PE5.05 Application of a real-time PCR and a Modified XLD agar for Detection of MDR S. typhimurium DT104 342.00

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Abstract - In Denmark, special legislation regarding multi drug resistant Salmonella typhimurium DT104 (MDR ST DT104) is in force. Isolates from all salmonella positive meat- or carcass-samples must be sent to the Salmonella Reference Laboratory (National Food Institute, Technical University of Denmark) for further characterization (antibiotic resistance, sero and phage typing, PFGE). Meat contaminated with MDR Salmonella typhimurium resembling the typical MDR ST DT104 clone must be recalled or heat treated to inactivate salmonella. To avoid recalls, if any, the meat industry has requested a rapid method for detection of MDR ST DT104. At the Danish Meat Research Institute (DMRI) a real-time PCR was developed targeting the gene complex responsible for the antibiotic resistance in MDR ST DT104. Also a modification of Xylose Lysine Desoxycholate agar (XLD) containing chloramphenicol, ampicillin, and spectinomycin (CAS-XLD) was developed. 1273 salmonella isolates from Danish slaughterhouses were tested using the real-time PCR method and CAS-XLD agar. Using the PCR method, 24 samples (1.9%) were positive for the MDR gene complex. The same isolates were able to grow on CAS-XLD agar. Additional 13 isolates were negative using PCR, but grew on CAS-XLD agar. The confirmed results obtained from the National Food Institute corresponded to the results from the PCR method. All 24 samples positive in the PCR method were either MDR ST DT104, MDR ST U302 or MDR ST phagetype non-typeable. More important, none of the PCR negative isolates were returned from the reference laboratory as MDR ST DT104.

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Index Terms - Antibiotic resistance, DT104, MDR, real-time PCR, Salmonella.

I. INTRODUCTION

A ntibiotic resistance in food borne pathogens has attracted increased attention in the public as well as within the Food Authorities. Danish legislation contains directions of actions to be taken for meat contaminated with MDR Salmonella typhimurium DT104 [1, 2]. A rapid and reliable method for detection of MDR ST DT104 is of interest to avoid distribution of contaminated meat. At DMRI, two different screening methods were developed for detection of MDR ST DT104 isolates. A real-time PCR method was developed targeting the "integrase-spacer-lactamase" gene sequence which is quite unique for MDR ST DT104. For internal amplification control (IAC) primers and probe targeting the Salmonella enterica specific invA gene [3] was constructed. Another method using XLD agar containing chloramphenicol, ampicillin, and spectinomycin was developed simultaneously. The existing method for detection of MDR ST DT104 uses sero- and phage-typing, determination of the antibiotic resistance pattern and in some cases PFGE (pulsed field gel-electrophoresis). This method is quite time consuming, and requires several days to complete. Therefore, the existing method is not suited for foods with a short shelf-life, such as fresh meat.

