

# *Salmonella enterica* Serotype Napoli is the First Cause of Invasive Nontyphoidal Salmonellosis in Lombardy, Italy (2010–2014), and Belongs to Typhi Subclade

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## Abstract

*Salmonella enterica* serotype Napoli (*S. Napoli*) is currently emerging in Europe and particularly in Italy, where in 2014 it caused a large outbreak associated with elevated rates of bacteremia. However, no study has yet investigated its invasive ability and phylogenetic classification. Here, we show that between 2010 and 2014, *S. Napoli* was the first cause of invasive salmonellosis affecting 40 cases out of 687 (invasive index: 5.8%), which is significantly higher than the invasive index of all the other nontyphoidal serotypes (2.0%,  $p < 0.05$ ). Genomic and phylogenetic analyses of an invasive isolate revealed that *S. Napoli* belongs to Typhi subclade in clade A, Paratyphi A being the most related serotype and carrying almost identical pattern of typhoid-associated genes. This work presents evidence of invasive capacity of *S. Napoli* and argues for reconsideration of its nontyphoidal category.

**Keywords:** *Salmonella* Napoli, *Salmonella* Typhi, invasive salmonellosis, phylogeny, bacteremia

## Article

**S**ALMONELLA ENTERICA SEROTYPE Napoli (*S. Napoli*) is currently considered a nontyphoidal serotype that has strongly emerged in Europe (Fisher *et al.*, 2009), particularly affecting northern Italy (Graziani *et al.*, 2013). *S. Napoli* has caused notable outbreaks in the past, all of which related to consumption of Italian food products including chocolate bars (Gill *et al.*, 1983), horse salami (Costa *et al.*, 1986), and raw rucola lettuce (EFSA Panel on Biological Hazards (BIOHAZ), 2013). The most recent *S. Napoli* outbreak occurred in Milan (2014) and was associated with the ingestion of contaminated ham, causing elevated rates of hospitalization (30%) and bacteremia (13%) (Huedo *et al.*, 2016). Nevertheless, this serotype remains yet poorly studied, as it seems to be an important health problem mainly restricted to northern Italy.

In this study, we have analyzed the invasive salmonellosis cases of *S. Napoli* during 2010–2014 in Lombardy (10,000,000 inhabitants) and investigated its phylogenetic classification, for the first time.

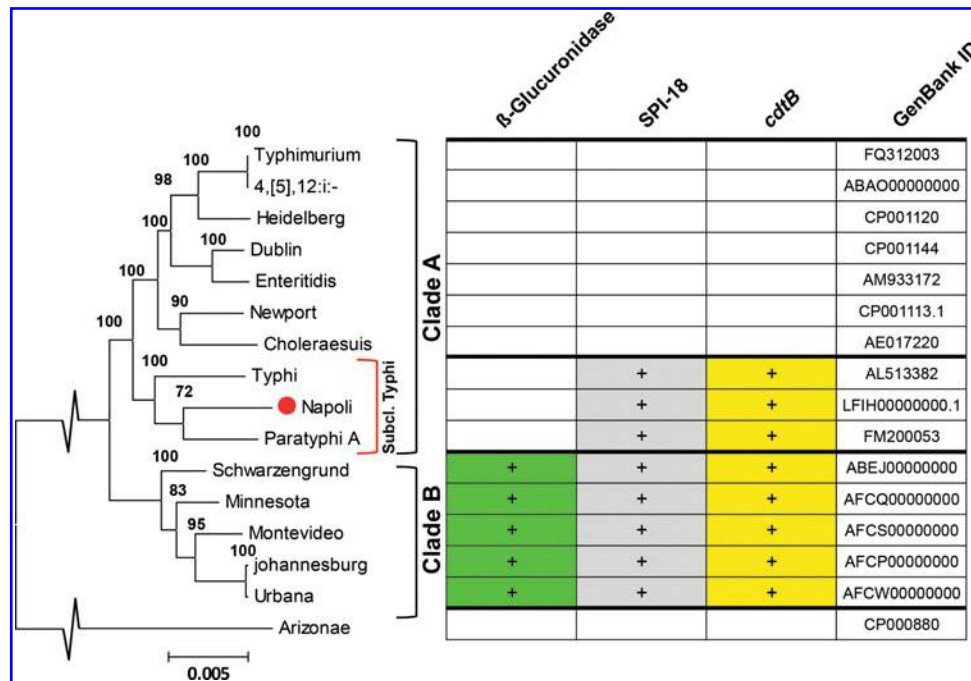
Data of human salmonellosis cases in Lombardy during 2010–2014 were obtained from IT-ENTER-NET ([www.iss](http://www.iss)

