Salmonella Typhimurium: confirmation monophasic variation 4,[5],12:i:- by RealTime PCR

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GeneDia

Importance of serovar 4,[5],12:i:-

Salmonella enterica subsp. enterica serovar **4,[5],12:i:-**, defined as monophasic variant of **Salmonella Typhimurium**, appears to be of increasing importance in many EU Member States (MSs) because it was identified as the cause of large number of infections in both human and animals bred for food.

Thus a misclassification of S. Typhimurium and of its monophasic variant could have significant public health consequences.

Salmonella traditional serotyping

Salmonella culture usually is tested with:

- O-antisera to identify the serogroup
- H-antisera to perform the agglutination
- The dominant flagellar antigen (phase 1) is then repressed to allows the expression of phase 2 flagellar antigen (phase inversion)

Salmonella serotyping

Disadvantages (part 1)

- The phase inversion must be repeated if the strain is positive for the phase 1 flagellar antigen, but not for the phase 2 in order to confirm a monophasic strain
- The number of times that the phase inversion should be repeated is not standardized

Salmonella serotyping

Disadvantages (part 2)

- It's impossible to be certain that a strain would be really monophasic rather than it expressed weakly the second H-phase
- The entire procedure requests many days before a complete serotyping

Reference of PCR assay

Tennant et al (2010): have developed a traditional agarose gel PCR to differentiate Salmonella Typhimurium and its monophasic variant from other serovars

A couple of primers (F-FLIB/R-FLIA) was used to amplifies the intergenic region fliB/fliA

While a second couple of primers (sense-59 antisense-83) was used to amplifies the fljB gene

Detection of Salmonella by PCR

Advantages related to use of PCR:

- PCR results can be obtained in an easier way
- PCR results are univocal
- The confirm of monophasic variant happens directly and not by repeated negative results as by traditional serotyping
- PCR requires shorter times of analysis

Detection of S. Typhimurium and 4,[5], 12:i:- by RealTime PCR

It was possible to develop the traditional protocol of PCR based on detection by gel electrophoresis in 2% agarose, as described by Tennant et al, in an assay of RealTime PCR that, matching the detection of amplicons in "real time" and the melting curve at the end of run, allows faster and easier results

Target genes for PCR assay (part 1)

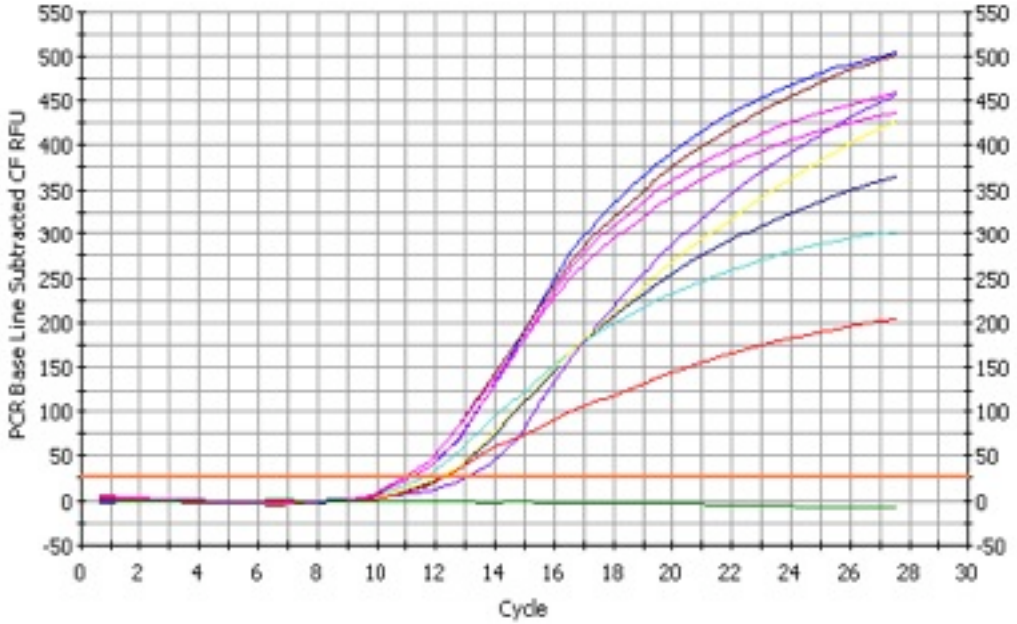
- **16S rna**: used as housekeeping gene in order to verify the correct procedure of extraction of bacterial DNA from the strains and absence of PCR inhibitors
- **fljB gene**: is absent in the monophasic variant 4,[5],12:i:-, while is present for Salmonella Typhimurium