II. MATERIALS AND METHODS

Control strain For each PCR run a positive control was used. The positive control consisted of a diluted, heat-inactivated culture of MDR Salmonella albany. 25 µl of the control was mixed with 200 µl lysis buffer and DNA was extracted in the same manner as for the isolates, but omitting centrifuging. Preparation of template DNA from Salmonella samples Isolates from samples found positive for salmonella at the slaughterhouse were received on Nutrient agar. A loop-full of bacterial material was suspended in 1 ml sterile 0.85% saline and centrifuged in Eppendorf tubes (5 min., 10,000 g, 4 °C). The supernatant was discarded, and the pellet added 200 µl KF lysis buffer from the Promega Magnesil KF Genomic system (Promega, MD 1460). The extraction of DNA for the PCR analysis was carried out as previously described [4]. Real-time PCR The real-time PCR was performed in the Stratagene Mx3005p using 0.2 ml Stratagene 96-well plates (Cat. no. 401425) with optical caps (cat. no. 401333). The 20 µl real-time PCR mixture contained 10 µl SureMaster PCR mix, 1 µl fluorescence detection enhancer (FDE) and 0.1 µl Platinum Taq polymerase (Congen Biotechnologie, C4003-2). All primers and probes were initially diluted to a concentration of 2.5 pmol/µl. For each reaction the following reagents were added: 1.6 µl forward primer (KEPO-1) 5′-GTGGTAACGGCGYAGTGG; 1.6 µl reverse primer 5′-GCCTTAACGTCTTGTTCAACTTGC (KEPO-2); 1.6 µl probe 5′-FAM-TCTATGCCTCGGGCATCCAAGCA-BHQ1 (KEPO-p); 0.4 µl forward primer (IAC-1) 5′-CTATGTTCG-TCATYCCATTACCTACCT; 0,4 µl reverse primer (IAC-2) 5′-CCAGACGAAAGAGCGTKGTAAT; 0.8 µl probe 5′-HEX-TCTGGTTGATTTCCTGATCGCACTGAA-BHQ2 (IAC-p); 1.6 µl template DNA, and 0.9 µl H2O to a total volumen of 20 µl. The cycle profile was as follows: initial denaturation at 95 °C for 10 min., followed by 40 cycles of 95 °C for 20 sec. and 60 °C for 1 min. Fluorescence was measured during annealing, and the amplification-curves were analyzed with MxPro 4.01 software. CAS-XLD agar method CAS-XLD agar was prepared from XLD agar (Oxoid, CM0469) containing an additional 16 µg/ml chloramphenicol (Fluka 23275), 16 µg/ml ampicillin (Fluka 10044) and 64 µg/ml spectinomycin (Fluka 85555). 8 mg chloramphenicol and 8 mg ampicillin was each dissolved in 500 µl 1:1 mixture of distilled water and ethanol, whereas 32 mg spectinomycin was dissolved in 500 µl distilled water, and autoclaved XLD agar was added until 500 ml at approx. 50°C. The prepared plates were surface inoculated using approx. 10 µl of the same suspension used for DNA extraction. The CAS-XLD agar plates were incubated at 37 °C for 24 hours. Final characterization of salmonella isolates. All the salmonella isolates were characterized at the National Food Institute, The Technical University of Denmark, being the Danish Salmonella Reference Laboratory by serotyping, phagetyping and determination of antibiotic resistance pattern. If the isolate is S. typhimurium and penta-resistant but no phagetype can be obtained, a PFGE is carried out. The PFGE is carried out in order to see if the isolate belong to the typical ST DT104 group, and thus covered by the special legislation.

III. RESULTS AND DISCUSSION

The position of the primers and probe for the detection of the “integrase-spacer-lactamase” gene sequence in MDR ST DT104 is shown in Figure 1. It can be seen from the figure that the amplicon is quite long for real-time PCR products (324 bp). In spite of the long amplicon, quite a good amplification rate was obtained for the MDR Salmonella albany as can be seen in Figure 2. To avoid depletion of PCR reagents, which could prevent detection of the MDR gene complex, a low concentration of primers were used for the internal amplification control (IAC). This results in a quite low fluorescence signal from the IAC probe (Fig. 2). The concentration of the antibiotics in the selective agar method was lower than the National Committee for Clinical Laboratory Standards (NCCLS) breakpoint for the 3 antibiotics. When the XLD agar was used together with the three antibiotics which had been added to the NCCLS breakpoint, the combined selectivity of the XLD agar and the antibiotics inhibited growth of MDR Salmonella. The concentrations of the three antibiotics were therefore optimized to allow growth of MDR Salmonella, while still suppressing growth of other multi drug resistant non-salmonella. The CAS-XLD agar was considered positive for MDR salmonella when red colonies with a diameter of >1 mm were observed. Blackening of the substrate due to H2S production was suppressed for several MDR Salmonella, probably because of the high selectivity of the

CAS-XLD. Blackening was therefore not considered necessary for positive identification of MDR Salmonella. 1273 salmonella isolates from Danish slaughterhouses were tested with the real-time PCR method and examined for growth on CAS-XLD agar. Using the PCR method, 24 isolates were positive for the "integrase-spacer-lactamase" gene sequence. The same isolates were able to grow on CAS-XLD agar. Additional 13 isolates were negative using PCR, but able to grow on CAS-XLD agar. The confirmed results obtained from the National Reference Laboratory corresponded to the results from the PCR method. Table 1 shows that out of 24 isolates positive for MDR with the PCR method, 17 isolates were confirmed as typical MDR ST DT104 by PFGE, 2 isolates were MDR ST U302, whereas 5 isolates were MDR ST non-typeable. All 24 MDR isolates belonged to the classical ACSSuT penta-resistant group (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline). The 7 isolates that were found positive using the PCR method but not resembling the typical MDR ST DT104 clone by PFGE, should then be considered as false positive. But more important, none of the PCR negative isolates were returned from the reference laboratory as typical MDR ST DT104. Therefore, no false negative results among the 1273 isolates tested were obtained neither with the PCR nor the CAS-XLD method. Out of 13 isolates growing on CAS-XLD that were negative in the PCR, two isolates belonged to the classical ACSSuT penta-resistant group, but did not have a typical ST DT104 PFGE profile (Table 1). Therefore these two isolates are not covered by the special legislation and do not belong to the target-organisms. The remaining 11 isolates were all S. derby. Both methods had sensitivity at 100 %, meaning that no false negative results were seen. The PCR method had a frequency of false positive of 29 % whereas the CAS-XLD agar method had a frequency of false positive results which were 49 %.

