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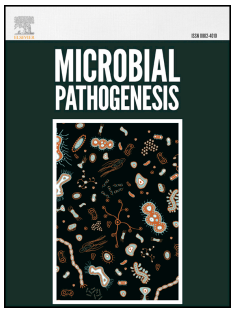

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Prevalence, molecular and antimicrobial resistance of *Salmonella* isolated from sausages in Meknes, Morocco

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Abstract

Salmonella is among the most important food borne pathogens worldwide contaminating a wide range of animal products including meat products. The aims of this study go through two steps: The first step is to estimate the proportion of sausages products contaminated by *Salmonella* in Meknes city (Morocco), which were collected from various shopping sites: butchery, street vendors, supermarket and souk (Weekly market combines the population of the small villages around Meknes city). The second one is to identify serovars, to determine the antimicrobials resistance patterns of isolates and to detect the *invA* and *spvC* genes. 34 (21.79%) *Salmonella* was isolated, recovered 4 serogroups and 12 serotypes. The most prevalent serotypes were *Salmonella* Corvallis (23.53%) and *Salmonella* Kentucky (17.65%). All *Salmonella* isolates were tested for their susceptibility to 18 selected antimicrobials agents, of which 100% were resistant to at least one antimicrobial, 85.30% (29/34) were resistant to two or more antimicrobials and 44.12% (15/34) were resistant to at least three antimicrobials. All *Salmonella* are resistant to ampicillin, 76.47% to streptomycin, 20.59% to sulphonamides, 17.65% to Tetracycline and 11.77% to Ofloxacin. The "ACSSuT" penta-resistance pattern was observed in tow of the *Salmonella* Typhimurium strains. In addition, ~~our~~ this study showed that all *Salmonella* strains (34) were positive for invasion gene *invA* and negative for the virulence gene *spvC*.

Key words: *Salmonella*, Antimicrobials resistance, Sausages, *invA*, *spvC*

Introduction

Food-borne diseases imposes a substantial economic burden and threatens the public health on society causing an acute morbidity and chronic sequelae [1,2]. Worldwide, the consumption of contaminated food is responsible for two million deaths per year [3]. Furthermore, the non-typhoidal *Salmonella* is the main cause of food-borne diseases, which pose a big problem for medicine and agribusiness; it's associated in most cases with the consumption of animal products [4,5]. However, some studies show that even of plant origin may be a vector for transmission of *Salmonella* to humans [6,7].

Worldwide, *Salmonella* is responsible for the millions cases of foodborne diseases each year [8]. The incidence of salmonellosis ranged between 14.53 and 17.55 cases per 100,000 people in the United States [9]. Moreover, *Salmonella* was the second most commonly reported zoonoses in the European Union with a total of 91,034 confirmed cases of human salmonellosis in 2012 [10]. The true incidence of salmonellosis in both humans and animals is difficult to evaluate in developing countries because of the lack of epidemiological surveillance systems [11]. The bacterial resistance to antimicrobial agents, including *Salmonella* is a major problem of food safety; it affects both the veterinary and public health by transfer of resistant strains to humans through the food chain [12,13]. The multi-resistant strains can sometimes be the cause of international outbreaks raising fears therapeutic impasses and increased morbidity and mortality for simple food poisoning [4].

In Morocco, the consumption of meat products has experienced a sharp increase in recent years and the presence of *Salmonella* in these products is usual [14–16], which increases the population exposure to the pathogenic agent. Also, the manufacture of sausages is done under conditions that do not comply with good hygiene practices. In Meknes city, sausages are sold in supermarkets, butchery, street vendors and souk. The street vendors are located mainly in neighborhoods, they have generally a very low cultural level and are unconscious of good

hygiene practices and work in illegal conditions without control and without regulatory legislation. As regards the souk, sausages are presented on parts in contact with air and dust at ambient temperatures, without any respect for good hygiene practices. Those who make a deadly food and a potential transmission vector of pathogenic bacteria to humans.

