

Monophasic variant of *Salmonella* Typhimurium 4,[5],12:i:- outbreak: an investigation by the Competent Authority

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Abstract

Foodborne diseases represent a significant public health issue, regarding both collective health and the economy, with implications for healthcare costs and agribusiness. This paper shows the

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Key words: official control, foodborne disease outbreaks, monophasic variant of *Salmonella* Typhimurium, porchetta.

Contributions: all the authors made a substantial intellectual contribution, read and approved the final version of the manuscript, and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare no potential conflict of interest.

Ethics approval and consent to participate: not applicable.

Funding: none.

Availability of data and materials: data and materials are available from the corresponding author upon request.

Conference presentation: this paper was presented at the XXXII National Conference of the Italian Association of Veterinary Food Hygienists (AIVI), September 13-15, 2023, Maierato, Italy.

Received: 28 March 2024.

Accepted: 20 May 2024.

Early access: 3 June 2024.

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Italian Journal of Food Safety 2024; 13:12522

doi:10.4081/ijfs.2024.12522

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description and results of the investigation of a *Salmonella enterica* subsp. *enterica* foodborne outbreak that occurred in the Marche Region (Italy) in 2022, which was linked to the consumption of a roasted pork product (*porchetta*). As part of the outbreak investigations discussed in this article, molecular analysis to evaluate the genomic correlation between clinical, food, and environmental origin strains was carried out. All *Salmonella* strains of different origins were serotyped as monophasic variant of *Salmonella* Typhimurium with an antibiotic-resistance pattern and an allelic profile such as to define the "cluster strain" allowing the correlation between clinical and food/environmental strains, definitively confirmed by whole genome sequencing analysis. Following the laboratory evidence, corrective measures at the *porchetta* processing plant and at the retail stores involved were carried out by the Local Animal Based Food Hygiene Service. The results of this study show that effective intervention is only possible if efficient data exchange, standardized procedures, and staff training are guaranteed. The latter aspect also concerns the food business operator, who must take appropriate measures to minimize the risk.

Introduction

Foodborne diseases are a serious public health concern and are still one of the most important causes of economic losses and social costs (Hoffmann and Ahn, 2021).

In many cases, outbreaks are not recognized, reported, and/or epidemiologically investigated. In addition, the food source or the responsible pathogen remains unknown, so traceback procedures that could quickly stop the consumption of the contaminated product are not activated. This means that many outbreaks go undetected, not allowing for prevention and control measures. According to the European Union's One Health 2022 Zoonoses Report (EFSA and ECDC, 2023), in 70 (40%) outbreaks out of 175 total reported in Italy in 2022, the causative pathogen remained unknown, a figure in line with the European situation (46.1%). As far as the Italian situation is concerned, salmonellosis remains the most frequently reported zoonosis in humans, followed by campylobacteriosis, in contrast to the European trend.

In 2022, Italy notified the European Food Safety Authority of a total of 175 foodborne epidemic outbreaks involving 1604 human cases and resulting in 303 hospitalizations and 15 deaths.

Salmonella was the pathogen responsible for most of the epidemic outbreaks (52 out of 175) and also caused a high number of human cases (212 out of 1604). In 12 outbreaks (37.5 %) out of a total of 32 (18.3%), the food source involved was meat and meat products, mainly of pork origin. At the European Union level, the

European Community established procedures for the surveillance and collection of information on zoonoses as early as 1999 with Directive 2003/99/EC (European Parliament and Council of the European Union, 2003), adopted in Italy by Legislative Decree No. 191/2006, which provides for the obligation to investigate foodborne outbreaks [Italian Republic, 2006 - Article 7(2)].

As a result, an epidemiological surveillance network with updated infectious agents for surveillance was created, and a new information system for reporting infectious diseases called “PRE-MAL” was set up in 2017 with the decree of March 3 (Italian Republic, 2017). The Marche Region has also actively addressed the management of foodborne disease outbreaks and included it in the strategic macro-objective M06-9 of the 2020-2025 prevention plan: “ensure the identification of the food responsible for a foodborne outbreak so that the alert system can be activated to withdraw/recall the dangerous or potentially dangerous food from the market” (Marche Region, 2021).