IV. CONCLUSION

Two rapid and reliable methods for detection of MDR ST DT104 were developed. Of 1273 isolates tested, no false negative results were obtained neither with the PCR nor the CAS-XLD agar method. The percentage of false positive results was higher for the CAS-XLD agar method than for the real-time PCR method.

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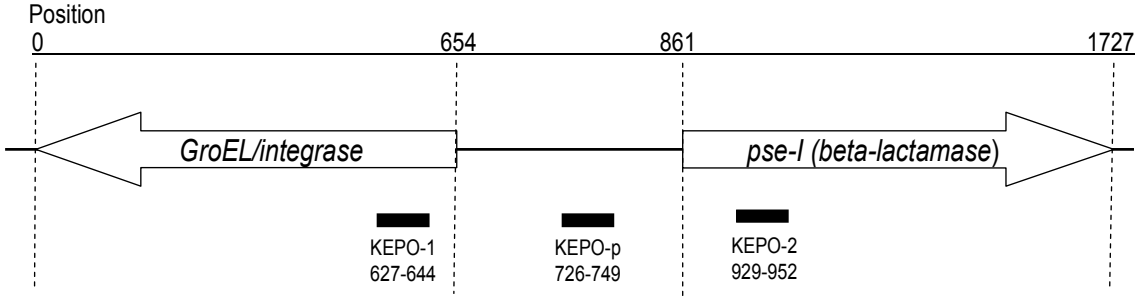

Figure 1. Position of forward primer (KEPO-1), reverse primer (KEPO-2) and probe (KEPO-p) on the "integrase-spacer-lactamase" gene cassette.