The aim of this study is to determine the prevalence of *Salmonella* in sausages made from beef meat, turkey meat and artisanal sausages "Merguez" trades in Meknes city (Morocco), using the conventional method, by biochemical and serological means. The molecular identification ~~confirmation~~ by PCR was performed to detect the *invA* and *spvC* genes. The sensitivity and resistance of *Salmonella* to antimicrobials agents were studied. It should be mentioned that "Merguez" is a small raw sausage generally made of lean and fat beef mixed with condiments and stuffed into natural casings of sheep and beef; it is generally sold in street vendors.

Material and methods

1. Samples Collections

From March 2014 until February 2015, 156 samples of sausages (60 of Turkey sausages, 60 of Beef sausages, 36 of Artisanal sausages "Merguez") were collected from various sites: 72 from butchery, 36 from street vendors, 24 from Supermarket and 24 from Souk (Weekly market) in Meknes city in Morocco, with a frequency of 13 samples per month (5 of Turkey sausages, 5 of Beef sausages, 3 of Artisanal sausages "Merguez"). The collected amount of each sample of sausage is about 40 grams, which they were transferred immediately in a cooler to microbiology laboratory at the Faculty of Science Meknes.

2. Isolation of *Salmonella*

~~25 g~~ Twenty-five grams of sausages were pre-enriched into 225 mL of sterile buffered peptone water (Oxoid, England) and incubated at 37°C for 18 to 24 hours. 0.1 mL of pre-enrichment culture was inoculated into 10 mL of Rappaport-Vassiliadis broth (Biokar) and 1

mL was inoculated into 10 mL of Selenite Cysteine Broth (Biokar), the cultures were incubated at 42°C and 37°C, respectively, for 24 hours. From them, a streak culture is performed on Hektoen enteric agar (Biokar) and *Salmonella-Shigella* agar (Biokar), respectively; the inoculated plates were incubated at 37°C for 24 hours. The suspect colonies of *Salmonella* were inoculated into Hanja Kligler agar (Biokar), Simmons citrate agar (Oxoid), mannitol motility agar (Bio-Rad), lysine decarboxylase broth (Scharlab), Indole urea (bio Merieux RSA) and incubated at 37°C for 18 to 24 hours. The oxidase discs (in vitro diagnostics) and ONPG (Oxoid Limited) are also tested. All the isolates were confirmed by biochemical tests using an API 20E (Bio Mérieux RSA).

3. Molecular identification of *Salmonella*

All *Salmonella* strains collected in this study were tested for the invasion gene *invA* and virulence gene *spvC* (Table 1), using the conventional polymerase chain reaction (PCR) as described by Karraouan et al. [15].

4. Determination of *Salmonella* Serogroup and Serotype

The serogroup and serovars of the *Salmonella* isolates were determined by slide agglutination tests with O antigen and H antigen antiserums obtained from Pasteur Institute of Casablanca city in Morocco. The results were interpreted according to the Kauffmann-White scheme [17].

5. Antimicrobial Susceptibility Testing

The agar dilution method with Mueller-Hinton agar was used to test the susceptibility of *Salmonella* to 18 antimicrobials (Table 2). The results were interpreted according to the recommendation of the Clinical and Laboratory Standards Institute [18]. In this study, the isolates showing a decrease in susceptibility (intermediate) were considered as resistant and *Escherichia coli* ATCC 25922 was used as control strains.

Results

1. Prevalence of *Salmonella* in sausage samples

Salmonella was detected in 21.79% (34/156) of samples. Artisanal sausages "Merguez" are the most contaminated 30.55% (11/36), followed by Turkey sausages 23.33% (14/60) and Beef sausages 15% (9/60). Considering the area of marketing, sausages sold in Street Vendors are the most contaminated (30.55%), followed by souk (29.16%), butchery (18.05%) and supermarket (12.5%). Invasion gene operon *invA* was detected in all *Salmonella* isolates in ~~our~~ this study. However, *spvC* was not detected in any strain (Figure 1).

2. Determination of serogroups and Serotypes of isolated *Salmonella*

The diversity of raw material and the conditions of manufacturing sausages influence the distribution of serogroups and serotypes. In this study, 4 serogroups and 12 serotypes were identified in 34 *Salmonella* isolates with an abundance of *Salmonella* and *Salmonella* Kentucky (Table 3).

