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Machine Learning-Powered ATR-FTIR Spectroscopic Clinical Evaluation for Rapid Typing of *Salmonella enterica* O-Serogroups and *Salmonella* Typhi

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Abstract

Clinical manifestations of salmonellosis in humans typically include acute gastroenteritis, abdominal pain, diarrhea, nausea, and fever. Diarrhea and anorexia may persist for several days. In some cases, the organisms may invade the intestinal mucosa and cause septicemia, even in the absence of significant gastrointestinal symptoms. Most clinical signs are attributed to hematogenous dissemination of the pathogen. As with other microbial infections, disease severity is influenced by the serotype of the organism, bacterial load, and host susceptibility. Serotyping analysis of *Salmonella* spp. using the White–Kauffmann–Le Minor scheme remains the gold standard for strain typing. However, this method is expensive, time-consuming, and requires significant expertise and visual interpretation by trained personnel, which is why it is typically restricted to regional or national reference laboratories. In this study, we evaluated a spectroscopic technique coupled with chemometrics and multivariate machine learning algorithms for its ability to discriminate the main *Salmonella* spp. serogroups in a clinical routine setting. We analyzed 95 isolates of Salmonella that were randomly selected, including four strains of *S.* Typhi. The I-dOne Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) system (Alifax S.r.l., Polverara, Italy) also shows promising potential for distinguishing *Salmonella* Typhi within the D serogroup. The I-dOne system enables simultaneous identification of both species and subspecies using the same workflow and instrumentation, thus streamlining the diagnostic process.

Keywords: attenuated total reflectance Fourier transform infrared spectroscopy; ATR-FTIR; *Salmonella*; *S.* Typhi

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1. Introduction

The epidemiology of *Salmonella* and other foodborne pathogens has evolved considerably over the recent decades, driven by major shifts in food production and consumption. Key contributing factors include the increasing industrialization and globalization of the food supply chain, the growing demand for ready-to-eat and raw food products, demographic changes such as aging populations, a rising proportion of immunocompromised individuals, and the widespread use of antibiotics in food animal production [1–3]. *Salmonella* infection is estimated to cause approximately 2.8 billion cases of diarrheal disease globally

each year. *Salmonella enterica* serovar Typhi (*S.* Typhi), the etiological agent of typhoid fever, is responsible for 16 to 33 million infections annually, resulting in 500,000 to 600,000 deaths. Non-typhoidal *Salmonella* (NTS) infections account for approximately 90 million cases and 155,000 deaths worldwide each year [4]. However, the true global burden of salmonellosis is likely underestimated. For every laboratory-confirmed case, it is estimated that seven additional cases occur in the community but remain unreported [5]. In the United States, *Salmonella* is the principal cause of foodborne infections, accounting for 1.4 million cases each year and associated medical burden of $2.17 billion in 2010 [6]. Surveillance and outbreak detection tools have substantially improved in recent years: for instance, PulseNet has enhanced the ability to detect clusters of foodborne illness that may signal emerging *Salmonella* outbreaks [7]. The application of advanced epidemiological methods has driven safety improvements in both regulatory frameworks and food industry practices. In the European Union (EU), surveillance of zoonoses is governed by the Directive 2003/99/EC 1, which mandates Member States (MSs) to collect and report data on zoonoses, antimicrobial resistance, and foodborne outbreaks. Despite these developments, the incidence of salmonellosis has remained persistently high and, in some regions, has even increased over time. The 2023 EFSA/ECDC (European Food Safety Authority/European Centre for Disease Prevention and Control) annual report confirms that campylobacteriosis and salmonellosis remain the two most frequently reported zoonoses in humans in the European Union [8]. In that year, 77,486 confirmed cases of human salmonellosis were notified, a notification rate of 18 cases per 100,000 population-representing a 16.9% increase over the 2022 rate. Shiga toxin–producing *Escherichia coli* (STEC) ranked third among reported zoonotic agents, followed by *Yersinia enterocolitica* and *Listeria monocytogenes*. Although West Nile virus and *L. monocytogenes* infections were less common, they caused the highest size of hospitalizations and mortality rates. Among *Salmonella* serovars, *S.* Enteritidis remained the predominant cause of both sporadic cases and foodborne outbreaks. The "laying hens-eggs" vehicle accounted for the largest number of *S.* Enteritidis-related outbreaks and was second only to larger multi-food events in hospitalizations. In the "broilers-broiler meat" and "bovine meat" categories, *S.* Enteritidis was the third most common serovar, whereas *S.* Infantis dominated the broiler-meat source and ranked among the top four serovars across all animal-food matrices. Notably, the majority of *Salmonella* outbreaks reported in 2023 were attributable to these two serovars [8].

