

## RELATION BETWEEN THE PRESENCE OF EXTRACHROMOSOMAL DNA AND VIRULENCE FEATURES IN *SALMONELLA ENTERICA* STRAINS

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### Introduction

Although several attempts to control its diffusion, *Salmonella* is still the first responsible of foodborne disease all over the world. The considerable incidence of salmonellosis can be frequently connected to the consumption of food of animal origin; among these, poultry meat, pork and eggs are often involved.

The International Surveillance Network for the Enteric Infections (Enter-Vet, 2004) registered 1.925 isolates from foodstuffs in Italy. The strains, mostly coming from pigs (40.10%) and from poultry (21.87%), belonged to *S. Typhimurium* (24.78%), *S. Derby* (14.10%), *S. Enteritidis* (5.6%), *S. Infantis* (3.58%) and *S. Virchow* (0.46%) serotypes.

No significant variations in the prevalence of the main serotypes were noticed about pork isolates, regarding biennium 2002-2003, while in poultry meat a meaningful increase in the frequency of *S. Livingstone* and a decrease of *S. Virchow* were observed (9).

*Salmonellas* ingested with food, in order to be able to survive and replicate in hosts, must possess chromosomal and plasmidic codificated virulence factors. However, phenotypic expression of the virulence genotype depends on the environment and on the growth phase of the microorganism.

The presence of one or more nucleotidic virulence sequences can influence the pathogenicity of the various serotypes and even of different strains belonging to the same serovar (4). Particularly, as reported in the literature, the serotype's infectious dose may be influenced also by the presence of extrachromosomal stuff like, for example, the virulence plasmid pKDSC50 (49.503 bp).

It is assessed that strains belonging to serovars whose virulence is well known, just as *S. Choleraesuis*, *S. Dublin*, *S. Enteritidis*, *S. Pullorum* and *S. Typhimurium*, own differently sized plasmids (50 – 90 Kb).

These plasmids carry both serovar-specific virulence genes, and common genic sequences; among these, *spv* region (6-8 Kb), comprising *spvR* positive regulon and four structural genes (*spvABCD*) (1, 2, 8).

Experimental studies suggested that the presence of *spv* region increases *Salmonella*'s intracellular increase in extraintestinal tissues and favours the interaction between the microorganism and the host's immune system, even if the molecular mechanism is not still completely clear (5, 7).

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In this purpose, the aim of this research was to detect, genic fragments contained in *spv* region (*spvC* and *spvR* genes) in *Salmonella enterica* strains isolated from poultry meat and pork. Subsequently, the presence of virulence plasmid was evaluated, in order to check the possible integration of plasmidic genes in the bacterial chromosome.

## Materials and methods

A total of 25 pork (“cotechino” and sausage) and 20 poultry samples (breast, legs, leg quarters, kebabs of meat and vegetables) were collected from different shops of the large-scale retail trade, in Parma and Reggio Emilia provinces. The isolation of *S. enterica* was carried out by ISO Standard 6579/1993 method by a reference laboratory; the strains were then serotyped. InstaGene™ Matrix – Biorad (10) chelating resin was used to extract the genomic DNA from cultures.

The plasmidic genic sequences were amplified in GeneAmp PCR System 2400 (Perkin Elmer) thermal cycler, using primer couples Spvc for/Spvc rev (699 bp) and SPVR1/SPVR2 (891 bp), respectively for *spvC* and *spvR* genes (3).

The extraction and the subsequent purification of the plasmid, firstly applied to the positive control *E. coli* K12 strain (NTCC 50192-39R861), was carried out by MiniPrep kit (Invitrogen). The method starts from the preparation of a culture, added with different buffers (Resuspension Buffer, Lysis Buffer, Precipitation Buffer).

The DNA linking and washing step was performed into a purification column, added with Wash Buffer and Elution Buffer, in order to obtain the elution of the genetic stuff.

The amplified fragments and the plasmid extracted from the cultures were visualized by the transluminator, after electrophoresis on agarose gel, respectively 1.6% and 0.8% (Agarose Standard, Eppendorf).

## Results

A total of 41 *S. enterica* strains (11 from poultry and 30 from pork) were isolated. The serotype identification, performed by serological tests, led to the individuation of 12 different serovars (*S. Enteritidis*, *S. Typhimurium*, *S. Virchow*, *S. Derby*, *S. Livingstone*, *S. Senftenberg*, *S. Infantis*, *S. Coeln*, *S. Agona*, *S. Bredeney*, *S. Brandenburg* and *S. Bovis-morbificans*).