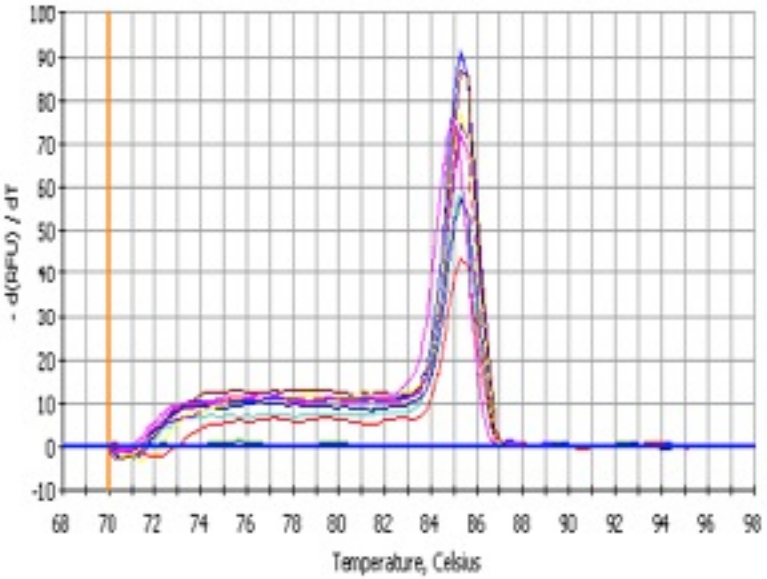
Target genes for PCR assay (part 2)

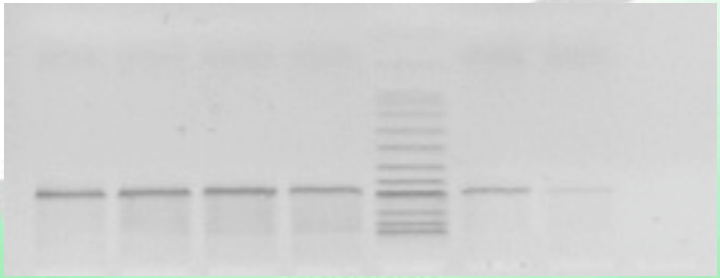
- **fliB-fliA intergenic region**: Salmonella Typhimurium and its variant present a specific IS200 fragment within the flagellin gene cluster. This fragment, peculiar for these two serovars, is located downstream of the fljB gene and upstream of the fliA gene region

Detection of 16S rna (RealTime PCR)

Example of amplification plot, melting curve and agarose gel for the region 16S rna :