From a microbiological standpoint, the genus *Salmonella* comprises Gram-negative, rod-shaped, facultatively anaerobic bacteria within the family *Enterobacterales*. It is currently divided into two species—*S. enterica* (itself subdivided into six subspecies) and *S. bongori*—which together include many serovars defined by combinations of O (somatic) and H (flagellar) antigens. The vast majority of human disease is caused by a small subset of *S. enterica* subspecies I serovars, among which *S.* Enteritidis, *S.* Typhimurium and its monophasic variant (1,4,[5],12:i:-) have accounted for over 70% of EU-acquired cases since 2014, rising to 84.8% in 2023 [8]. *Salmonella enterica* is classified into six subspecies based on distinct biochemical properties: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). Serological differentiation of *Salmonella* serovars relies on the White–Kauffmann–Le Minor scheme [9], the gold-standard method for antigenic characterization. Three principal antigenic structures are targeted: the O-antigen (somatic), the H-antigen (flagellar), and the Vi-antigen (capsular). By profiling combinations of these surface antigens, over 2600 serovars of *S. enterica* have been identified worldwide [10]. Most *Salmonella* serovars are motile via peritrichous flagella; notable exceptions is *S.* Gallinarum which is non-flagellated and therefore non-motile (*S.* Gallinarum includes two biovars: *S. enterica* subsp. *enterica* ser. Gallinarum biovar Gallinarum and *S. enterica* subsp. *enterica* ser. Gallinarum biovar Pullorum). Despite lacking this classic virulence factor, these two

biovars cause severe systemic infections in poultry, leading to substantial economic losses, particularly in low- and middle-income countries [11,12]. Ecologically, *Salmonella* spp. are adapted to the gastrointestinal tracts of humans and animals. Consequently, their detection in water, environmental samples, or food products typically signals fecal contamination and poses a risk for further transmission.

In clinical microbiology laboratories, identification of *Salmonella* is typically performed at the genus level using mass spectrometry (e.g., MALDI-TOF MS) or biochemical assays. Classical serological classification of an unknown *Salmonella* isolate, however, is a laborious task: whole serotype assignment requires sequential agglutination tests with multiple antisera over several days [13]. Consequently, in-depth serovar discrimination, despite its clinical and epidemiological relevance, is largely confined to reference laboratories due to its complexity, cost, and duration.

Recently, vibrational spectroscopy techniques, particularly infrared spectroscopy, coupled with chemometrics and multivariate machine learning algorithms, emerged as promising tools for fast and precise characterization of microbes at various taxonomic levels, also for clinical microbiology purposes [14–20]. Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) spectroscopy analyzes the absorption of infrared light by cellular biomolecules, producing a metabolic fingerprint that reflects the macromolecular composition, nucleic acids, proteins, lipids, and carbohydrates, in the 700–1500 cm^{-1} region [20–22]. ATR-FTIR offers rapid data acquisition, minimal sample preparation, and low sample-volume requirements. Qualitative interpretation of spectral patterns is pivotal for accurate microbial identification.

Here, we report the first clinical evaluation of a vibrational spectroscopic workflow using the I-dOne software (AlifaxS.r.l., IT, Polverara, Italy). This certified platform was assessed for both identification (CE-IVD marked) and serogroup/serovar typing of non-typhoidal *Salmonella* (NTS) within principal *S. enterica* serogroups, as well as typhoidal salmonellae (*S.* Typhi and *S.* Paratyphi B), as Research Use Only (RUO) application v1.0.