3. The antimicrobial susceptibility of isolated *Salmonella*

From the total of 34 *Salmonella* isolates evaluated for resistance against 18 selected antimicrobial agents (Table 4), all *Salmonella* tested (100%) are resistant to at least one antimicrobial, 85.30% (29/34) are resistant to two or more antimicrobials and 44.12% (15/34) are resistant to three or more antimicrobials (Table 5). The analysis of antimicrobials susceptibility showed four strains (4/34) of *Salmonella* Kentucky are resistant to fluoroquinolone and two strains (2/34) of *Salmonella* Typhimurium are resistant to five antimicrobials agents (Tetracycline, Sulfonamides, Streptomycin, Chloramphenicol, and Amoxycillin). ~~and the absence of beta-lactamase extended spectrum (ESBL).~~

Discussion

The prevalence of *Salmonella* in sausages is 21.79% (34/156). It's in agreement to those reported in Brazil (24.4%) [19] and Botswana (26%) [20]. However, this value is less than that found in Algeria (46.77%) [21] and higher than that found in Morocco (0.097%) [6] and Italy (3.5%) [22]. A study done in Tunisia showed the absence of *Salmonella* in Merguez

sausages [23]. This comparison should be made with suspicion, because the results may be influenced by several factors such as the level of development between different countries, the location and climate of the sites, the origin of the raw material used, the sampling seasons, the hygienic quality of production units and isolation methods.

The origin of the raw material influences the risk of contamination of sausages. Artisanal sausages "Merguez" are the most contaminated by *Salmonella* (30.55%), followed by Turkey sausages (23.33%) and Beef sausages (15%). This difference can be explained by the precarious health conditions of preparation and sale of Artisanal sausages "Merguez", including cross-contamination offal and raw material can occur. Furthermore, turkey meat is generally considered a very important reservoir for *Salmonella* contamination [15,16,24]; other studies have shown the presence of *Salmonella* in beef meat products [25–28]. This contamination increases all along the production chain, because of cross-contamination during slaughter, cutting and further processing [29]. So it’s important to monitor the occurrence of *Salmonella* throughout the production chain, in order to avoid the contamination of finished products.

The present study shows a wide distribution of *Salmonella* serotypes due to the diversity of the raw material, the conditions of preparation, storage and distribution modality. Twelve serotypes were identified; *Salmonella* Corvallis (23.53%) and *Salmonella* Kentucky (17.65%) are the most abundant. In Morocco *Salmonella* Corvallis was detected in food [30] and ground turkey [15], *Salmonella* Kentucky was isolated in turkey carcasses [16] and food [31]. However, these serotypes differ from those published in other studies [6,14].

The increase and accumulation of the antimicrobials resistance in pathogenic bacteria are a major public health problem, thus *Salmonella* acquires their resistance to multiple antimicrobial agents in animals farm before being transmitted to humans through the food chain [5]. Several studies in livestock farms showed the presence of *Salmonella* resistant to

multiple antimicrobials [5,32–34], other studies have shown the relationship between the use of antimicrobial agents in the veterinary field and humans and increased bacterial resistance [35].

~~Our~~ This study indicates that 100% of isolated *Salmonella* were resistant to at least one antimicrobial; these results are higher than those found previously in Morocco (75.43%) [14], and those recorded in Algeria (90.32%) [21], Tunisia (20%) [23], Italy (84.2%) [28] and China (74%) [36]. 85.30% (29/34) of isolated *Salmonella* are resistant to two or more antimicrobials and 44.12% (15/34) are resistant to three or more antimicrobials. These results are superior to those found in Botswana (20.3%) [20], ~~and~~ but lower than those found in Egypt (100%) [26].

The rate of resistance to streptomycin and tetracycline was 76.47% and 17.65%, respectively, the resistance to these antimicrobials is common in *Salmonella* isolates and has been observed previously in Morocco [14], Algeria [21], Tunisia [23], Egypt [37], Iran [38], Malaysia [39] and China [29]. The low percentage of tetracycline resistance compared to that of streptomycin, can be explained by the prohibition of the use of this drug in poultry farms and cattle. The amount of the sulfonamides resistance is significant (20.5%) and could be the result of widespread use of these antimicrobials in livestock; this result is lower than that found in Morocco (64.1%) [15] and Algeria (90.32%) [21], but higher than that found in Tunisia (1.2%) [23].