Detection of S. Typhimurium

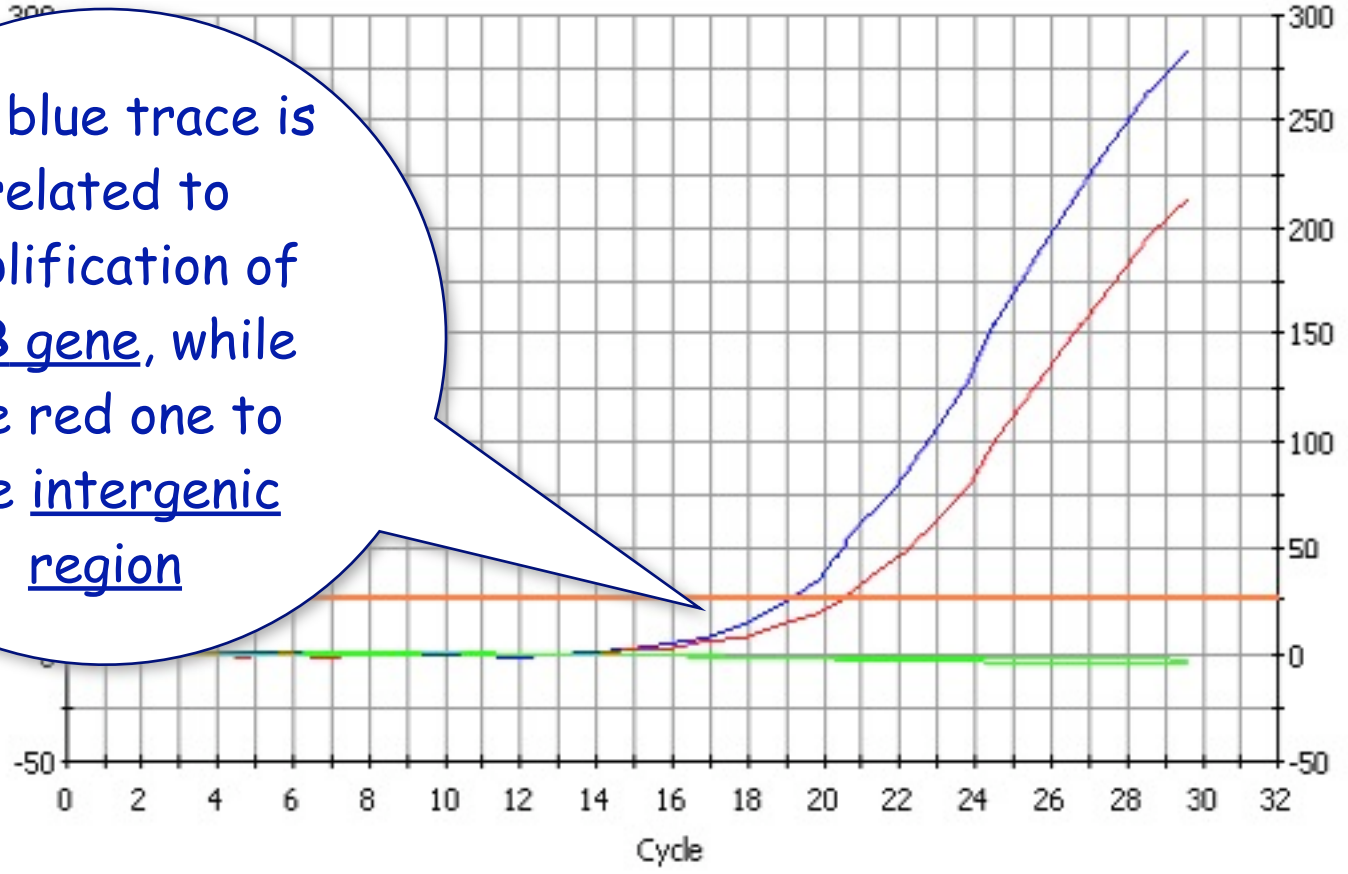
S.Typhimurium is identified by 2 amplicons:

- 1000 bp (for intergenic region)
- 1389 bp (for fljB gene)

By RealTime PCR is possible to identify these 2 amplicons with a signal of fluorescence both for intergenic region and for fljB gene

Amplification of S. Typhimurium

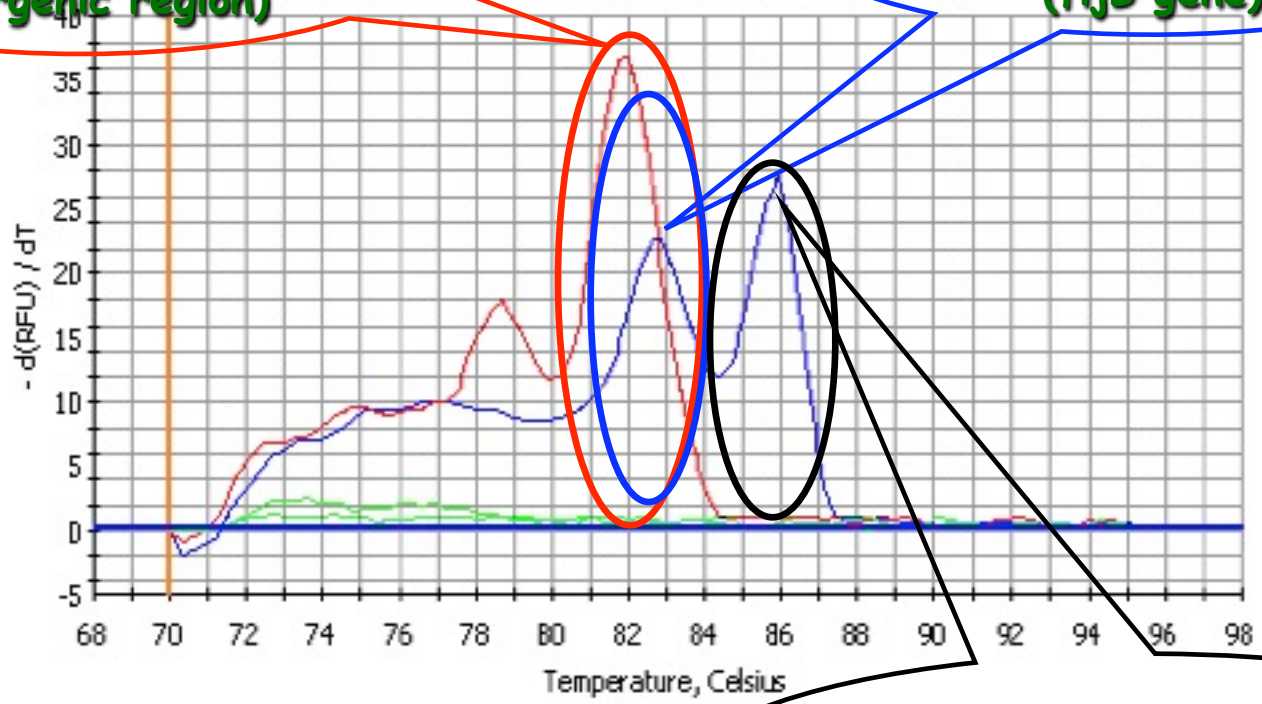
The blue trace is related to amplification of fljB gene, while the red one to the intergenic region