2. Methods

2.1. Setting and Surveillance System

The Azienda Ospedaliero-Universitaria Pisana (Italy) is a tertiary-care university hospital, where approximately 50,000 patients are hospitalized each year. The hospital includes General Wards, Cardiology, Endoscopy, Hematology, Gynecology, Pediatrics, a Neonatal Unit, a Maternity Ward, Surgical Units, a Burn Unit, an Emergency Department, and nine Intensive Care Units. In addition, it provides services for a broad area comprising non-hospitalized patients. The hospital operates a robust surveillance system for monitoring multidrug-resistant (MDR) microorganisms, particularly carbapenemase-producing *Enterobacterales*, carbapenemase-producing *Acinetobacter baumannii* complex, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* complex, and in blood cultures *Candida* spp. Upon a positive result, an alert is automatically generated by the OpenLIS software v21.1.0 (Engineering Software Laboratory), triggering notification emails to the respective ward, clinical microbiologists, infectious disease clinicians, the infection control staff, and the medical direction. Foodborne infections are also reported to the Medical Direction, the Public Hygiene Unit, and the regional reference center for foodborne infections (CERRTA —Centro di Riferimento Regionale sulle Tossinfezioni Alimentari) for epidemiological tracking and further investigation. Pathogens under surveillance include *Salmonella* spp., *Listeria* spp., *Campylobacter* spp., *Shigella* spp., *Yersinia* spp., *Vibrio* spp., and Shiga toxin-producing *Escherichia coli* (STEC).

2.2. Bacterial Isolates and Culture Conditions

Ninety-five biological samples were cultured on common isolation media in routine diagnostics and incubated at 37 °C overnight. The internal protocol for stool samples consists of direct seeding on selective agar plates for *Campylobacter* spp., which are then incubated at 42 °C for 72 h. A portion of stool is also inoculated in selenite broth and, after 8–12 h at 37 °C, specimens are cultured on selective agar for *Salmonella* spp. and *Shigella* spp. growth (Figure 1). Colonies are identified at the genus level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics GmbH, Bremen, Germany). Antimicrobial susceptibility testing is performed by Phoenix M50, panel 503 (Becton Dickinson, Franklin Lakes, NJ, USA). Minimum inhibitory concentrations (MICs) are interpreted according to the latest European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. O-group antigen is characterized using latex agglutination test. Briefly, pure colonies, cultured on a blood agar plate, are emulsified to a drop of water on a glass slide, and a drop of each antiserum is added. Agglutination is visually observed against a dark background. Upon clinicians' request, a subculture is sent to the reference laboratory, the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana M. Aleandri, for O-group antigen confirmation and serovar characterization. The strains are frozen in brain heart infusion broth supplemented with 10% glycerol for convenient storage. When needed, a 10 µL sterile loop is used to streak a small portion of the frozen bacterial broth onto a blood agar plate, followed by overnight incubation at 37 °C.

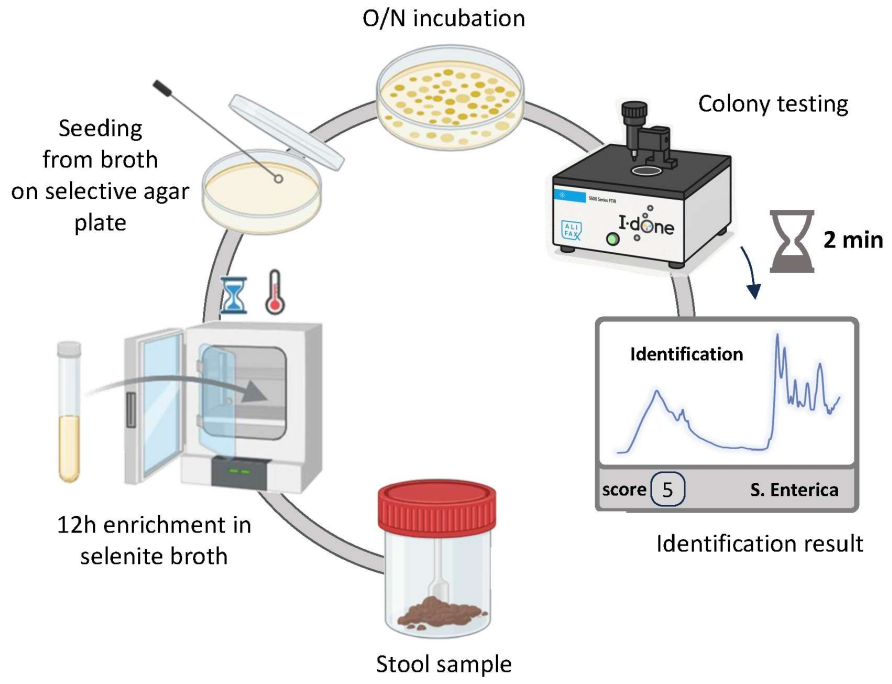

Figure 1. The operative workflow from stool sample to *Salmonella* typing using IdOne system.