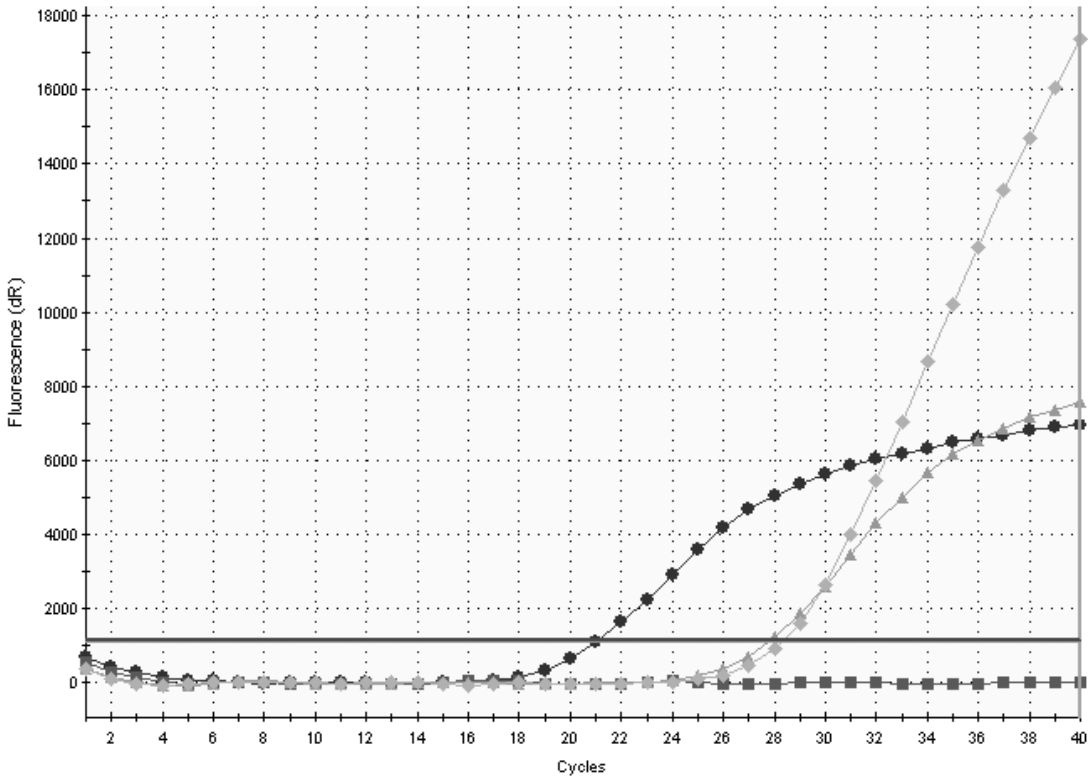

Figure 2. Amplification plot showing amplification curves from 2 different *Salmonella* (MDR *Salmonella albany* respectively a non-resistant *Salmonella spp*). (●) HEX signal from IAC probe from *Salmonella spp*; (■) FAM signal from KEPO probe from *Salmonella spp;* (▲) HEX signal from IAC probe from MDR *Salmonella albany;*

(♦) FAM signal from KEPO probe from MDR *Salmonella Albany*.

	Assays performed at DMRI		Assays performed at the National Food Institute			
Sample no.	PCR result	CAS-XLD result	Sero-type	Resistant (ACSSuT)	Phage typing	PFGE
1	Pos	Pos	*S. typhimurium*	Yes	DT104	
2	Negative	Pos	*S. derby*	No		
3	Negative	Pos	*S. derby*	No		
4	Negative	Pos	*S. derby*	No		
5	Negative	Pos	*S. derby*	No		
6	Pos	Pos	*S. typhimurium*	Yes	NT	Not typical DT104 profile
7	Pos	Pos	*S. typhimurium*	Yes	DT104	
8	Pos	Pos	*S. typhimurium*	Yes	DT104	
9	Pos	Pos	*S. typhimurium*	Yes	DT104	
10	Negative	Pos	*S. typhimurium*	Yes	NT	Not typical DT104 profile
11	Pos	Pos	*S. typhimurium*	Yes	NT	Not typical DT104 profile
12	Pos	Pos	*S. typhimurium*	Yes	NT	Not typical DT104 profile
13	Pos	Pos	*S. typhimurium*	Yes	DT104	
14	Negative	Pos	*S. derby*	No		
15	Negative	Pos	*S. derby*	No		
16	Pos	Pos	*S. typhimurium*	Yes	DT104	
17	Pos	Pos	*S. typhimurium*	Yes	DT104	
18	Pos	Pos	*S. typhimurium*	Yes	NT	Not typical DT104 profile
19	Pos	Pos	*S. typhimurium*	Yes	NT	Not typical DT104 profile
20	Negative	Pos	*S. derby*	No		
21	Pos	Pos	*S. typhimurium*	Yes	DT104	
22	Pos	Pos	*S. typhimurium*	Yes	DT104	
23	Pos	Pos	*S. typhimurium*	Yes	DT104	
24	Negative	Pos	*S. derby*	No		
25	Pos	Pos	*S. typhimurium*	Yes	U302	Not typical DT104 profile
26	Pos	Pos	*S. typhimurium*	Yes	U302	Not typical DT104 profile
27	Pos	Pos	*S. typhimurium*	Yes	DT104	
28	Pos	Pos	*S. typhimurium*	Yes	DT104	
29	Negative	Pos	*S. typhimurium*	Yes	NT	Not typical DT104 profile
30	Pos	Pos	*S. typhimurium*	Yes	DT104	
31	Negative	Pos	*S. derby*	No		
32	Negative	Pos	*S. derby*	No		
33	Negative	Pos	*S. derby*	No		
34	Pos	Pos	*S. typhimurium*	Yes	DT104	
35	Pos	Pos	*S. typhimurium*	Yes	DT104	
36	Pos	Pos	*S. typhimurium*	Yes	DT104	
37	Pos	Pos	*S. typhimurium*	Yes	NT	Typical DT104 profile

Pos = Positive
NT = non-typeable

Table 1. List of 37 isolates found positive for multi drug resistance with either the real-time PCR method or the CAS-XLD agar method. The results from the PCR and the agar method are in agreement with the results from the Danish Salmonella Reference Laboratory (National Food Institute). 29 % of the samples were false positive with the PCR method and 49 % were false positive with the CAS-XLD agar method. No false negative results were obtained among the 1273 isolates tested.