Melting curve of S. Typhimurium

Red peak (TM=82°C): amplicon of 1000 bp (intergenic region)

Blue peak (TM=83°C): amplicon of 1389 bp (fljB gene)



Second blue peak (TM=86°C): probably formation of etheroduples (for fljB gene)

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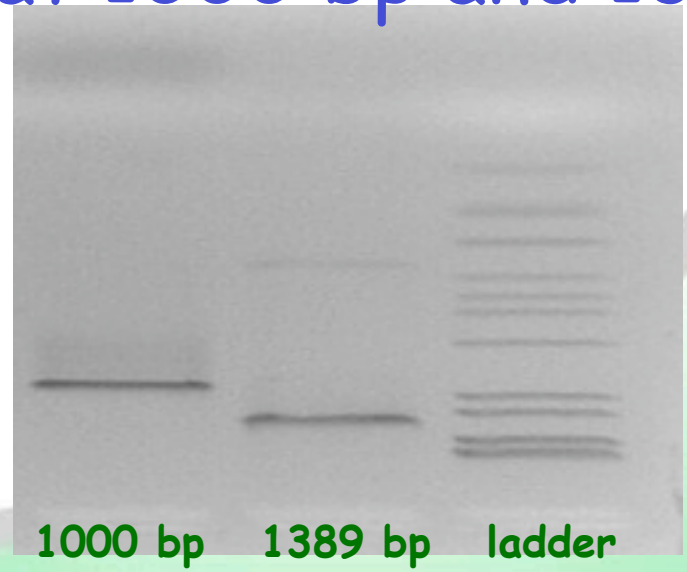
Agarose gel of S. Typhimurium

All the amplicons can also detect by gel electrophoresis in 2% agarose.
S.Typhimurium produces the two expected bands at 1000 bp and 1389 bp