2.3. ATR-FTIR Spectroscopy

Spectra were acquired as previously described [22] using an ATR-FTIR spectrometer (5500a Series, Agilent, Santa Clara, CA, USA) working in the mid-infrared range (4000–650 cm^{-1}). The data were recorded at room temperature (25 ± 2 °C) via 64 scans at a resolution of 4 cm^{-1}. Spectra identification and typing was carried out using I-dOne RUO software v.1.0.1, based on Alifax patented machine learning prediction models. The complete procedure flows automatically and involves (a) crystal cleanliness check, (b) background acquisition, (c) sample deposition and spectral profile check, and (d) spectrum

acquisition and identification. After each measurement, the ATR crystal was cleaned with a few drops of 70% v/v ethanol and wiped with tissue paper to avoid inter-sample cross-contamination. Data were collected from pure isolates on solid culture, Mac Conkey or blood agar plates. Briefly, a small amount of colony is picked with a 1 µL loop and spread on the ATR crystal during the sample deposition phase. The software itself checks the quality of the sample signal and allows for the automatic acquisition of the spectrum. If it complies with the criteria defined by the manufacturer the spectrum is acquired and compared with the spectra present into the database for the identification process. Each acquisition takes less than two minutes, and the final result is reported with a reliability score and relative color scale and comments. The score is related to the quality of the prediction with respect to the database, ranging from 0 (Not Identifiable strain) to 5 (Excellent match with reference library). It is up to the user to accept or discard an intermediate score. In this paper, identifications with scores lower than 2.25 (Good match with reference library) were rejected and the test was repeated. Figure 1 reports the operative workflow from stool sample to *Salmonella* typing. Figure 2 reports an example of the ATR-FTIR spectra of a *Salmonella'* s strain.

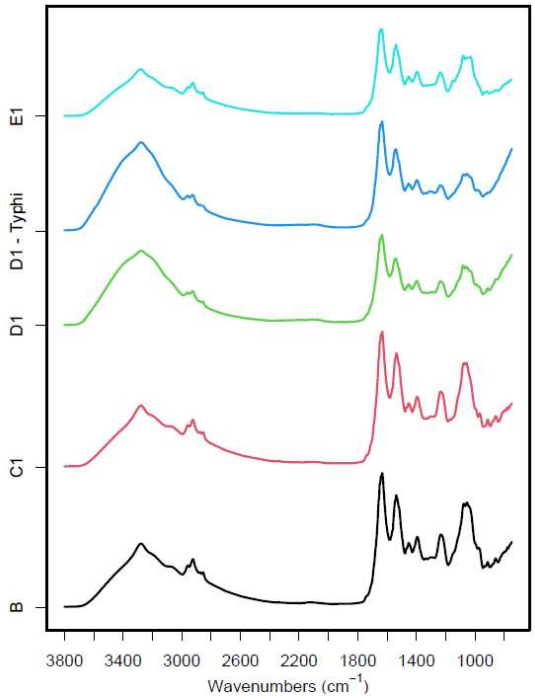

Figure 2. Example of rough ATR-FTIR spectra of a *Salmonella'*s O-groups E1, D1-Typhi, D1, C1, B. The main differences are evident in the carbohydrate area (800–1200 cm^{-1}).

2.4. Ethical Considerations

No human or animal subjects were involved in this study. All experimental procedures were performed on bacterial cultures handled using DPI in compliance with institutional biosafety regulations. Strains were taken from remnants of patients' standard samples and used anonymously. For this type of study, no written informed consent was required.

3. Results and Discussion

Salmonellosis is a globally prevalent disease affecting both humans and animals. *Salmonella* spp. are ubiquitous in nature and are commonly detected in water or food contaminated with animal or human excreta. Fecal-oral transmission is the principal way of infection spreading between animals and from humans to humans and animals to humans. For instance, rat feces can remain infectious for up to 148 days at room temperature [23].

