automated ELISA method based on the detection of *Salmonella* using specific antibodies coated on the inner surface of a tip-like

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disposable pipette which is introduced into the Vidas system along with the Vidas *Salmonella* strip containing the boiled *Salmonella* culture (Curiale *et al.* 1997; Keith 1997). The Vidas system enables high-throughput and rapid screening of large number of samples for presence of *Salmonella*. Recently, iQ-Check real-time PCR was introduced for rapid detection of *Salmonella* in foods. The iQ-Check real-time PCR is an updated version of the Probelia™ PCR System. The iQ-Check PCR is a qualitative homogenous real-time PCR assay using a specific fluorescent probe, a molecular beacon, which hybridizes to the amplified product during the PCR annealing step and allows the determination of PCR product in real-time whereas the Probelia™ PCR kit was based on DNA amplification by PCR followed by probe hybridization in a 96-well plate for colorimetric detection. The Probelia™ PCR system has been the subject of comparative evaluations and has been shown to generate results that are consistent with the standard culture method (Fach *et al.* 1999; Wan *et al.* 2000). Recent advancements in the PCR technology have enabled real-time detection of the PCR product eliminating the need of post-PCR processing (Cockerill and Smith 2002). Real-time PCR can be carried out entirely in sealed PCR tubes thus reducing the risk of cross-contamination which is a major drawback for the introduction of PCR methods in a routine microbiological laboratory. Real-time PCR has been developed for rapid *Salmonella* detection using different fluorescent-based systems for detection of PCR products. Eyigor *et al.* (2002) used the double-stranded DNA binding dye SYBR Green I for real-time monitoring of *Salmonella* PCR product and implemented the real-time PCR assay for detection of *Salmonella*-positive poultry flocks. Chen *et al.* (1997) evaluated the 5′ nuclease real-time PCR (Taqman) for the detection of *Salmonella* in foods of animal origin. Knutsson *et al.* (2002) further investigated the robustness of the 5′ nuclease real-time PCR for optimization in combination with an enrichment procedure for *Salmonella* detection. A highly sensitive and specific molecular beacon assay was developed by Chen *et al.* (2000) to detect the presence of *Salmonella*, however, no applications of molecular beacon based real-time PCR for detection of *Salmonella* in foods were reported. The purpose of this study was to compare the iQ-Check PCR *Salmonella* with the Vidas *Salmonella* method and the cultural method using the semi-solid DIASALM medium for *Salmonella* detection in artificially and naturally contaminated food and environmental field samples.

MATERIALS AND METHODS

Culture media, Vidas reagents and PCR reagents

Salmonella enterica subsp. *enterica* serotype Enteritidis (*Salmonella* Enteritidis) LMG 10395 was obtained from the BCCM Culture Collection of the Laboratory of Microbiology (University of Gent). All media were obtained from Oxoid (Basingstoke, UK) except for the diagnostic semi-solid *Salmonella* medium (DIASALM; LabM, IDG, Bury, UK). A *Salmonella* working stock was maintained at 4°C on nutrient agar (NA). Fresh cultures were prepared by growing the culture in brain–heart infusion (BHI) broth at 37°C for 18–20 h. All reagents for the Vidas ELISA kit were supplied with the Vidas *Salmonella* sp. Kit (BioMérieux, Marcy-l'Etoile, France) and all reagents for the preparation of bacterial DNA and the PCR were supplied with the iQ-Check *Salmonella* sp. Kit (Bio-Rad, Marnes La Coquette, France) and were used in accordance with the protocols recommended by the manufacturer.