The predefined program PP09, “Environment, Climate and Health”, defines measures on various aspects: i) legal acts for the establishment of the regional reference laboratory; ii) the development and implementation of a guide for foodborne diseases; iii) the publication of information material for citizens; iv) the training/information of official control officers; v) food business operators (FBOs) and primary production; vi) health personnel and others involved in notification.

In this article, we present the description and the results of the investigation of a foodborne outbreak of *Salmonella enterica* subsp. *enterica* that occurred in the Marche Region (Italy) in 2022, as part of several foodborne illnesses linked to the consumption of a roasted pork product (*porchetta*).

Materials and Methods

Between July and September 2022, several notifications for suspected food-related infections were shared by the Hygiene and Public Health Service (HPHS) in Fermo and neighboring provinces with the Animal Based Food Hygiene Service (ABFHS, hereafter Service) of the Fermo Local Health Unit (LHU). All the cases had in common enteric symptoms and feces confirmed positive for *Salmonella*; some of them also declared the consumption of roasted pork product (*porchetta*) bought and/or consumed in different places and times but produced at the same food-processing plant located in the Fermo area (site A) and recognized under EC Regulation No. 853/2004 (European Parliament and Council of the European Union, 2004a).

ABFHS inspected site A and also the two retail stores (sites B and C) recognized under EC Regulation No. 852/2004 (European Parliament and Council of the European Union, 2004b). A total of 3 samples of *porchetta* and 23 environmental samples (using the sponge swab method) were collected for *Salmonella* detection. As *porchetta* does not fall within the scope of Regulation (EC) No. 2073/2005 (Commission of the European Communities, 2005 - Annex 1, Chapter 1, paragraph 8), the sampling strategy was changed. Annex 7 - Table 20 of the Deliberation No. 1508/2016 in which the “guide values” for the matrices that do not fall within the scope of the European standard are defined, was used as the legal basis (Marche Region, 2016). In this particular case, *porchetta* was considered a ready-to-eat product.

From the combination of the provisions of the regional regulation and Legislative Decree no. 27/2021 (Italian Republic, 2021 - Annex 1, point 2), a legal single aliquot and a single sample unit of

porchetta were collected for a single non-repeatable analysis. Positivity for *Salmonella* spp. was considered as a food safety criterion. For surface sampling, 7.5×3.5 cm cellulose sponges were used, moistened with 10 mL buffered peptone water in the case of unsanitized surfaces or with neutralizing Dey-Engley broth in the case of sanitized surfaces. The choice of sponges rather than swabs was necessary to sample a larger area and ensure more effective removal of bacterial cells. A single, non-repeatable analysis was performed on the surface samples to also ensure the operator’s right of defense.

In addition to the sampling, an assessment of the hygienic conditions of the premises and equipment was carried out in all sites (A, B and C) and an assessment of the procedures implemented by the FBO based on hazard analysis critical control point (HACCP) principles was carried out at site A.

The environmental and food samples collected at sites A, B, and C were analyzed for *Salmonella* at the food safety laboratory of the *Istituto Zooprofilattico Sperimentale dell’Umbria e delle Marche* (IZSUM) according to the UNI EN ISO 6579-1:2020 standard (ISO, 2020).

Salmonella spp. strains isolated from *porchetta*, environmental swabs, and human clinical samples were serotyped at the Regional Reference Center for Enteric Pathogens for the Marche Region (CRRPE) of IZSUM to ISO/TR 6579-3:2014 (ISO, 2014), and subsequently confirmed by multiplex polymerase chain reaction (PCR) for *fljB* (flagellar antigen H:2) gene (Rahn *et al.*, 1992).

Further sub-characterization analysis of a monophasic variant of *Salmonella* Typhimurium (MVST) strains was performed. In particular, antimicrobial susceptibility testing was performed by disk diffusion method (Kirby-Bauer test) according to the guidelines of the Clinical and Laboratory Standards Institute (2023).