In our epidemiological study, a total of 212 clinical *Salmonella* strains were isolated from patients between January 2014 and December 2024. Among these, 50.9% belonged to group B, 18.9% to group C (subdivided into 11 isolates in subgroup C1, 28 in C2, and 2 in C), 20.3% to group D, and 2.8% to group E1. Additionally, three isolates belonged to group Y, and one to group F. Fifty-five of these strains were submitted to a reference laboratory for serovar characterization; the results are summarized in Table 1. Regarding the source of isolation, 75% of the strains were recovered from stool samples, 11% from blood cultures, 9% from urine samples, and the remaining 5% from other body sites. Among the blood culture isolates, 33% belonged to group B (including one identified as *S.* Paratyphi B), 33% to group C1, 25% to group D (including one identified as *S.* Typhi), while the remaining isolates were assigned to groups F and Y. Table 2 reports the main hospital wards from which positive samples were obtained. The highest proportion of isolates was recovered from patients in Pediatric Wards (22%), followed by patients referred to our laboratory by general practitioners (20%), primarily presenting with symptoms of diarrhea. In recent years, the burden of antimicrobial resistance also among foodborne pathogens has been associated with a high mortality rate, prolonged hospital stays, and higher treatment costs due to therapeutic failure. The first report of antibiotic-resistant *Salmonella* dates back to the early 1960s and involved resistance to chloramphenicol [24]. Since the late 1990s and early 2000s, several multidrug-resistant (MDR) *Salmonella* clones have emerged and subsequently spread globally [25–35]. This global spread has been closely linked to the misuse, overuse, and easy accessibility of antimicrobials in both human and veterinary medicine. In addition, the higher prevalence of MDR *Salmonella* strains, particularly those resistant to fluoroquinolones and third-generation cephalosporins, has become a major public health worry [26,28,36–43]. In our study, 50% of the isolates were resistant to ampicillin, 22% to ciprofloxacin, and 14% to trimethoprim-sulfamethoxazole. Encouragingly, only four isolates (1.9%) exhibited resistance to third-generation cephalosporins.

As reported in Table 1, the majority of *Salmonella enterica* isolates belonged to *S.* Typhimurium and the monophasic variant of *S.* Typhimurium (4,12:i:-) (Group B), followed by *S.* Enteritidis (Group D1) and *S.* Infantis (Group C1). Our prevalence data are consistent with the top five European Union-acquired *Salmonella* serovars associated with human infections, namely: *S.* Enteritidis (70.8%), *S.* Typhimurium (8.9%), monophasic *S.* Typhimurium (1,4,[5],12:i:-) (5.1%), *S.* Infantis (2.0%), and *S.* Coeln (0.77%) [8]. We detected in our hospital one isolate of *S.* Typhi (Group D) and one of *S.* Paratyphi B (Group B), both recovered from blood cultures of patients with systemic infections. These serotypes are highly adapted to humans and have no known reservoir in nature [44]. In contrast, serotypes such as *S.* Typhimurium (Group B) and *S.* Enteritidis (Group D1) have a broad host range and are capable of infecting various animal species, including humans [45]. Other serotypes show host specificity but may occasionally infect humans. Examples include S. Choleraesuis (adapted to swine) [46–48], S. Dublin (adapted to cattle) [49], and *Salmonella enterica* subsp. *arizonae* (primarily associated with reptiles) [50,51]. These NTS serotypes can cause a wide spectrum of clinical manifestations in humans, ranging from gastroenteritis to bacteremia. *Salmonella* Choleraesuis, for example, is a host-adapted pathogen responsible for swine paratyphoid [46,48], but is also pathogenic to humans, where it typically causes septicemic illness with minimal intestinal involvement [52,53]. The strain collection of 95 isolates analyzed by ATR-FTIR spectroscopy included not only the most frequently isolated global serovars (*S.* Enteritidis, *S.* Typhimurium, and the monophasic variant of *S.* Typhimurium), but also clinically significant serotypes (*S.* Typhi, *S.* Paratyphi B, *S.* Choleraesuis), as well as emerging ones such as *S.* Infantis and *S.* Senftenberg.