Samples and methods comparison study for detection of *Salmonella*

Poultry carcass samples (neck skin, 10 g) and swabs from pork carcasses (600 cm^2 by combining four swabs each covering 150 cm^2) were collected from several local abattoirs in the frame of the national *Salmonella* surveillance programme, environmental swabs were collected from a laying hen farm. Retail samples of poultry carcasses/poultry parts (100 cm^2 skin), poultry meat and meat preparations (25 g), and pork/beef ground meat (25 g) were obtained from the distribution centre of a large food chain in Belgium. Neck skin samples were added to 100 ml buffered peptone water (BPW); swabs were put into 225 ml BPW. The 100 cm^2 skin excised from poultry carcasses/poultry parts were transported to the lab in 100 ml saline/peptone solution (1 g peptone and 8·5 g NaCl per litre) and on arrival in the lab added to 100 ml double strength BPW. The 25 g samples of poultry meat and meat preparations were diluted 10-fold in BPW (final volume 250 ml). Subsequently, samples were homogenized and incubated for 24 h at 37°C. The pre-enriched samples were tested for *Salmonella* using the DIASALM method (Farghaly *et al.* 2001), the Vidas assay and were also used in the iQ-Check PCR assay. For the DIASALM method, three drops of pre-enriched BPW were inoculated in one spot into the centre of a DIASALM plate. After incubation for 18–24 h at 42°C, DIASALM plates were examined for a motility zone with a purple/black colour change. A loopful of the motile zone which is the farthest from the sample inoculum was subcultured onto xylose lysine desoxycholate (XLD) agar incubated for 18–24 h at 37°C. Presumptive colonies on XLD were purified on NA and confirmed biochemically by inoculating into triple sugar iron agar slopes, urea broth, lysine decarboxylase broth and tryptone water with final confirmation carried out using a Crystal ID EF (Becton Dickinson, Franklin Lakes, NJ, USA) identification. For the Vidas assay, 0·1 ml

pre-enriched BPW was transferred to 10 ml Rappaport-Vassiliadis soya peptone (RVS) broth, incubated for 20–24 h at 42°C, 0·1 ml of enriched RVS was transferred to 10 ml M-broth (Becton Dickinson, Sparks, MD, USA), incubated for 6 h at 42°C, 1 ml of enriched M-broth was boiled for 15 min and 0·5 ml transferred to the Vidas *Salmonella* strip for automated detection of *Salmonella* in the miniVidas apparatus.

iQ-Check PCR amplification and detection

Preparation of bacterial DNA. Bacterial cells were harvested by centrifugation (10 000 *g*, 10 min) from either 1-ml aliquots of serial dilutions of an overnight culture of *Salmonella* Enteritidis or from 1-ml aliquots of food samples pre-enriched in BPW. The cell pellet was resuspended in 200-μl cell lysis reagent (supplied with the iQ-Check PCR kit) and cell lysis was effected by heating the samples at 56°C for 15 min followed by 100°C for 8 min. After cooling to room temperature, the supernatant was collected by centrifugation (10 000 *g*, 10 min) and used as template DNA for the iQ-Check PCR amplification. DNA extracted from food samples was 10-fold diluted with DNase and RNase free water (Sigma, St Louis, MO, USA) in order to dilute inhibitory compounds which might interfere with the PCR reaction.

PCR protocol and detection of PCR products. The bacterial DNA preparation (5 μl) was added to 40 μl of *Salmonella* amplification solution (containing PCR buffer, nucleotides, *Salmonella*-specific primers, *Taq* DNA polymerase and internal control) and supplemented with 5 μl specific fluorescent probes. Amplification was performed in a dedicated thermocycler associated to a camera and a software (iCycler; Bio-Rad). The thermocycling conditions were 50°C for 2 min, 95°C for 5 min, followed by five cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 15 s, 35 cycles of 95°C for 15 s, 57°C for 15 s, 72°C for 15 s and one final cycle of 72°C for 5 min, as recommended by the manufacturer. The more amplified product appears during PCR amplification, the more fluorescence intensity increases because of hybridization of the molecular beacon probe to the PCR product. The iCycler iQ camera takes pictures of the plate during the annealing step of each cycle of the PCR and the associated software plots automatically during the amplification the relative fluorescent unit (RFU) *vs* number of cycle. This allows the detection in real-time of the PCR product and thus the presence of *Salmonella* in the sample. In the software an initial baseline for the RFU is calculated but needs to be recalculated in function of fluorescence noise and is based on the RFU value observed at the cycle number located just before the first curve rises (the fluorescence increases). When adjusting the graph to have log RFU in *y*-axis vs the cycle number in the *x*-axis it should be verified that the threshold cycle C_t (the point at which the fluorescence rises appreciably above background) is located in the linear part of the graph (corresponding to the PCR exponential phase as the scale is in log). A C_t value > 0 indicates presence of *Salmonella* in the sample.