[.it/salm/?lang=1&id=1&tipo=4](http://www.iss.it/salm/?lang=1&id=1&tipo=4)). General nontyphoidal salmonellosis (NTS) case definition was “an isolate of *Salmonella enterica* with identified nontyphoidal serovar from a human sample.” Invasive NTS (iNTS) case definition was “an isolate of *Salmonella enterica* with identified nontyphoidal serovar from human blood sample.” Total NTS and iNTS cases with a focus on the top five serotypes were examined. Mean annual incidences per 100,000 inhabitants were computed referring to the population living in Lombardy from 2010 to 2014 (<http://demo.istat.it>). Trends of general and invasive salmonellosis (mean annual increments) were estimated using a generalized-linear model (Poisson distribution, log link function, and logarithm of mean population as offset variable). Comparison of invasive indexes (percentage of invasive salmonellosis from all cases in a serotype) was done using chi-square test.

To infer phylogeny of *S. Napoli*, 93 core-genome genes (den Bakker *et al.*, 2011) from the draft genome of the outbreak isolate SN310 (Huedo *et al.*, 2015) were selected and concatenated, generating a DNA sequence of 73,761 nucleotides. The same orthologous sequence of 15 different serotypes of *Salmonella enterica* subsp. *enterica*—a representative sample of clade A and clade B serotypes—was

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**FIG. 1.** Phylogenetic relationships of 15 *Salmonella enterica* subsp. *enterica* serovars and one *S. enterica* subsp. *arizonae* isolate based on sequence data for 93 core-genome genes (den Bakker *et al.*, 2011) using 200 bootstrap replications. The percentage of trees in which the associated taxa clustered together is shown above the branches. The blocks to the right of the phylogenetic tree indicate the presence of the  $\beta$ -glucuronidase operon (green), SPI-18 (gray), and the *cdtB* gene (yellow) in their genomes.

extracted from a previous work (den Bakker *et al.*, 2011). The orthologous sequence of *S. enterica* subsp. *arizonae* was used as the out-group. All DNA sequences are available in the Supplementary Data and Genbank accession numbers are listed in Figure 1. Phylogenetic analysis was done using the Maximum Likelihood method based on the Tamura–Nei model using 200 bootstrap replications in MEGA 6.0. Presence of the typhoid-associated genes *hlyE* and *taiA* (SPI-18), *cdtB*, *pltA*, and *pltB* (CdtB-Islet), and the GICT18/1 islet (den Bakker *et al.*, 2011) in the genome of *S. Napoli* was determined using BLAST.

A total of 10,690 cases of NTS occurred in Lombardy during 2010–2014. Total cases, mean annual incidence, and mean annual increment of the top five serotypes causing NTS are reported in Table 1. The annual number of isolates significantly decreased from 2,441 in 2010 to 1,834 in 2014 (mean annual incidence decrement of  $-8.5\%$ ,  $p < 0.05$ ). During this period, the only serotypes that showed an increment of NTS reports were 4,[5],12:i:- (from 416 cases in 2010 to 713 in 2014) and Napoli (from 122 cases in 2010 to 151 in 2014). Remarkably, *S. Napoli* has reached the third position in 2014, overtaking *S. Enteritidis* (119 cases) (Figure 2). Nonetheless, a

**TABLE 1.** TOTAL CASES, MEAN ANNUAL INCIDENCE, MEAN ANNUAL INCREMENT, AND INVASIVE INDEX OF THE TOP FIVE SEROTYPES CAUSING NONTYPHOIDAL SALMONELLOSIS AND INVASIVE NONTYPHOIDAL SALMONELLOSIS IN LOMBARDY (ITALY) DURING 2010–2014