Table 1. *Salmonella enterica* serovars isolated at Pisa University Hospital. The number of each characterization is contained in brackets.

Group	Serovar
B	S. Abony
	S. Agona (2)
	S. Derby (2)
	S. Paratyphi B
	S. Typhimurium (6)
	Monophasic variant of S. Typhimurium (16)
C1	S. Choleraesuis (2)
	S. Infantis (6)
	S. Virchow
	S. Thomson
C2	S. Goldcoast
	S. Manhattan
D	S. Enteritidis (6)
	S. Typhi
	S. Napoli (2)
E	S. Senftenberg
	S. Goelzau
	S. Give
F	S. Veneziana
G	S. Poona
Y	*S. enterica* subsp. *diarizonae*

Table 2. Wards where the isolation of *Salmonella* spp. occurred.

Wards	Isolates (%)
Pediatric	22%
External patients	20%
General Medicine	11%
Hematology	8%
Surgery	7%
Emergency Department	6%
Infectious Disease	4%

The sensitivity and accuracy performances of this new technology based on the 95 isolates are reported in Tables 3 and 4, together with the confusion matrix (Table 5). For all the strains, the identification score was successful in all cases, with no need to repeat the test. A 100% agreement was observed between the manual agglutination test results and those generated by the I-dOne software, considering all the serogroups currently identifiable with the RUO software (100% specificity, 100% sensitivity). Additionally, four strains of *S.* Typhi were correctly classified. However, a few strains belonged to serogroups not currently identifiable through the I-dOne RUO Salmonella software and were not included in the current database. In particular, the *S.* Goldcast (C2) and *S.* Senftenberg (E4)

strains were identified as C1 and E1 serogroups, respectively. Indeed, C1 and C2 share the same O:6 somatic antigen, while E1 and E4 share the somatic antigen O:3. These similarities may be reflected in the ATR-FTIR spectra, resulting in the identification being the closest match within the database. Despite the limited clinical impact of these misclassifications, this confirms the need to extend the database towards other classes or include the possibility of reporting borderline identification, instead of forcing a potentially incorrect match, in a future version of the prototype. In this perspective, class sensitivity for C2 and E4 is null, while C1 and E1 serogroups specificity is >98%.

Table 3. I-dOne group results compared with the agglutination test group results.

N°	Serovar	Group (Agglutination Test)	Group (I-dONE)	Score (I-dONE)
1	S. Abony	B	B	5
2	S. Agona	B	B	5
3	S. Agona	B	B	5
4	S. Choleraesuis	C1	C1	5
5	S. Choleraesuis	C1	C1	5
6	S. Derby	B	B	5
7	S. Derby	B	B	5
8	S. Enteritidis	D1	D1	5
9	S. Enteritidis	D1	D1	5
10	S. Enteritidis	D1	D1	5
11	S. Enteritidis	D1	D1	5
12	S. Enteritidis	D1	D1	5
13	S. Enteritidis	D1	D1	5
14	S. Goelzau	E	E	5
15	S. Goldcoast	C2	C2	5
16	S. Infantis	C1	C1	5
17	S. Infantis	C1	C1	5
18	S. Infantis	C1	C1	5
19	S. Infantis	C1	C1	5
20	S. Infantis	C1	C1	5
21	S. Infantis	C1	C1	5
22	S. Napoli	D1	D1	5
23	S. Napoli	D1	D1	5
24	S. Poona	G	G	5
25	S. Senftenberg	E	E	5
26	S. Thomson	C1	C1	5
27	S. Typhi	D1	D1-*S.* Typhi	5
28	S. Typhimurium	B	B	5
29	S. Typhimurium	B	B	5
30	S. Typhimurium	B	B	5
31	S. Typhimurium	B	B	5