Molecular typing by multiple-locus variable-number tandem repeat analysis (MLVA) according to the European Center for Disease Prevention and Control standard laboratory operating procedure for MLVA of *Salmonella enterica* serotype Typhimurium (ECDC, 2011), was carried out by the Department of Infectious Diseases of *Istituto Superiore di Sanità* and the National Reference Center for Salmonellosis of *Istituto Zooprofilattico Sperimentale delle Venezie* for human and for food and environmental samples, respectively. Whole genome sequencing was performed on a representative selection of 30 strains, among the total collected clinical, food and environmental strains, based on the MLVA results and epidemiological information (for further details, see Napoleoni *et al.*, 2023).

Results

Positivity for *Salmonella* spp. was detected both from environmental swabs (4 out of 23 total) and from *porchetta* (1 out of 3 total) (Table 1). The positive swabs regarded unsanitized surfaces in contact with the roasted pork: transporting board for cooked *porchetta* (site A), teflon chopping board for supporting and cutting *porchetta* (site B), wooden chopping board for *porchetta* and *porchetta* knife (site C). *Porchetta* sample, which tested positive for *Salmonella*, was collected at site C.

Salmonella spp. strains isolated from food *porchetta* (n=1), environmental swabs (4 out of 5 total positives for *Salmonella* spp.), and human clinical cases involved (n=64) were serotyped and confirmed by multiplex PCR as MVST 4,[5],12:i:-. 57 clinical strains of MVST out of 64 showed resistance to the following antibiotics: ampicillin (A), chloramphenicol (C), streptomycin (S),

sulfisoxazole (Su), gentamicin (Gm), trimethoprim (Tmp), and trimethoprim-sulfamethoxazole (Sxt) (ACSSu+Gm+Tmp+Sxt profile, *i.e.*, “cluster strain” antibiotic resistance type). The remaining 7 clinical strains showed different partial types of antibiotic resistance with respect to the type of cluster strain: ACSSu+Tmp+Sxt (n=3), ACSSu+Gm (n=1), ACSSu+Amc+Gm+Tmp+Sx (n=1), CSSu+Gm+Tmp+Sxt (n=1). 63 clinical strains of MVST out of 64 (57 with the cluster strain antibiotic resistance type and 7 with antimicrobial resistance types close to the cluster-strain type), presented 3-14-10-na-211 allelic profile while the remaining strain, a closely related allelic profile, *i.e.*, 3-13-10-na-211. Therefore, a total of 57 isolates from cases of human salmonellosis were identified as belonging to the same cluster of the MVST (antibiotic resistance type ACSSu+Gm+Tmp+Sxt and MLVA 3-14-10-na-211 or 3-13-10-na-211). Additionally, seven isolates from human cases were defined as being closely related to this cluster of the MVST.

All 5 food/environmental strains of the MVST (4 from environmental swabs and 1 from *porchetta*) had the antibiotic resistance type ACSSu+Gm+Tmp+Sxt and the MLVA profile 3-14-10-na-211, corresponding to those of the human cluster strains.

The core genome multilocus sequence typing (cgMLST) analysis showed that no significant allelic distance was highlighted between the clinical (n=24), food-related (n=1), and environmental (n=4) strains tested, except for one clinical strain that was shown to be 49 alleles distant from the main group and was therefore considered a different clone (for further details about the obtained results, see Napoleoni *et al.*, 2023).

An inspection conducted at the food-processing plant revealed structural, sanitation, and documentary deficiencies regarding the procedures for good manufacturing and hygiene practices and those based on HACCP principles. In particular, the full production package did not correctly identify critical control points (CCPs) in the *porchetta* processing steps by managing cooking and subsequent cilling as good manufacturing practices (GMPs). No structural and sanitation nonconformities were found at site B. The inspection at site C found a lack of hygiene and non-compliance with product storage procedures.