The rate of resistance to ofloxacin is 11.77%, this antimicrobial belonging to the fluoroquinolone; it's used in the treatment of severe salmonellosis cases for adults [4]. Unfortunately, *Salmonella* isolates were found resistant to other fluoroquinolone in Morocco [15,16], Sudan [40], Algeria [21], Tunisia [41] and China [29]. However, the resistance of *Salmonella* to this ~~such as~~ antimicrobial has a potentially negative impact on human health.

Two serotypes of *Salmonella* Typhimurium are resistant to ceftriaxone (5.88%), an antimicrobial that belongs to the third generation cephalosporins, it's used in the treatment of salmonellosis cases in children [4]. Other studies have also reported the resistance of *Salmonella* to the same antimicrobials family [36,42]. The resistance to chloramphenicol is detected in 5.88% of isolates, it should be noted that the chloramphenicol, have not been used in breeding for many years, this resistance may be due to cross-resistance or co-resistance mechanisms.

Conclusion

In this study, the high levels of contamination of sausages with *Salmonella* and the detection of several serotypes highlight the poor hygiene all along the chain of manufacture and sale of this product. Furthermore, our results indicate the widespread presence of *Salmonella* strains resistant to antimicrobials: the resistance of *Salmonella* Typhimurium "ACSSuT" and the resistance of *Salmonella* Kentucky to Ofloxacin. So it's time to monitor, control and rationalize the use of antimicrobial agents in livestock animals, to preserve the efficacy of existing drugs and to prevent the acquisition and increased resistance to recent molecules in order to fight against the vertical and horizontal transfer of multidrug resistant strains. It should be stressed also the need to implement proactive measures in hygiene practices and the application of Hazard Analysis Critical Control Point (HACCP) in the preparation and manufacturing of sausages, for reducing *Salmonella* contamination and the risk of food-borne diseases.

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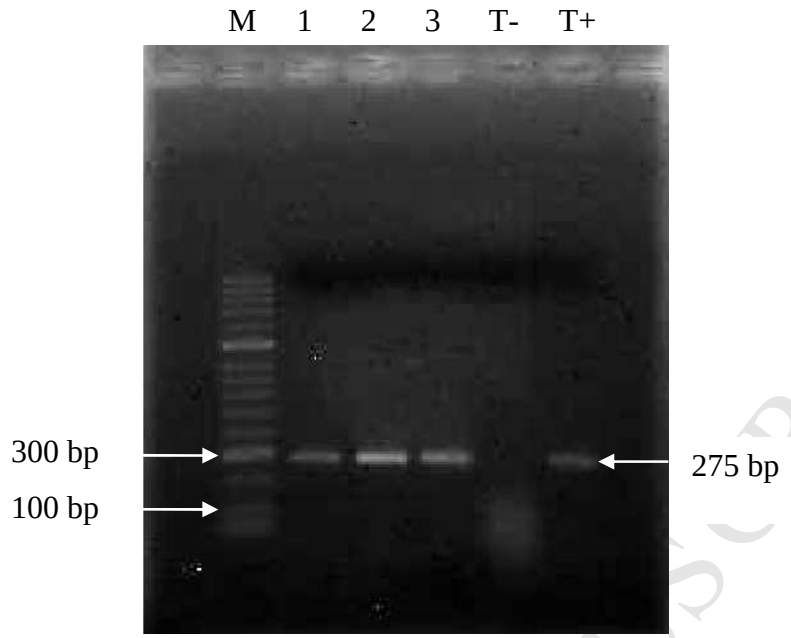

Figure 1: PCR amplification of the *invA* gene. T-: Negative control; T+: Positive control; M: Molecular weight marker 100 bp; Lanes 1-3: *Salmonella* isolates from (Turkey sausages samples, Beef sausages samples, Artisanal sausages samples) showing positive 275 bp DNA fragment of *invA* gene.