The research of plasmidic genes detected the presence of *spvR* gene in all the strains, while *spvC* gene was individuated only in one *S. Enteritidis* strain (2.4%) (Picture n. 1).

The results also show that 9 *S. enterica* strains (21.95%) own the pKDSC50 plasmid (Picture n. 2).

Particularly, plasmid between 63.4 Kb and 148 Kb were detected in *S. Enteritidis* (1/10), *S. Typhimurium* (4/13), *S. Virchow* (2/5), *S. Derby* (1/3) and *S. Infantis* (1/1) serotypes (Table n. 1).

The plasmid was mainly present in *S. enterica* strains isolated from pork (88.89%) than those from poultry meat (11.11%).

### Considerations and conclusions

This study showed a strong heterogeneity between different *S. enterica* strains regarding the presence of the virulence plasmid. On the contrary the plasmidic gene distribution appeared less diversified, instead; *spvR* gene was detected in all the strains, while *spvC* gene was always absent, with the exception of one only strain. It is important to remember that the presence of these genic fragments is not directly connected with bacterial virulence, since their phenotypical expression is strongly dependent on environmental and nutritional stress conditions.

The results showed that only 9 strains on 41 (21.95%) possess the plasmid, thus suggesting that the functions codified by extrachromosomal genic sequences are rarely indispensable for the bacterial survival, in optimum conditions. This could also explain why the bacteria can easily lose the plasmid, above all when the microorganism is sub-cultivated for a long period, as in our research (11).

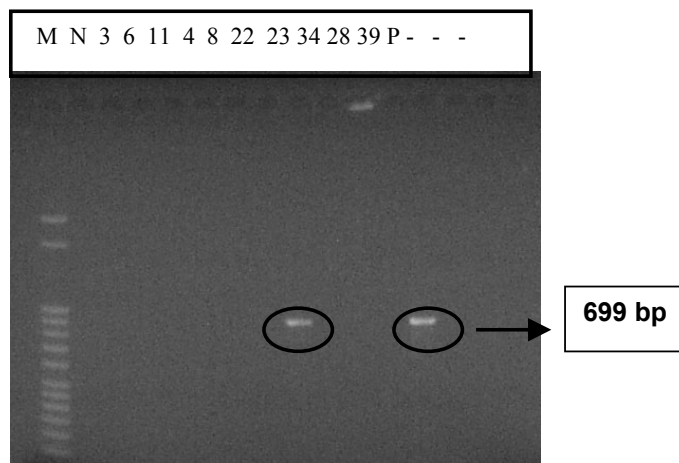
In plasmid-negative strains (78.05%) owing to the different serovars, *spvR* plasmidic gene was always detected. It is interesting to notice that one particular *S. Enteritidis* strain lacked the extrachromosomal stuff but presented both the gene *spvR* and *spvC*. This situation emphasizes the possibility of integration of these genic fragments in the bacterial chromosome. In this purpose must be remembered that plasmids can independently replicate independently but, frequently, they can also integrate themselves in the chromosome and multiply with it (episomes).

Finally it is interesting to observe the constant lacking of *spvC* gene in the 9 plasmid-positive strains, which was probably detached by the plasmid, because of successive cultural passages. Among the plasmid-positive strains, an high percentage is represented by to *S. Typhimurium* strains (30.76%). This feature is very significant because this serovar, which is the first responsible of human and animal infections, is frequently related to multiresistant phenotypes (e.g. *S. Typhimurium* DT104 phagetype), which is known to be plasmid-codified (6). In Italy Enter-Vet system detected an high incidence (75.76%) of multiresistant strains, just belonging to this serovar; the phenomenon also appeared quite significant for *S. Infantis* (13.21%), *S. Derby* (13.14%) and *S. Virchow* (10%) (9).

**Table n. 1:** Presence of virulence plasmid in strains belonging to different serovars.

Serotypes	no. tested strains		no. plasmid-positive strains
	Pork	Poultry meat	
<i>S. Enteritidis</i>	-	10	<b>1</b>
<i>S. Typhimurium</i>	13	-	<b>4</b>
<i>S. Virchow</i>	5	-	<b>2</b>
<i>S. Derby</i>	3	-	<b>1</b>
<i>S. Livingstone</i>	2	-	-
<i>S. Seftenberg</i>	2	-	-
<i>S. Coeln</i>	1	-	-
<i>S. Agona</i>	1	-	-
<i>S. Bredeney</i>	1	-	-
<i>S. Brandenburg</i>	-	1	-
<i>S. Bovis-morbificans</i>	1	-	-
<i>S. Infantis</i>	1	-	<b>1</b>
<b>Totale</b>	<b>30</b>	<b>11</b>	<b>9</b>