Table 3. *Cont.*

N°	Serovar	Group (Agglutination Test)	Group (I-dONE)	Score (I-dONE)
32	S. Typhimurium	B	B	5
33	S. Typhimurium	B	B	5
34	Monophasic variant of S. Typhimurium	B	B	5
35	Monophasic variant of S. Typhimurium	B	B	5
36	Monophasic variant of S. Typhimurium	B	B	5
37	Monophasic variant of S. Typhimurium	B	B	5
38	Monophasic variant of S. Typhimurium	B	B	5
39	Monophasic variant of S. Typhimurium	B	B	5
40	Monophasic variant of S. Typhimurium	B	B	5
41	Monophasic variant of S. Typhimurium	B	B	5
42	Monophasic variant of S. Typhimurium	B	B	5
43	Monophasic variant of S. Typhimurium	B	B	5
44	Monophasic variant of S. Typhimurium	B	B	5
45	Monophasic variant of S. Typhimurium	B	B	5
46	Monophasic variant of S. Typhimurium	B	B	5
47	Monophasic variant of S. Typhimurium	B	B	5
48	Monophasic variant of S. Typhimurium	B	B	5
49	Monophasic variant of S. Typhimurium	B	B	5
50	S. Virchow	C1	C1	5
51	Not known	C1/C2	C1	5
52	Not known	D1	D	5
53	Not known	C1	C	4.5
54	Not known	E G	E1	5
55	Not known	B	B	5
56	Not known	D1	D	5
57	S. Typhi	D1	D1-*S. Typhi*	4.75
58	Not known	D1	D	5

Table 3. *Cont.*

N°	Serovar	Group (Agglutination Test)	Group (I-dONE)	Score (I-dONE)
59	Not known	D1	D	5
60	Not known	B	B	5
61	Not known	D1	D	4.5
62	Not known	D1	D	5
63	Not known	D1	D	5
64	Not known	B	B	5
65	Not known	B	B	5
66	Not known	B	B	5
67	Not known	C1	C1	5
68	Not known	B	B	4.5
69	Not known	B	B	5
70	Not known	C1	C1	5
71	S. Anatum	E1	E1	5
72	S. Anatum	E1	E1	5
73	Not known	C1	C1	5
74	S. Anatum	E1	E1	5
75	S. Anatum	E1	E1	5
76	Not known	D1	D1	5
77	Not known	D1	D1	5
78	Not known	D1	D1	5
79	Not known	D1	D1	4.75
80	Not known	B	B	5
81	Not known	B	B	5
82	S. Typhi	D1	D1-*S.* Typhi	5
83	Not known	C1	C1	5
84	Not known	D1	D1	5
85	Not known	B	B	5
86	Not known	C1	C1	4, 5
87	Not known	D1	D1	5
88	S. Anatum	E1	E1	5
89	S. Anatum	E1	E1	5
90	S. Enteritidis	D1	D1	4.75
91	S. London	E1	E1	4.75
92	S. Anatum	E1	E1	5
93	S. Anatum	E1	E1	5
94	S. Typhi	D1	D1-*S.* Typhi	5
95	Monophasic variant of S. Typhimurium	B	B	5

Table 4. I-dOne *Salmonella* RUO metrics. Only one strain with uncertain reference class (E\G) was removed from the analysis, although the result E1 cannot be considered completely wrong.

Serogroup	Sensitivity	Specificity
B	100.0%	100.0%
C1	100.0%	98.7%
C2	0.0%	100.0%
D1	100.0%	100.0%
D1-S. Typhi	100.0%	100.0%
E1	100.0%	98.8%
E4	0.0%	100.0%
G	100.0%	100.0%

Table 5. Confusion matrix where reference data are compared with predicted data.

		Predicted Data					
		B	C1	D1	D1-S. Typhi	E1	G
Reference data	B	38	0	0	0	0	0
	C1	0	17	0	0	0	0
	C2	0	1	0	0	0	0
	D1	0	0	22	0	0	0
	D1-S. Typhi	0	0	0	4	0	0
	E G	0	0	0	0	1	0
	E1	0	0	0	0	10	0
	E4	0	0	0	0	1	0
	G	0	0	0	0	0	1

In recent years, researchers evaluated the potential of machine learning applied to the *Salmonella* detection and characterization [54–57]. In the present study, the novel capabilities of artificial intelligence were thoroughly investigated to develop a classifier capable of differentiating *Salmonella* isolates at the O-serogroup level, with particular emphasis on distinguishing *S.* Typhi within the D-group. In principle, the development of a method to fully replace time-consuming and costly serological analyses for *Salmonella* serotyping would be a highly desirable goal, and the differentiation of either a few or several *Salmonella* serovars by infrared spectroscopy has already been successfully demonstrated [58–60]. However, complete differentiation of the many known *Salmonella* serotypes would require extensive validation at the serovar level, which is now too complex and resource-intensive to be practically achievable. The accurate characterization of *Salmonella enterica* strains remain of great interest for many scopes.

