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Development of a PCR Method for Verification of S. Dublin

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Objective

In Denmark, special rules regarding absence of *Salmonella* Dublin in beef marketed as fresh meat is agreed on by the Danish Food Authorities and the Beef producing sector. Thus, the Danish Beef Producers need a method for rapid identification of *S.* Dublin in order to avoid distributing contaminated meat. Consequently, a method for verification of *S.* Dublin based on real-time PCR was developed.

Materials & Methods:

Briefly, 9 different primer pairs targeting the virulence plasmid or the *rfbE* and *fliC* genes were designed for identification of *S.* Dublin. The 9 primer pairs were initially evaluated using a real time SYBR-Green PCR against a test panel of 36 different bacterial strains including 14 strains of *S.* Dublin.

Two primer pairs showed 100% specificity (table 1). For these primer pairs TaqMan probes were designed in order to develop a robust real-time PCR analysis. The two PCR systems were further optimised with respect to primer and probe concentration, annealing temperature, addition of BSA, glycerol and $MgCl_2$. In order to develop a user-friendly PCR method, commercially available "ready-to-use mastermix" from different suppliers were examined. An Internal Amplification Control (IAC) comprising primers and probe targeting the Salmonella *invA* gene was also included. For DNA extraction, two methods were investigated: Heat extraction in TE-buffer pH = 8.0; 10 min. at 96°C was compared to the Promega Magnesil KF Genomic System using a KingFisher instrument.

Conclusion

A real time PCR method for verification of *S.* Dublin was developed, and subsequently validated by the NordVal protocol for use on pure cultures of *Salmonella*. In The NordVal comparative study, the method obtained 100% specificity and 100% sensitivity, while in the collaborative test (comprising 8 laboratories) the method showed a 99% specificity (1 false positive result) and 100% sensitivity (no false negative results).

The two optimised PCR systems were further evaluated using a test panel of 92 bacterial strains including 50 *S.* Dublin strains, strains of other *Salmonella* known to harbour the virulence plasmid and other *Salmonella* belonging to the D serogroup (O:9).

In order to obtain approval from NordVal, the final *S.* Dublin PCR method was validated according to the NordVal protocol. First a comparative study comprising 50 strains of *S.* Dublin, 20 strains of other *Salmonella* and 10 *Enterobacteriaceae* was carried out (table 2). The study compared the results from the PCR with the serotype obtained by the reference method (slide agglutination according to the Kauffmann-White Scheme at Statens Serum Institut, SSI). Secondly, a collaborative test was carried out comprising 8 laboratories, each analysing 20 "unknown" isolates of *Salmonella* by use of the new *S.* Dublin PCR method (table 3).

Results:

Table 1: Number of PCR positive (Ct < 36, duplicate samples) for 9 different primerpairs, tested against 14 S. Dublin and 22 non S. Dublin salmonella.

		S. Dublin	Non *S.* Dublin		Reactivity for *S.* Dublin	
Primer pair	target	(N = 14)	O:9 (N = 13)	Non O:9 (N = 9)	Sensitivity	Specificity
SalDub	***Plasmid pOU1113***	***14***	***0***	***0***	***100***	***100***
SPN	Plasmid pOU1113 (vagC)	14	1	0	100	95
SERO9	*rfbE*	14	13	0	100	41
FLAG	*fliC*	14	5	0	100	77
JEOA	***Plasmid pSVD***	***14***	***0***	***0***	***100***	***100***
JEOB	Plasmid pSVD	14	3	1	100	82
JEOC	Plasmid pSVD	3	0	0	21	100
JEOD	Plasmid pSVD	0	0	0	No PCR product	
JEOE	Plasmid pSVD	0	0	0	No PCR product	

Two primer pairs (SalDub and JEOA) showed 100% specificity/sensitivity (table 1) and were selected for further testing and optimization using a real-time TaqMan PCR.

Table 2: Number of PCR positive (Ct < 36, duplicate samples) for the two optimized real time PCR analyses (SalDub and JEOA). The testpanel consisted of 50 S. Dublin and + 20 non-S. Dublin salmonella and 10 Enterobacteriaceae

		S. Dublin	Other *Salmonella*	*Entero-bacteriaceae*	Reactivity for *S.* Dublin	
Primer pair	target	(N = 50)	(N = 20)	(N = 10)	Sensitivity	Specificity
SalDub	Plasmid pOU1113	50	0	0	100%	100%
JEOA	Plasmid pSVD	50	0	0	100%	100%

Both real time PCR analyses showed 100% specificity and sensitivity for *S.* Dublin, when compared to the serotype obtained by the reference method (slide agglutination acc. Kauffmann-White Scheme at Statens Serum Institut). However, the SalDub PCR had a slightly better Limit of Detection and produced the highest fluorescence signals, and this PCR was eventually selected for the final method.

The optimised PCR analysis:

1 colony is suspended in 200 µl of TE buffer, pH 8.0 and is heat treated at 96°C for 10 minutes. 2 µl is used as template in the PCR described below. The PCR analysis was optimised using a Stratagene MX 3005p PCR cycler.

Cycle profile: 95°C for 10 min, then 40 cycles at 95°C in 20 sec + 60°C for 60 sec.

0.5 µl PCR Water
11.1 µl SureMaster PCR Mix C4003, Congen Berlin
1.6 µl of a 2.5 µM primer-1 (SalDub-1): 5'-GGG TGA GCG AGC TGG AAA-3'
1.6 µl of a 2.5 µM primer-2 (SalDub-2): 5'-CGC CAT AAA GTC CGG GTC A-3'
1.6 µl of a 2.5 µM probe (SalDub-p): 5' FAM-TTT TTC GAG CTG CGC GAA CGA GC-BHQ1
0.4 µl of a 2.5 µM IAC-1: 5'-CTA TGT TCG TCA TYC CAT TAC CTA CCT-3'
0.4 µl of a 2.5 µM IAC-2: 5'-CCA GAC GAA AGA GCG TKG TAA T-3'
0.8 µl of a 2.5 µM IAC-p: 5' HEX-TCT GGT TGA TTT CCT GAT CGC ACT GAA-BHQ2
2.0 µl template DNA

Table 3: Collaborative Trial: Number of S. Dublin positive isolates from each laboratory as compared to the reference laboratory (10 S. Dublin and 10 non-S. Dublin salmonella)

laboratory	Non *S.* Dublin	*S.* Dublin	Correct results
A	0	10	20
B	0	10	20
C	0	10	20
D	0	10	20
E	0	10	20
F	1	10	19
G	0	10	20
H	0	10	20
Reference Lab (SSI)	*0*	*10*	

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