Internal positive control. An internal positive control (IPC) system is supplied with the iQ-Check PCR kit and included in each *Salmonella* PCR assay (containing IPC primers, IPC DNA template and IPC probe) in order to determine if PCR inhibitors were interfering with the PCR system. The internal control is amplified at the same time as the *Salmonella* target gene but detected by a second fluorophore. For each PCR assay, if either or both the IPC and *Salmonella* PCR have C_t values > 0, the result of the *Salmonella* PCR was regarded as valid. If both the IPC and *Salmonella* PCR C_t values = 0, an inhibitory effect was present and the PCR assay needed to be repeated with diluted DNA samples.

Sensitivity and detection-limit determination of iQ-Check PCR

To determine the sensitivity of the real-time PCR assay, DNA was prepared from appropriate initially 10-fold and finally twofold serial dilution of a BHI culture of *Salmonella* Enteritidis in order to obtain *ca* 200, 100 and 50 CFU ml^{-1}. DNA was prepared in duplicate for each inoculum level and each DNA template was used in triplicate as an input for the iQ-Check PCR assay. The exact inoculums in the dilutions was confirmed by spread plating in duplicate on NA.

To determine the detection limit in foods, artificially spiked samples with *Salmonella* Enteritidis were prepared. Briefly, 25 g samples of each chicken leg skin and pork/beef ground meat were weighed and inoculated with 0·5 ml of appropriate dilutions of a BHI culture of *Salmonella* in order to obtain *ca* 50 and 5 CFU per 25 g. Spiked samples and a noninoculated control sample were analysed by the DIA-SALM method, the Vidas assay and the iQ-Check PCR assay after 6 and 24 h at 7°C, and after 7 days at −18°C. Duplicate samples were prepared for each sampling time.

RESULTS

The capability of the iQ-Check PCR assay to detect *Salmonella* Enteritidis numbers as low as <10 CFU per PCR reaction, corresponding to *ca* 50–500 CFU ml^{-1}, was demonstrated by using dilutions of a pure culture (Fig. 1). After a 24 h pre-enrichment step, the iQ-Check PCR test could detect *Salmonella* in artificially contaminated food samples at the lower contamination level (*ca* 5 CFU per 25 g) in eight of 12 samples (Table 1). False-negative

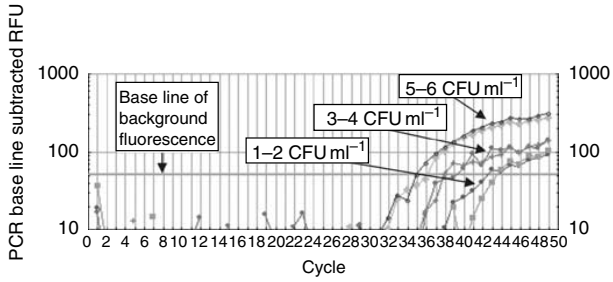

Fig. 1 Detection of low numbers of *Salmonella* (1–10 CFU ml^{-1}, duplicate experiments) using iQ-Check PCR *Salmonella* displayed in real-time (log RFU in *y*-axis vs the cycle number in the *x*-axis). The threshold cycle C_t is the cycle at which the fluorescence rises appreciably above background. A C_t value > 0 indicates presence of *Salmonella* in the sample