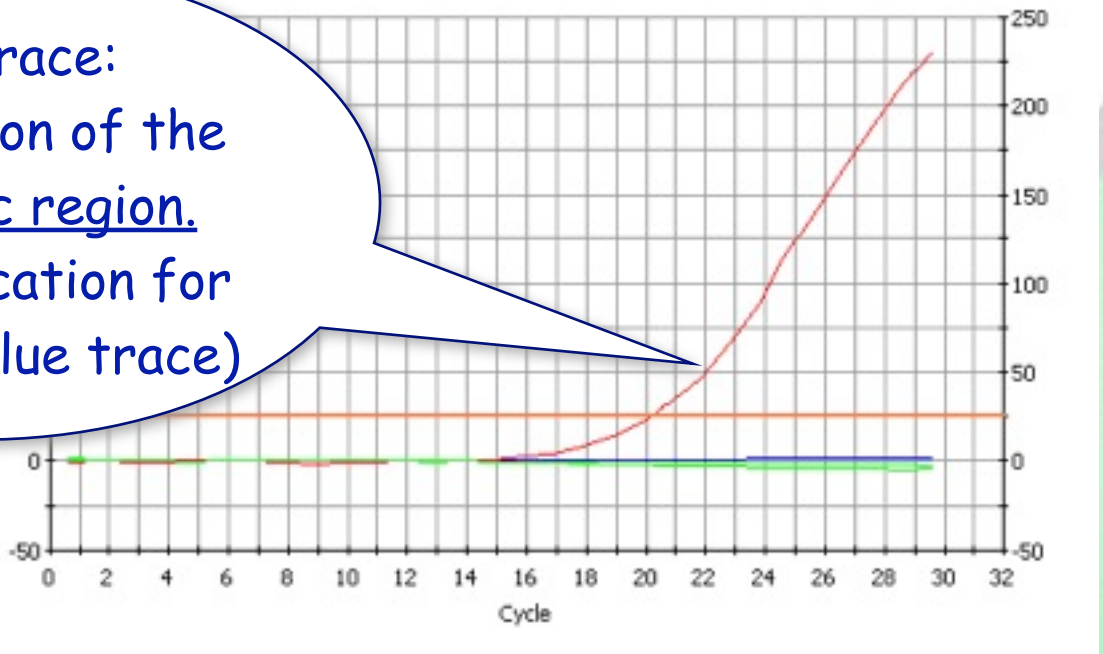
Detection of 4,[5],12:i:-

4,[5],12:i:- is characterized by only 1 amplicons of 1000 bp (for intergenic region) due to absence of the fljB gene

Consequently by RealTime PCR is possible to obtain only the signal of fluorescence related to the intergenic region

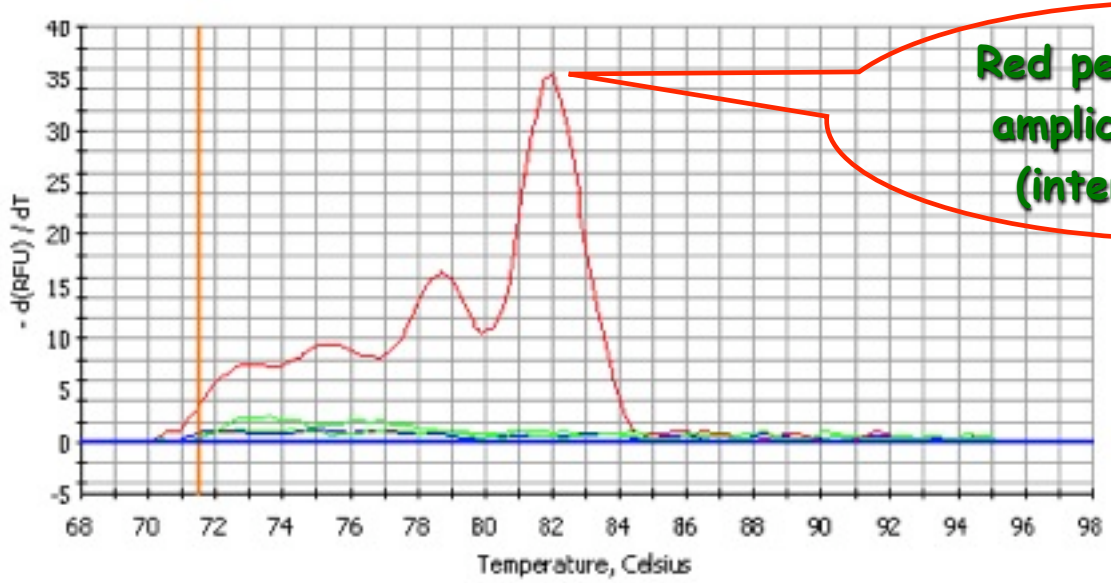
Amplification of 4,[5],12:i:-

Red trace: amplification of the intergenic region. No amplification for fljB gene(blue trace)



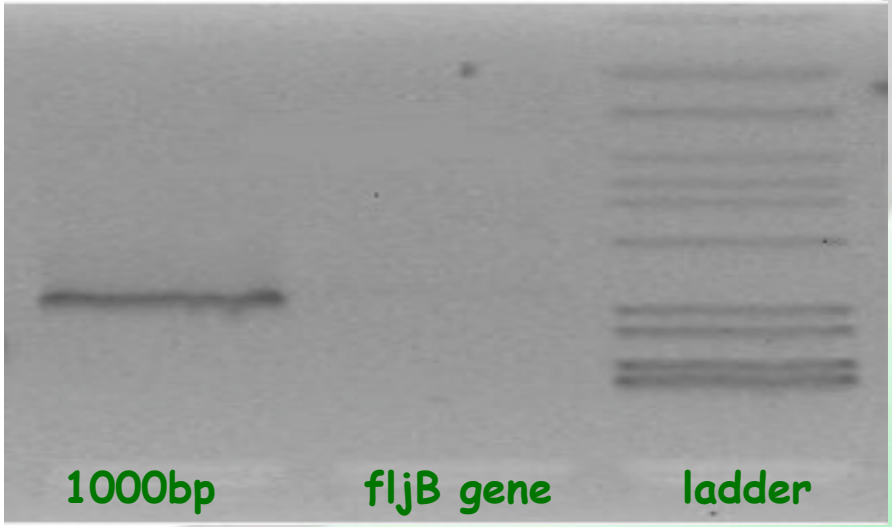
Melting curve of 4,[5],12:i:-

Also in melting curve the monophasic variant of S.Thyphimurium presents only one peak with a TM=82°C, related to the intergenic region