Following the results of the inspections and sampling, the service took the following actions: i) *porchetta* production was suspended according to Article 138 of EU Regulation No. 625/2017 (European Parliament and Council of the European Union, 2017); violations of Articles 4 and 5 of EC Regulation No. 852/2004 for hygiene and sanitation deficiencies and non-application of procedures resulted in the imposition of two administrative sanctions pursuant to Article 6, paragraphs 5 and 6, of Legislative Decree No. 193/2007 (European Parliament and Council of the European Union, 2004b; Italian Republic, 2007); ii) site C was informed of a minor non-compliance rule with corresponding compliance deadlines in relation to structural deficiencies and the implementation of self-checking procedures, resulting in a measure under Article 138 of EU Regulation No. 625/2017 with compliance dead-

lines and the imposition of two administrative sanctions for non-compliance with official control requirements and non-application of the procedure under Article 6(5) and (7) of Legislative Decree No. 193/2007 (Italian Republic, 2007; European Parliament and Council of the European Union, 2017); iii) pursuant to Article 347 of the Code of Criminal Procedure (Italian Republic, 1988), a criminal complaint was issued against the legal representative of the production plant for violation of Article 444 “trafficking in substances intended for food and harmful to public health” and Article 452 “offenses against public health” of the Criminal Code (Codice Penale, 1930); these measures were ordered following the isolation of the same serotype in all samples and clinical cases; iv) following Article 50 of Regulation (EC) No 178/2002 (European Parliament and Council of the European Union, 2002), an information notification was sent by the competent authority to the Rapid Alert System for Food and Feed, followed by a validation without triggering an alert, as the product was no longer on the market; v) case management report to the Veterinary and Food Safety Functional Unit of the Marche Region.

Discussion

The first interface in the food-related infections investigations is the HPHS, which evaluates warnings from various sources (Competent Authorities, Hospitals, other LHUs, *etc.*). In the case described, the reporting sources were both the emergency room of Fermo Hospital and the Departmental Services of neighboring provinces. The next phase of the investigation involved interconnected operational steps: epidemiological investigation, environmental and food samplings, laboratory analysis, and preparation of the final report. During the period of the outbreak, the regional foodborne diseases investigation guidelines (Marche Region, 2022a) that would have been available in 2022 with the following standardization of the operative flows (Figures 1 and 2), had not yet been published; however, the activity carried out did not deviate from what the document would have later provided.

The management of the epidemiological investigation and its subsequent sharing by HPHS with ABFHS was one of the critical issues highlighted in the case. The development of shared procedures and guidelines, as previously reported, will surely improve this critical point.

In general, another critical point related to the management of foodborne infection cases is the human samples sent to several laboratories, not allowing complete epidemiological investigations of the clinical cases.

In the Marche Region, the presence of a single reference laboratory, *i.e.*, the CRRPE, where strains of *Salmonella* spp. of clinical, veterinary, food and environmental origin are sent by the regional hospital analysis laboratories and the human/food/environmental private analysis laboratories, allowed for a rapid understanding of the extent of the outbreak at a regional level. The

Table 1. Results of the sampling.

Site	Environmental swabs			Roasted pork product (<i>porchetta</i>)		
	Negative	Positive	Total	Negative	Positive	Total
A	7	1	8	1	0	1
B	9	1	10	1	0	1
C	3	2	5	0	1	1
	19	4	23	2	1	3

Enteric Regional Reference Center, established by the Marche Regional Department of Health following a note of 12 September 2001 under the express request of the *Istituto Superiore di Sanità*, was subsequently formalized and further identified as the Regional Reference Laboratory for Foodborne Diseases in Humans in 2022 by Executive Decree No. 149 of October 7, 2022 (Marche Region, 2022b).

In the case described, the CRRPE also received clinical strains isolated at hospitals and private laboratories in the region, which allowed the detection of an increase in cases of salmonellosis in a given period (July-September 2022).