**Figure n. 1:** Electrophoretic course of the amplicon (699 bp) identifying *spvC* gene.



**Legenda:**

M = marker (Step Ladder 50 bp) (50-3.147 bp) (Sigma)

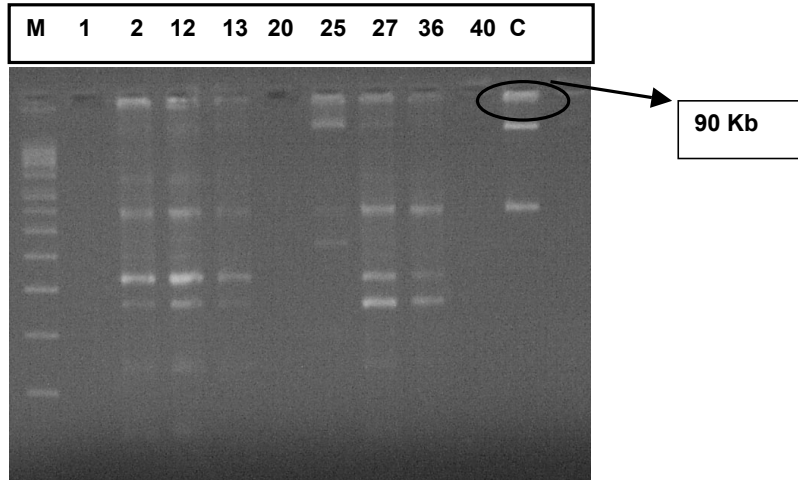
N = negative control

C = positive control

n. 23 = positive strain

nn. 3, 6, 11, 4, 8, 22, 34, 28, 39 = negative strains

**Figure n. 2** Electrophoretic course related to the presence of virulence plasmid.



**Legenda:**

M = Pulse marker (2,067-16,210 Kb) (Sigma)

C = positive control (*E. coli* K12, NTCC 50192-39R861)

nn. 2-12-13-25-27-36 = positive strains

**Key-words:** *Salmonella enterica*, virulence genes, plasmid, pork, poultry meat.

**Parole chiave:** *Salmonella enterica*, geni di virulenza, plasmidio, carne di suino, carne di pollo.

**Mots clés:** *Salmonella enterica*, genes de virulence, plasmide, viande de porc et poule.

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**Summary:** The presence of the virulence plasmid pKDSC50 was investigated in *Salmonella enterica* strains isolated from pork and poultry meat. Genic fragments (*spvC* and *spvR*) included in the homonymous plasmidic region were also detected by biomolecular technique (PCR). 41 *S. enterica* strains (30 from pork and 11 from poultry meat) were collected. Serotyping allowed to detect 12 different *S. enterica* serovars. *SpvR* gene was found in all the strains, while *spvC* gene was found only in one *S. Enteritidis* strain (2.40 %). The investigation on the virulence plasmid resulted in 9 positive *S. enterica* strains (21.95%). Particularly, plasmids sized from 63.4 Kb and 148 Kb were detected in *S. Enteritidis* (1/10), *S. Typhimurium* (4/13), *S. Virchow* (2/5), *S. Derby* (1/3) and *S. Infantis* (1/1).

**Riassunto:** È stata ricercata la presenza del plasmidio di virulenza pKDSC50 in stipiti di *Salmonella enterica*, isolati da matrici carnee avicole e suine e, con tecnica biomolecolare (PCR), sono stati individuati i frammenti genici (geni *spvC* e *spvR*) appartenenti alla regione plasmidica omonima. Sono stati isolati complessivamente 41 stipiti di *S. enterica*, 11 di provenienza avicola e 30 suina. L'indagine sierologica ha portato all'individuazione di 12 diversi serovar (*S. Enteritidis*, *S. Typhimurium*, *S. Virchow*, *S. Derby*, *S. Livingstone*, *S. Seftenberg*, *S. Infantis*, *S. Coeln*, *S. Agona*, *S. Bredeney*, *S. Brandenburg* e *S. Bovis-morbificans*). Il gene *spvR* è stato trovato in tutti i ceppi saggiati, mentre *spvC* è risultato presente solamente in uno stipite di *S. Enteritidis* (2,4%). La presenza del plasmidio di virulenza è stata evidenziata in 9 stipiti di *S. enterica* (21,95%); in particolare sono stati rilevati plasmidi, di dimensioni comprese tra 63,4 Kb e 148 Kb, nei sierotipi *S. Enteritidis* (1/10), *S. Typhimurium* (4/13), *S. Virchow* (2/5), *S. Derby* (1/3) e *S. Infantis* (1/1).