Agarose gel of 4,[5],12:i:-

4,[5],12:i:- detected by gel electrophoresis in 2% agarose produces only the expected band at 1000 bp (for intergenic region)



Detection of other S. serovars

Other S. serovars can be caracterized by one or two amplicons (if mono or bi-phasic).

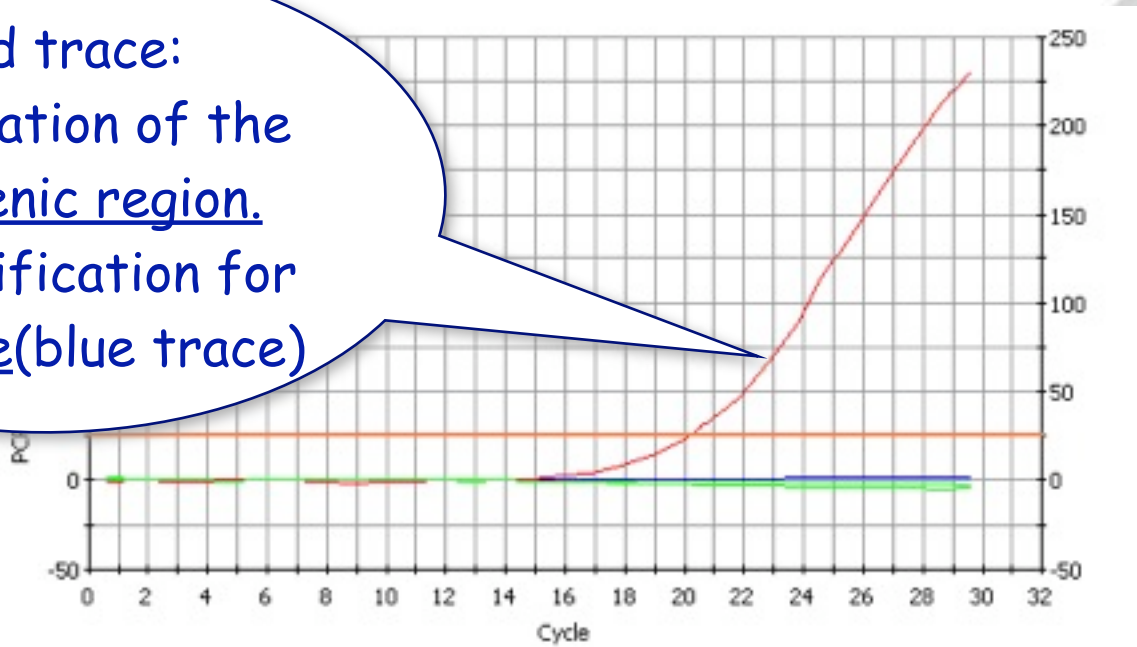
The size of amplicons are:

- 250 bp (for intergenic region)
- 1389 bp (for fljB gene) (only for biphasic strains)

By RealTime PCR is possible to identify these two amplicons with a signal of fluorescence both for intergenic region, and for fljB gene if present