samples occurred most often when *Salmonella* cells were severely stressed, e.g. by freezing at −18°C for 7 days. Also the Vidas method failed to detect *Salmonella* in three of eight artificially contaminated samples (*ca* 5 or 50 CFU g^{-1}) subjected to freeze-stress. Both the iQ-Check PCR and the Vidas method detected *Salmonella* in one of the poultry control samples which showed to be naturally contaminated with low numbers of *Salmonella*. The C_t value of the iQ-Check PCR for this sample was 40·52. A high C_t value indicates that fluorescence accumulating during PCR only increased above the baseline after a long number of thermocycles indicating few target DNA molecules and thus few *Salmonella* cells being present at the PCR input. The C_t values obtained at the low inoculum level (*ca* 5 CFU g^{-1}) for the artificially contaminated samples ranged from 27·64 to 39·09. The more target DNA (in the present PCR this is *Salmonella* DNA) present at the beginning of the reaction the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background and thus lower the C_t value. The present iQ Check *Salmonella* real-time PCR is a qualitative test and preceded by an enrichment in BPW. The variation in outgrowth of potential sub-lethal injured *Salmonella* cells present in the food matrix during enrichment accounts for variation in the numbers of *Salmonella* used as an input for PCR sample preparation and thus Salmonella DNA at the start of the PCR reaction and subsequently the C_t values obtained.

The results of the method's comparison study with 120 naturally or noncontaminated foods are shown in Table 2. Of the total number of naturally contaminated field samples analysed ($n = 120$), 45 samples gave rise to isolation of a biochemical confirmed *Salmonella* isolate according to the DIASALM cultural method which was taken as the reference method ($n+ = 45$). Thus 75 samples were regarded as being noncontaminated. The relative accuracy defined according to ISO 16140 (Anon. 2002) as the agreement percentage with the reference method was 92% for the iQ-Check PCR and 95% for the Vidas method respectively. The specificity being an indication of the method's ability to discriminate negative samples was respectively 95% for the iQ-Check PCR and 96% for the Vidas method. The sensitivity being an indication of the method's ability to detect positive samples was respectively 87% for the iQ-Check PCR and 93% for the Vidas method. C_t values of iQ-Check PCR ranged from 16·59 to 42·63.

Table 1 Comparison of *Salmonella* detection from artificially contaminated samples by diagnostic semi-solid *Salmonella* medium (DIASALM) culture method, Vidas and iQ-Check PCR

Sample type	Inoculation level	Conditions before analysis	No. of positive samples		
			DIASALM	Vidas	iQ-Check PCR
Chicken leg meat	50 CFU per 25 g	6 h at 7°C	2/2	2/2	2/2
		24 h at 7°C	2/2	2/2	2/2
		7 days at −18°C	2/2	1/2	2/2
	5 CFU per 25 g	6 h at 7°C	2/2	2/2	2/2
		24 h at 7°C	1/2	1/2	2/2
		7 days at −18°C	2/2	0/2	1/2
	Control	6 h at 7°C	0/2	0/2	0/2
		24 h at 7°C	1/2	1/2	1/2
		7 days at −18°C	0/2	0/2	0/2
Pork/beef ground meat	50 CFU per 25 g	6 h at 7°C	2/2	2/2	2/2
		24 h at 7°C	2/2	2/2	2/2
		7 days at −18°C	2/2	2/2	1/2
	5 CFU per 25 g	6 h at 7°C	2/2	2/2	1/2
		24 h at 7°C	1/2	1/2	1/2
		7 days at −18°C	2/2	2/2	1/2
	Control	6 h at 7°C	0/2	0/2	0/2
		24 h at 7°C	0/2	0/2	0/2
		7 days at −18°C	0/2	0/2	0/2

Table 2 *Salmonella* detection from naturally contaminated samples using diagnostic semi-solid *Salmonella* medium (DIASALM) culture method versus Vidas and iQ-Check PCR

		DIASALM	Vidas				iQ-Check PCR			
Sample type	No. of samples	Positive samples	Positive agreement	Negative agreement	False-positive	False-negative	Positive agreement	Negative agreement	False-positive	False-negative
Poultry carcasses (neck skin, per 10 g)	40	19	18	20	1	1	16	19	2	3
Poultry carcasses and poultry parts (skin, per 100 cm^2)	30	6	5	24	0	1	5	24	0	1
Poultry meat and meat preparations (per 25 g)	15	5	4	10	0	1	5	10	0	0
Pork/beef ground meat (per 25 g)	10	4	4	5	1	0	4	6	0	0
Pork carcasses (swab)	11	2	2	9	0	0	2	7	2	0
Laying hen stable (swab)	14	9	9	4	1	0	7	5	0	2
All sample types	120	45	42	72	3	3	39	71	4	6