Table 1: PCR primers used for amplification of virulence genes in *Salmonella* isolates.

Virulence genes	Primer sequence 5'-3'	Size (bp)	Reference
invA	F- TATCGCCACGTTCGGCAA	275	[43]
	R- TCGCACCGTCAAAGGAACC		
spvC	F- CGGAAATACCATCAAATA	669	[44]
	R- CCCAAACCCATACTTACTCTG		

Table 2: Antimicrobial agents and the range of concentrations tested.

Antimicrobial agents	Code	Concentration Disc
Ampicillin	AMP	10 µg
Amoxicillin	AML	25 µg
Ticarcillin-clavulanic acid	TIM	85 µg
Imipenem	IPM	10µg
Ceftriaxone	CRO	30 µg
Cefuroxime sodium	CXM	30 µg
Ceftazidime	CAZ	30 µg
Cefamandole	MA	30µg
Colistin sulfate	CT	50 µg
Kanamycin	K	30 µg
Ofloxacin	OFX	5 µg
Nalidixic acid	NA	30 µg
Sulfonamide	SSS	200 µg
Gentamicin	CN	30 µg
Chloramphenicol	C	30 µg
Tetracycline	TE	30 µg
Streptomycin	S	10 µg
Trimethoprim–Sulfamethoxazole	SXT	1.25 µg/ 23.75 µg

Table 3: Serovar distribution of isolated *Salmonella*

Serogroupe	Serotype	Beef	Turkey	Artisanal	Total
Group B (n=4)	Typhimurium	0	2	0	2
	Agona	0	1	0	1
	Saintpaul	0	1	0	1
Group C1 (n=8)	Mbandaka	3	0	1	4
	Montevideo	0	3	0	3
	Livingstone	0	0	1	1
Group C2-C3 (n=16)	Corvallis	2	1	5	8
	Kentucky	1	5	0	6
	Bovismorbificans	2	0	0	2
Group E1 (n=6)	Give	0	0	4	4
	Anatum	1	0	0	1
	Muenster	0	1	0	1

Table 4: Antimicrobial resistance percentages of *Salmonella* isolated from sausages.

		No. of *Salmonella* isolates (n = 34)	
		S	R
Amoxycillin (25 µg)	AML	27 (79.41%)	7 (20.59%)
Ampicillin (10 µg)	AMP	0 (0%)	34 (100%)
Ticarcillin/ Clavulanic Acid (85 µg)	TIM	30 (88.23%)	4 (11.77%)
Imipenem (10µg)	IPM	34 (100%)	0 (0%)
Ceftriaxome (30 µg)	CRO	32 (94.12%)	2 (5.88%)
Cefuroxime Sodium (30 µg)	CXM	31 (91.18%)	3 (8.82%)
Ceftazidime (30 µg)	CAZ	34 (100%)	0 (0%)
Cefamandole (30µg)	MA	30 (88.23%)	4 (11.77%)
Colistin Sulfate (50 µg)	CT	23 (67.65%)	11 (32.35%)
Kanamycine (30 µg)	K	32 (94.12%)	2 (5.88%)
Ofloxacin (5 µg)	OFX	30 (88.23%)	4 (11.77%)
Nalidix acid (30 µg)	NA	29 (85.30%)	5 (14.70%)
Sulfonamides (200 µg)	SSS	27 (79.41%)	7 (20.59%)
Gentamicin (30 µg)	CN	34 (100%)	0 (0%)
Chloramphenicol (30 µg)	C	32 (94.12%)	2 (5.88%)
Tetracycline (30 µg)	TE	28 (82.35%)	6 (17.65%)
Streptomycine (10 µg)	S	8 (23.53%)	26 (76.47%)
Trimethoprim/Sulfamethoxazole (1.25µg/ 23.75µg)	SXT	32 (94.12%)	2 (5.88%)

S: Susceptible; R: Resistant

Table 5: Antimicrobial resistance profiles of *Salmonella* serovars isolated from sausages.