Serotype	NTS			iNTS			Invasive index <sup>c</sup>
	No. of cases (%)	Mean annual incidence <sup>a</sup>	Mean annual increment <sup>b</sup> (%)	No. of cases (%)	Mean annual incidence <sup>a</sup>	Mean annual increment <sup>b</sup> (%)	
4,[5],12:i:-	3290 (30.8)	6.7	9.4 <sup>d</sup>	32 (13.5)	0.06	37.3 <sup>d</sup>	1.0 <sup>d</sup>
Typhimurium	2644 (24.7)	5.5	$-32.8^d$	23 (9.7)	0.05	$-5.4$	0.9 <sup>d</sup>
Enteritidis	824 (7.7)	1.7	$-18.2^d$	19 (8.0)	0.04	$-18.3$	2.3
Napoli	687 (6.4)	1.4	3.5	40 (16.9)	0.08	19.8	5.8 <sup>d</sup>
Derby	314 (2.9)	0.6	$-5.0$	2 (0.84)	—	—	0.6
Other serotypes	2931 (27.4)	6.0	$-11.2^d$	121 (51.1)	0.25	$-14.8^d$	4.1
All serotypes	10,690	22.0	$-8.5^d$	237	0.49	$-1.3$	2.2

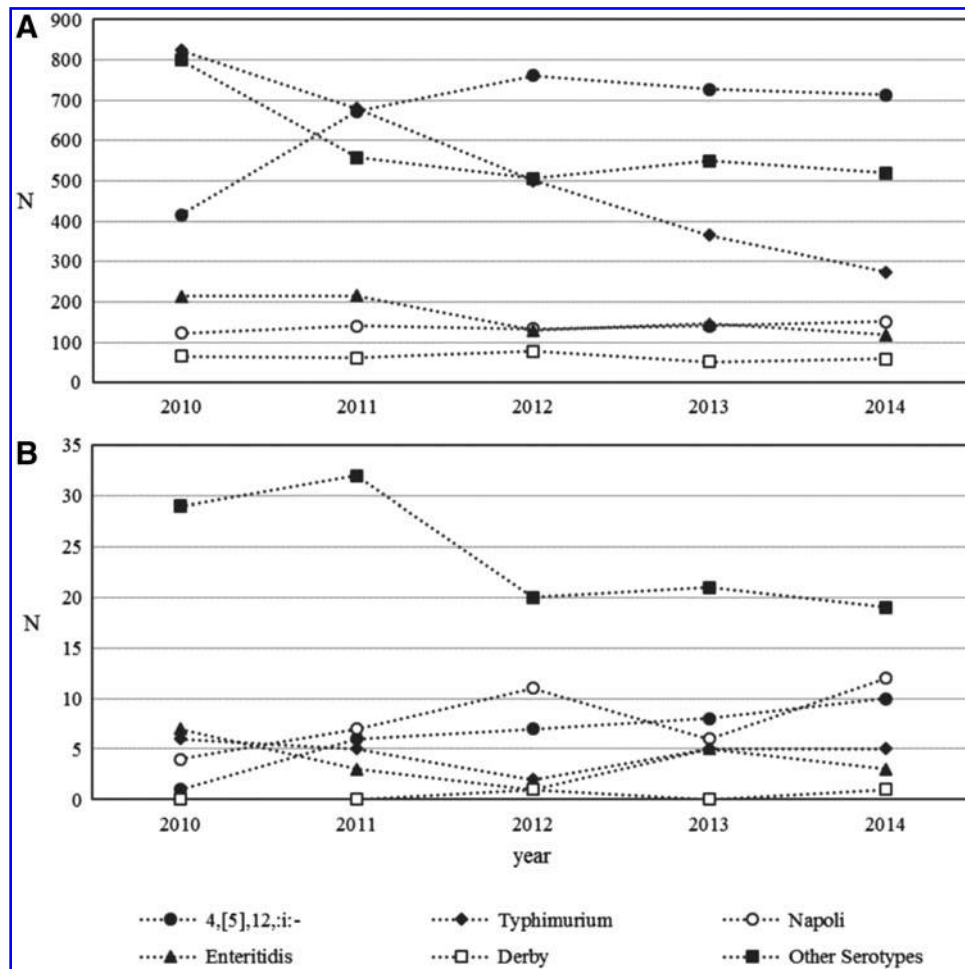
<sup>a</sup>Mean of incidences (cases per 100,000 inhabitants) of all years (2010 to 2014).