High C_t values were obtained for the four iQ-Check PCR-positive deviating samples (C_t ranging from 35·36 to 42·63). Upon repetition of the iQ-Check PCR starting from the same DNA extract the four false-positive results obtained with iQ-Check PCR showed a negative result. This indicated that the iQ-Check PCR result of these four samples was not reproducible and that these samples were situated near the detection limit of the iQ Check PCR assay. Repetition of the iQ-Check PCR test for the false-negative samples starting from the same DNA extract did not result in detection of *Salmonella*.

DISCUSSION

The iQ-Check *Salmonella* system is based on the amplification of the *iag*A gene (involved in the bacteria invasion process) of *Salmonella* spp. The specificity of the primer set was demonstrated by the manufacturer using over 100 *Salmonella* and non-*Salmonella* strains (Schindler *et al.* 2002). Molecular beacon possesses differential fluorescent properties based on the relative stability between its two duplex forms (hairpin and the probe-target hybrid). In the hairpin configuration, the fluorescent reporter is immediately adjacent to the quencher, so that the intensity of the background signal is minimized. When the probe encounters a single-strand target, it forms a hybrid with the target, undergoing a spontaneous conformation change that forces the arm sequences apart and causes fluorescence to occur. This concept incurs high specificity to molecular beacon probes (Tyagi *et al.* 1998). Specificity of the iQ-Check PCR and Vidas method was comparable. The Vidas method showed a better sensitivity. An unsatisfactory number of false-negative results were obtained with the iQ-Check PCR method. Positive detection of *Salmonella* was demonstrated when ≥50 CFU ml^{-1} was present in the samples used for DNA extraction. However, as in the case of food samples, the DNA extract was always 10-fold diluted before use as template DNA in PCR in order to eliminate inhibition of the PCR reaction, *Salmonella* cells present in the food samples will need to grow to minimum 500 CFU ml^{-1} during the pre-enrichment step in BPW. The overnight pre-enrichment period may not be sufficient to guarantee sufficient outgrowth for iQ-Check PCR samples especially for stressed *Salmonella* cells as shown in the artificially contaminated samples subjected to freeze-stress. Stressed cells have an increased lag time and especially if low numbers of cells are present the lag-time may vary considerably (Stephens *et al.* 1997). When cells are exposed to stress, a restricted enrichment time may decrease the overall sensitivity of the combined enrichment-PCR assay (Uyttendaele *et al.* 1998). Another reason for false-negative results can be as follows: salmonellae found in these natural samples may show atypical profile lacking motility and may not be detected by the semi-solid DIASALM culture method. In a comparison of a PCR with end-point detection and standard culture method for *Salmonella* detection in raw poultry (neck skin samples), PCR failed to detect *Salmonella* on seven samples from which the pathogen was cultured (Whyte *et al.* 2002). It has been suggested that the sensitivity of PCR assays is lower in naturally contaminated foods compared with those which have been artificially inoculated (Gouws *et al.* 1998).

It should be noted that the overall prevalence of *Salmonella* in these naturally contaminated samples (37·5%) was rather high. This can be explained because samples were chosen on the basis of former analysis results of these abattoirs and production sites and these sites were known to provide *Salmonella*-positive samples regularly.

In conclusion, the iQ-Check PCR enables rapid and specific detection of *Salmonella* in food and environmental samples. A minimum of technical expertise is needed and the fact that the PCR reaction is carried out in sealed PCR

tubes avoids the requirement of special dedicated laboratories to eliminate cross-contamination. As a rapid screening method, however, it is important in order to guarantee public health to restrict the number of false-negative results. For false-positive results, bacteriology (including identification and typing of the isolates) should be performed for confirmatory and epidemiological purposes.

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