Origin	*Salmonella* serovars	Antimicrobials resistance profile	MAR index
T.S	Agona	AMP	0.05
T.S	Muenster	AMP	0.05
A.S	Livingstone	AMP	0.05
B.S	Anatum	AMP, S, CT	0.16
A.S	Give	AMP, S	0.11
A.S	Give	AMP, S	0.11
A.S	Give	AMP, S	0.11
A.S	Give	AMP, S	0.11
B.S	Bovismorbificans	AMP, S	0.11
B.S	Bovismorbificans	AMP, S	0.11
A.S	Mbandaka	AMP	0.05
B.S	Mbandaka	AMP	0.05
B.S	Mbandaka	AMP, S, CT	0.16
B.S	Mbandaka	AMP, S, CT	0.16
T.S	Montevideo	AMP, S, CT	0.16
T.S	Montevideo	AMP, S, CT	0.16
T.S	Montevideo	AMP, S, CT	0.16
A.S	Corvallis	AMP, S	0.11
A.S	Corvallis	AMP, S	0.11
B.S	Corvallis	AMP, S	0.11

A.S	Corvallis	AMP, CT	0.11
B.S	Corvallis	AMP, CT	0.11
T.S	Corvallis	AMP, S	0.11
A.S	Corvallis	AMP, S, AML, CXM	0.22
A.S	Corvallis	AMP, AML, SSS	0.16
T.S	Saintpaul	AMP, S, CT, SSS, SXT	0.27
T.S	Kentucky	AMP, S	0.11
T.S	Kentucky	AMP, S	0.11
T.S	Kentucky	AMP, S, TE, OFX, NA	0.27
B.S	Kentucky	AMP, S, AML, SSS, TE, MA, OFX, NA	0.44
T.S	Kentucky	AMP, S, AML, SSS, TE, TIM, OFX, NA	0.44
T.S	Kentucky	AMP, S, AML, SSS, TE, TIM, MA, OFX, NA	0.5
T.S	Typhimurium	AMP, S, CT, AML, SSS, TE, TIM, MA, CXM, CRO, C, K, SXT, NA	0.77
T.S	Typhimurium	AMP, S, CT, AML, SSS, TE, TIM, MA, CXM, CRO, C, K, SXT	0.72

T.S: Turkey Sausages; B.S: Beef Sausages; A.S: Artisanal Sausages; AMP: Ampicillin; AML: Amoxicillin; S: Streptomicin; CT: Colistin sulfate; CXM: Cefuroxime sodium; SSS: Sulfonamide; SXT: Trimethoprim-Sulfamethoxazole; TE: Tetracycline; NA: Nalidixic acid; OFX: Ofloxacin; MA: Cefamandole; TIM: Ticarcillin-clavulanic acid; CRO: Ceftriaxone; C: Chloramphenicol; K: Kanamycin.

Highlight

- *Salmonella* were detected in 34 of the 156 samples (21.79%).
- 4 serogroups and 12 serotypes were identified; *Salmonella* Corvallis and *Salmonella* Kentucky were most prevalent.
- Multidrug resistance was found among the 15 (44.12%) *Salmonella* isolates.
- The "ACSSuT" penta-resistance pattern was observed in tow strains of the *Salmonella* Typhimurium.
- The resistance to Ofloxacin was observed in 4 strains of the *Salmonella* Kentucky.

Highlights

The theme in this work is related to the hygienic quality and contamination with Salmonella, in sausages marketed in the city of Meknes in Morocco. The prevalence, determining antibiotic resistance profile, biochemical identification, serological and research of invA virulence gene were performed and determined.

- Salmonella was detected in 34 of the 156 samples (21.79%).
- Four serogroups and 12 serotypes were identified; Salmonella Corvallis and Salmonella Kentucky were most prevalent.
- Multidrug resistance was found among the 15 (44.12%) *Salmonella* isolates.
- The ''ACSSuT'' penta-resistance pattern was observed in tow strains of the *Salmonella typhimurium*.
- The resistance to Ofloxacin was observed in 4 strains of the Salmonella Kentucky
- All *Salmonella* strains collected were tested for the virulence genes invA and spvC using the conventional polymerase chain reaction (PCR).