The territorial surveillance of the circulation of enteric pathogens is provided by the Enter-Net and Enter-Vet surveillance networks. The former, coordinated at the national level by the Department of Infectious Diseases of the *Istituto Superiore di Sanità*, deals with European surveillance of infections caused by bacteria such as *Salmonella* spp., *Campylobacter* spp., *Shigella* spp., *Yersinia* spp., *Aeromonas* spp. and *Vibrio* spp. and has been incorporated into the European surveillance system for “Food and Waterborne Diseases” coordinated by the European Center for Disease Prevention and Control. The latter, coordinated at the national level by the National Reference Center and World Organization for Animal Health Reference Laboratory for

Salmonellosis of the *Istituto Zooprofilattico Sperimentale delle Venezie*, collects data on *Salmonella* spp. isolates from animals, food and the environment.

In the Marche Region, both networks, Enter-Net and Enter-Vet, are powered with data by the Regional Reference Center in collaboration with the peripheral laboratories of human, veterinary, food, and environmental microbiology that send the strains to it.

In cases of outbreaks or international alerts, but also in the management of sporadic cases, the CRRPE, supported by the National Reference Centers, is in charge of analyzing and comparing bacterial isolates of different origins providing real-time surveillance data on the circulation of enteric pathogens in the regional territory.

The above aspects show the strategic importance of the simultaneous management of animal, food, environmental, and human origin strains according to a “One Health” approach.

Regarding the type and methodology of sampling used in the case study described, supporting the epidemiological investigation with environmental sampling made it possible to correlate the source of infection (*porchetta*) with the clinical cases. In addition, the positivity for *Salmonella* obtained from environmental samples carried out in a single, unrepeatable analysis allowed the ABFHS to apply the measures provided by the legislation.

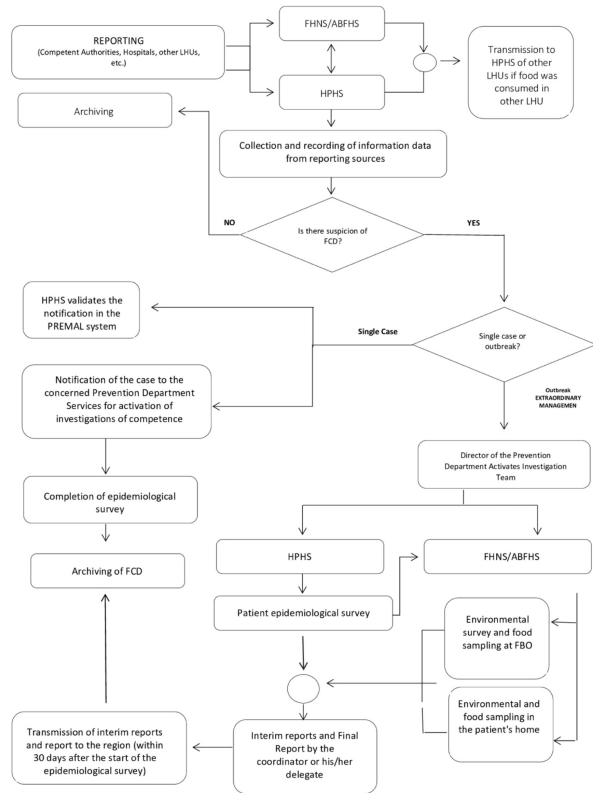


Figure 1. Flowchart of single case and outbreak investigation as proposed by the regional foodborne diseases investigation guidelines. Source: Marche Region, 2022a (Decree No. 98/2022). FHNS, Food Hygiene and Nutrition Service; ABFHS, Animal Based Food Hygiene Service; LHU, Local Health Unit; HPHS, Hygiene and Public Health Service; FCD, foodborne disease; FBO, food business operators.