<sup>b</sup>Mean annual increment (%) estimated using a Poisson model.

<sup>c</sup>Invasive index = (invasive infections/total cases)  $\times$  100; each invasive index is compared with the invasive index of all the other serotypes using a  $\chi^2$  test.

<sup>d</sup> $p < 0.05$ .

NTS, nontyphoidal salmonellosis; iNTS, invasive nontyphoidal salmonellosis.



**FIG. 2.** Total cases of (A) general non-typhoidal salmonellosis (NTS) and (B) invasive non-typhoidal salmonellosis (iNTS) caused by the top-five non-typhoidal serotypes in Lombardy during the period 2010–2014.

statistically significant increasing annual trend was only observed for *S. 4,[5],12,i:-* (mean of 9.4%,  $p < 0.05$ ).

Of all NTS cases, 237 (2.2%) corresponded to iNTS (Table 1). From these, 116 (48.9%) were caused by the top five serotypes. The inter-annual trend of total iNTS remained stable. Among the top five serotypes, *S. 4,[5],12,i:-* and *S. Napoli* showed an increment of iNTS notifications. iNTS caused by *S. 4,[5],12,i:-* increased from 1 case in 2010 to 10 cases in 2014. *S. Napoli* iNTS reports increased from 4 cases in 2010 to 12 in 2014. As for NTS, only *S. 4,[5],12,i:-* showed a statistically significant increase in the iNTS annual trend (mean of 37.3%,  $p < 0.05$ ) (Figure 2).

Most importantly, during this period, *S. Napoli* was the first cause of iNTS affecting 40 cases (16.9% of total iNTS) out of 687, with an invasive index of 5.8% (95% CI, 4.2–7.8), which is more than twofold higher to that observed for the other nontyphoidal serotypes (2.0%,  $p < 0.001$ ) (Table 1).

One limitation of this analysis is that all cases had been considered sporadic, because ENTER-NET database does not discriminate between outbreak-related and sporadic salmonellosis. Therefore, we could not determinate the exact number of cases derived from outbreaks in different years for all serotypes analyzed. The only outbreak that we could detect through our routine genotyping analysis was that caused by *S. Napoli* recently in Milan (Huedo *et al.*, 2016) and no

further PFGE clusters were identified among *S. enterica* isolates during the period under study. However, such possibility cannot be discarded, because not all isolates from the cases notified in the ENTER-NET database were available for genotyping.

Given the elevated incidence and invasive capacity observed for the serotype *Napoli*, we aimed to investigate its phylogenetic classification using a large multilocus sequence analysis comprising 93 core-genome genes and covering circa 73 Kb (den Bakker *et al.*, 2011). Our phylogenetic analysis (Fig. 1) revealed that *S. Napoli* belongs to Typhi subclade in clade A with a strong bootstrap support (100%), Paratyphi A being the most related serotype (bootstrap value 72%). Likewise, the genome of *S. Napoli* carries the *cdtB*/SPI-18 combination and the putative virulent cluster composed of genes STY3343-3346. As for typhoid and clade B serotypes, the genome of *S. Napoli* carries the *sapABCDF* operon interrupted by the GICT18/1 islet (den Bakker *et al.*, 2011). Contrary to clade B and in agreement with typhoid serotypes, *S. Napoli* lacks the uncommon  $\beta$ -glucuronidase operon (den Bakker *et al.*, 2011).

From the recent *S. Napoli* outbreak investigation (Huedo *et al.*, 2016), it has been noted that infections caused by *S. Napoli* and typhoid serotypes (Crump *et al.*, 2015) present important similar characteristics. For instance, both

cause prolonged incubation periods (7–14 days), common typhoid symptoms including high fever and bacteremia, and elevated rates of hospitalizations. In addition, both typhoid and *S. Napoli* serotypes require a very low infective dose to cause infection (Greenwood and Hooper, 1983; Crump *et al.*, 2015).

Although our work is restricted to a limited geographical area and only one genome has been studied in detail, our results strongly suggest that *S. Napoli* and typhoid serotypes are highly related. More in-depth epidemiological and phylogenetic studies will be needed to further validate such relation. Meanwhile, the emergence of *S. Napoli* should be taken into serious consideration and its nontyphoidal classification could be discussed.

#### Disclosure Statement

No competing financial interests exist.

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