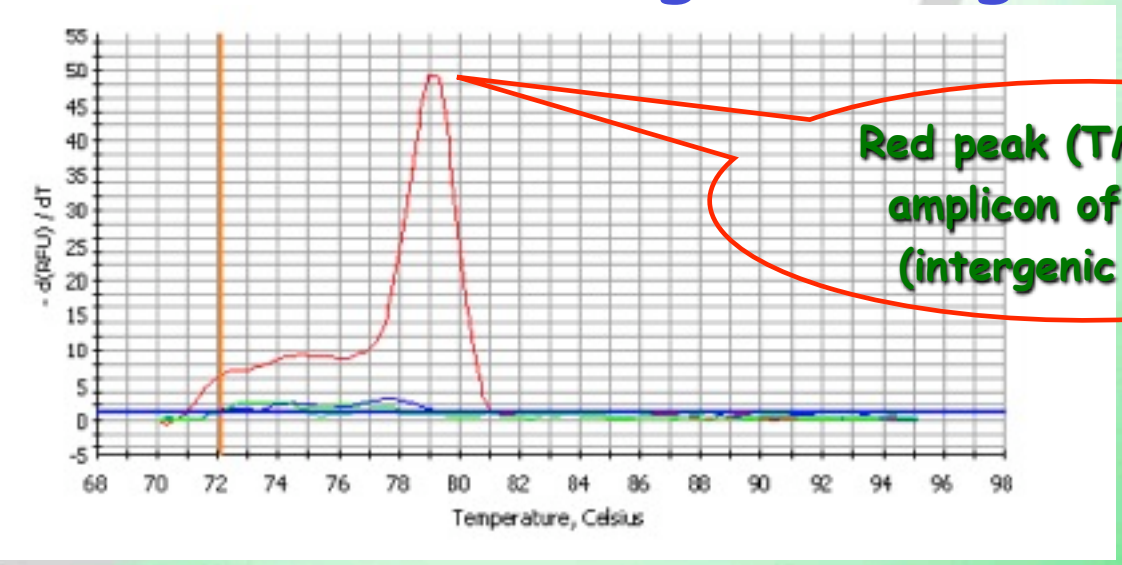
Amplification of other monophasic Salmonella serovars

Red trace: amplification of the intergenic region. No amplification for fljB gene(blue trace)



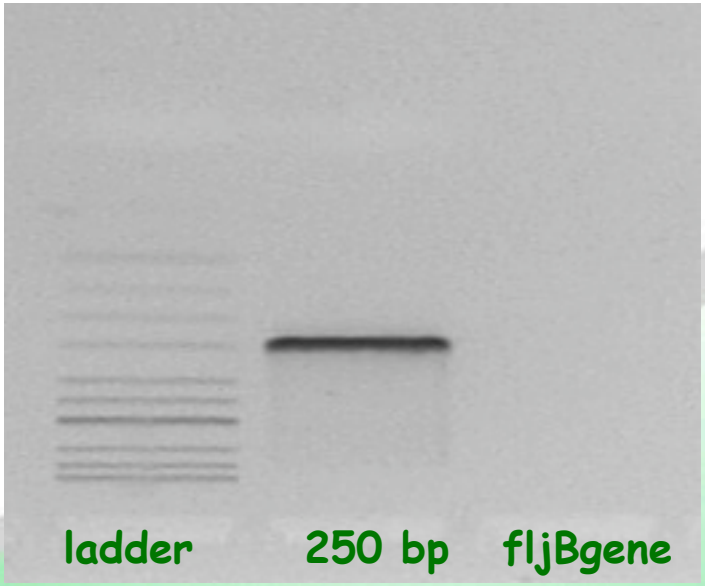
Melting curve of other monophasic Salmonella serovars

In melting curve such monophasic strains presents only one peak with a TM=79°C, related to the intergenic region



Agarose gel of other monophasic Salmonella serovars

These other monophasic Salmonella serovars in 2% gel agarose produce only one band at 250 bp (for intergenic region)



Validation of method

The assay in RealTime PCR was tested and validated on a selection of 56 strains isolates, purchased by ASL of Brescia

These strains were firstly classified by traditional method (O/H antisera seotyping) and then by RealTime PCR

The results are reported in the two following tables:

Table 1

n°	ID	traditional method	PCR
1	366658	S. 4, [5],12:i:-	S. 4, [5],12:i:-
2	366672	S. Agama	Other serovars (immobile or monophasic)
3	366673	S. 4, [5],12:i:-	S. 4, [5],12:i:-
4	366674	S. Derby	Other serovars (immobile or monophasic)
5	366675	S.Typhimurium	S.Typhimurium
6	366678	S. 4, [5],12:i:-	S. 4, [5],12:i:-
7	366679	S.Typhimurium	S.Typhimurium
8	366682	S. 4, [5],12:i:-	S. 4, [5],12:i:-
9	366683	S. 4, [5],12:i:-	S. 4, [5],12:i:-
10	366686	S. 4, [5],12:i:-	S.Typhimurium
11	366714	S. 4, [5],12:i:-	S. 4, [5],12:i:-
12	366715	S. 4, [5],12:i:-	S. 4, [5],12:i:-
13	366719	S. 4, [5],12:i:-	S. 4, [5],12:i:-
14	366720	S. 4, [5],12:i:-	S. 4, [5],12:i:-
15	366722	S. 4, [5],12:i:-	S. 4, [5],12:i:-
16	366723	S. 4, [5],12:i:-	S.Typhimurium
17	366724	S. 4, [5],12:i:-	S. 4, [5],12:i:-
18	366726	S. 4, [5],12:i:-	S. 4, [5],12:i:-
19	366727	S. 4, [5],12:i:-	S. 4, [5],12:i:-
20	366728	S. 4, [5],12:i:-	S. 4, [5],12:i:-
21	366730	S. 4, [5],12:i:-	S. 4, [5],12:i:-
22	366731	S. 4, [5],12:i:-	S. 4, [5],12:i:-
23	366732	S. 4, [5],12:i:-	S. 4, [5],12:i:-
24	366733	S. 4, [5],12:i:-	S. 4, [5],12:i:-
25	366737	S. 4, [5],12:i:-	S. 4, [5],12:i:-
26	351568	S. 4, [5],12:i:-	S. 4, [5],12:i:-
27	351571	S. 4, [5],12:i:-	S. 4, [5],12:i:-
28	351572	S. Napoli	Other serovars (immobile or monophasic)