Different levels of typing are required for different needs. The O-grouping system is useful in epidemiological studies and can also support genus identification in clinical settings; however, it does not allow for the rapid identification of whether an organism is likely to cause enteric fever, as considerable cross-reactivity occurs among serogroups. For example, serotype Infantis, which causes gastroenteritis, and serotype Choleraesuis, an important cause of invasive infections, both belong to group C1. Likewise, serotype Enteritidis, which cause gastroenteritis, and serotype Typhi, the causative agent of enteric fever, both belong to group D.

From a clinical perspective, differentiation of typhoidal serovars is of utmost importance to evaluate their etiological significance, to target the treatment, and to rapidly identify nosocomial outbreak. The main advantage of the I-dOne method lies in its ability to discriminate *S.* Typhi within group D, which is essential both from clinical and epidemiological standpoints. The method is also cost-effective, rapid, and accurate, meaning that it may be used also in low resource settings. However, this technique is not intended to fully replace conventional analytical methods, at least at the current stage. Rather, it may serve as a complementary tool for the rapid screening of *Salmonella* serogroups in the routine clinical laboratory practice, and for the presumptive classification of *S.* Typhi. Additionally, the potential for refining the identification of *S.* Paratyphi B variant Java among other group B strains [22]. This serovar exhibits spectral characteristics similar to other group B isolates, as previously noted by Cordovana et al. [61], and preliminary data from the current database indicate a sensitivity of 80% in distinguishing the Paratyphi B variant Java from other group B serovars [22]. Nevertheless, due to the high degree of spectral similarity, misclassifications in both directions can occur. Further studies including a broader range of serogroups, additional isolates of paratyphoid strains (*S.* Paratyphi A, *S.* Paratyphi B, and *S.* Paratyphi C), and rare serogroups will be essential to enhance the typing capabilities of the I-dOne software and to further consolidate the robustness of the method. Given its high discriminatory power at the microbial subtyping level, FTIR spectroscopy has been evaluated for tempestive outbreak investigations [62]. Species-level identification of bacteria remains a significant challenge in clinical microbiology laboratories, particularly for phylogenetically related species with similar phenotypic and genotypic profiles. Many commercially available phenotypic identification systems were developed decades ago and have not been substantially updated, resulting in a high rate of misidentification, especially among recently described taxa. These limitations have driven growing interest in alternative approaches for accurate bacterial typing, e.g., MALDI TOF MS [20]. In this context, infrared (IR)-based spectroscopic techniques present several advantages, including rapid analysis time, low operational cost, minimal sample preparation, and the absence of chemical reagents. Moreover, the IR spectral profile of a microorganism reflects its overall biomolecular composition, providing valuable information on cellular components such as lipids, carbohydrates, proteins, and nucleic acids, enabling effective discrimination at the species and subspecies levels.

4. Conclusions

The novel I-dOne technology represents a promising approach for the rapid and cost-effective differentiation of *Salmonella* serogroups, with a particular advantage in discriminating *S.* Typhi within group D. While this method cannot fully replace conventional serological analyses at present, it offers substantial benefits as a complementary tool in clinical and epidemiological practice. The ability to quickly screen for *Salmonella* serogroups and presumptively identify *S.* Typhi holds significant potential for improving the speed of diagnosis and treatment, particularly in outbreak scenarios and in low-resource settings. Despite the promising results, challenges remain, particularly with regard to spectral overlap between closely related serovars, such as *S.* Paratyphi B variant Java and other group B strains. Further refinement of the I-dOne software and the inclusion of additional serogroups and rare isolates will be crucial to enhance the method's accuracy and broaden its applicability. Moreover, the integration of this technology into routine clinical and environmental monitoring could facilitate early detection and better management of *Salmonella*-related diseases.

Given its high discriminatory power, particularly in the context of microbial subtyping, FTIR spectroscopy and AI-driven tools such as I-dOne represent a forward-looking solution

for improving surveillance, outbreak control, and the overall understanding of *Salmonella* epidemiology and, not least, a precious tool for fast microbiology.

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