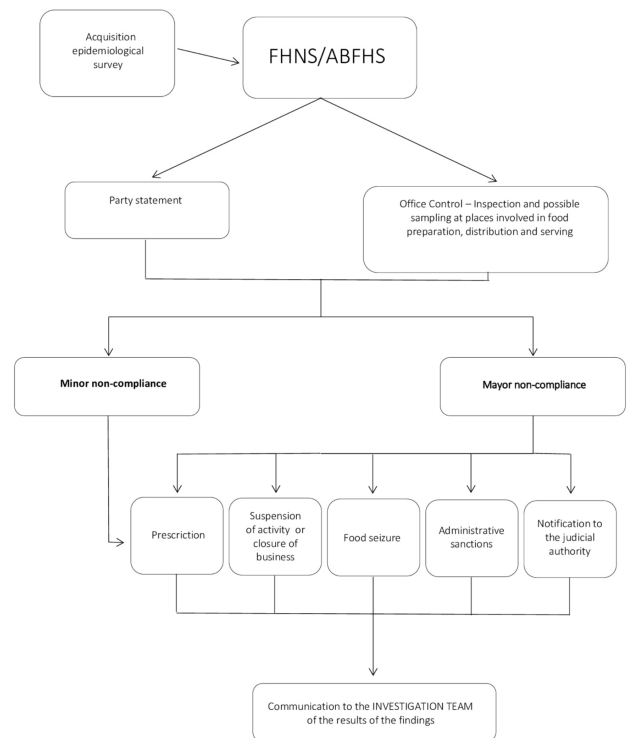


Figure 2. Flowchart of the food safety measures adopted by the competent authority as proposed by the regional foodborne diseases investigation guidelines. Source: Marche Region, 2022a (Decree No. 98/2022). FHNS, Food Hygiene and Nutrition Service; ABFHS, Animal Based Food Hygiene Service.

The result of the inspection revealed the wrong application of HACCP principles by the FBO. As laid down in Article 5 of Regulation (EC) No. 852/2004 (European Parliament and Council of the European Union, 2004b), the operator must develop, implement, and maintain one or more permanent procedures based on the principles of HACCP; one of the most important steps is to identify the CCPs at the level(s) where control proves essential to prevent or eliminate the risk or reduce it to an acceptable level. In the *porchetta* production HACCP plan, the only CCP highlighted by the FBO was cold storage, while cooking and subsequent refrigeration were treated as GMP. Cooking at the appropriate time and temperature is considered the only phase that can confer adequate sanitary and hygienic properties to the final product due to the inactivation of all non-spore-forming pathogens; subsequent blast chilling and storage at refrigeration temperature do not allow the proliferation of heat-resistant bacteria. Therefore, the simple application of good cooking and blast chilling practices does not provide a sufficient guarantee against microbiological hazards such as *Salmonella* spp.

Conclusions

Over the last three decades, a new *Salmonella* serotype has emerged worldwide, namely the MVST with the antigenic formula 4, [5],12:i:-. This serotype was first identified in chicken carcasses in Portugal in 1980 and gradually spread through the swine chain, especially in Europe and the United States (Machado and Bernardo, 1990).

The description of this outbreak shows how important the collaboration between the various institutions involved (medical and veterinary services, regional authorities, *Istituto Zooprofilattico Sperimentale*) is to carry out effective official control to identify the source of foodborne cases and then to take effective and appropriate measures. Therefore, the multidisciplinary approach is important for a successful epidemiological investigation but requires trained and specialized personnel. The training activity also concerns the FBOs, as the case described has shown that, although more than 20 years have passed since the mandatory introduction of procedures based on HACCP principles, FBOs are still not fully aware of the importance of proper implementation of hygiene and health risk prevention.

In conclusion, rapid intervention is essential to act effectively, both for the accurate and timely collection of relevant information and for the subsequent conduct of inspections and sampling. In addition to intensifying data exchange networks, it is necessary to create appropriate standardized procedures for conducting epidemiological investigations, collecting food, environmental and biological samples, and archiving the data in a common way, allowing an appropriate evaluation by the staff involved. At the local level, a joint procedure is currently being prepared between the services of the Prevention Department of the LHU of Fermo with the involvement of the microbiology laboratory of the Regional Hospital and the CRRPE of IZSUM. The next planned step is the involvement of other professionals (*e.g.*, general practitioners, pediatricians, private laboratories) to increase the percentage of foodborne outbreaks where the food source of infection can be identified, as defined in the Marche Region Prevention Plan.

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