Table 2

n°	ID	traditional method	PCR
29	351573	S. 4, [5],12:i:-	S. 4, [5],12:i:-
30	351574	S. 4, [5],12:i:-	S. 4, [5],12:i:-
31	359402	S. 4, [5],12:i:-	S. 4, [5],12:i:-
32	359404	S. 4, [5],12:i:-	S. 4, [5],12:i:-
33	359413	S. 4, [5],12:i:-	S. 4, [5],12:i:-
34	366567	S. 4, [5],12:i:-	S. 4, [5],12:i:-
35	366582	S.Typhimurium	S. 4, [5],12:i:-
36	366584	S. 4, [5],12:i:-	S. 4, [5],12:i:-
37	366586	S. 4, [5],12:i:-	S. 4, [5],12:i:-
38	366587	S. 4, [5],12:i:-	S. 4, [5],12:i:-
39	366589	S. 4, [5],12:i:-	S. 4, [5],12:i:-
40	366590	S. 4, [5],12:i:-	S. 4, [5],12:i:-
41	366593	S. 4, [5],12:i:-	(negative for intergenic region; positive for fljB gene)
42	366594	S. 4, [5],12:i:-	S. 4, [5],12:i:-
43	366597	S. 4, [5],12:i:-	S. 4, [5],12:i:-
44	366598	S. 4, [5],12:i:-	(negative for intergenic region; positive for fljB gene)
45	366599	S. 4, [5],12:i:-	S. 4, [5],12:i:-
46	366735	S.Typhimurium	S.Typhimurium
47	366736	S.Typhimurium	S.Typhimurium
48	366738	S.Typhimurium	S.Typhimurium
49	366739	S.Typhimurium	S.Typhimurium
50	366740	S.Typhimurium	S.Typhimurium
51	366741	S.Typhimurium	S.Typhimurium
52	366744	S. 4, [5],12:i:-	S. 4, [5],12:i:-
53	366748	S. 4, [5],12:i:-	S. 4, [5],12:i:-
54	371498	S.Typhimurium	S.Typhimurium
55	371499	S.Typhimurium	S. 4, [5],12:i:- :
56	371500	S. 4, [5],12:i:- :	S. 4, [5],12:i:- :

Results obtained (part 1)

S. Typhimurium

- 9 strains serotyped as S.Typhimurium were confirmed by PCR (82%)
- 2 strains serotyped as S.Typhimurium were classified by PCR as monophasic variant (18%)

(Possible explanation:not correct serotyping)

Other Salmonella Serovars

- 3 strains were detected as other Salmonella monophasic serovars both by traditional method and by PCR (100%)

Results obtained (part 2)

S.4,[5],12:i:-

- 38 strains serotyped as monophasic variant of S.Typhimurium were confirmed by PCR (90%)
- 2 strains serotyped as S. 4, [5],12:i:- were classified by PCR as S.Typhimurium (5%)

  (Possible explanation:not correct serotyping)
- 2 strains serotyped as S. 4, [5],12:i:- were not classified by PCR (5%) because they generated only the amplicon of 1389 bp (fljB gene), but not the amplicon of 1000 bp (intergenic region)

  (Possible explanation: polymorphism for the specific PCR primers binding site in the intergenic region)

Conclusions

The results of evaluation of a novel method by RealTime PCR to identify Salmonella Typhimurium and its monophasic variant (4,[5],12:i:-) showed that this assay was highly specific, easier and less time consuming, compared to the traditional serotyping

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