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Multidrug Resistance of *Salmonella* Isolated from Shellfish Samples, Morocco

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Authors' contributions

This work was carried out in collaboration between all authors. Author RB designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors BB, MA, BK, MK and AL managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aims to determine prevalence of *Salmonella* in shellfish and to study their resistance to antibiotics.
Samples: Three species of shellfish consisted of cockles, clams and mussels were sampled monthly in six sites during two years 2008 and 2012.
Methodology: As many as 272 samples of shellfish were examined for presence of *Salmonella*. Positives strains were confirmed for presence of invA gene, serotyping and tested for drug susceptibility.
Results: Up to 7.7% of samples were positive for *Salmonella* and a total of 90 *Salmonella* isolates belonging to 4 serovars (*S.* Kentucky, *S.* Glostrup, *S.* Newport and *S.* Reading) were tested for their susceptibility to a panel of 12 antimicrobial agents. Many resistant isolates were detected with

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75.8% of isolates resistant to at least one antimicrobial agent. Isolates demonstrated resistant to streptomicin, chloramphenicol, nalidixic acid, ciprofloxacin, amoxicillin, respectively with (90.6%; 51.6%; 31.6%; 30.5% and 19%). The most common pattern of multiple drug resistance included resistance to chloramphenicol, ciprofloxacin, nalidixic acid.

Conclusion: It seemed that strains isolated of *Salmonella* were multidrug resistant and almost one third of strains were resistant to quinolone.

The results emphasize the need of a monitoring programme of bacterial pathogenes *Salmonella* on shellfish to protect human health.

Keywords: Shellfish; Salmonella; multidrug resistance; cockles; clams; mussels.

1. INTRODUCTION

Salmonella serovars isolated from seafood are considered most problematic of human health. They can cause illness, diarrheic, fever and can lead to collective toxi-infection. To date, 2541 *Salmonella* serovars have been identified worldwide and cause more outbreaks of foodborne illnesses than any other bacteria [1]. Shellfish are consumed raw or lightly cocked, so if they are produced from polluted areas can transmitted pathogen bacteria to consumers. Prevalent studies of *Salmonella* in food (meat, poultry, eggs, dairy products) are numerous in Morocco. But few studies were undertaken in seafood and especially in shellfish including mussels [2,3]. This study aimed to determine *Salmonella* prevalence and multi Drug resistance of serovars identified.

2. MATERIALS AND METHODS

A total of 272 samples of shellfish consisted of mussels (*Mytilus galloprovincialis)*, cockles (*Acanthcardia tuberculatum)* and clams (*Callista chione*) were analysed for *Salmonella*. They were collected monthly during two years: 2008 and 2012 from harvested areas of the northwest Moroccan mediterranean coast.

2.1 Sampling Sites

Six sites were choosen in producing areas of shellfish (Fig. 1). This area is extended from Fnideq at the northwest to Oued Laou at the southeast. Sites were: Fnideq, Kabila, Corniche Martil and Oued Laou for the first year 2008 and sites of Riffiene, Oued Negro were added to period study 2012. The majority of sites were situated near or in front of river outlets at 400 metres away. Cockles and clams were naturaly lived in all sites, but for mussels, they were rearing in plateforms at -25 m deep from two sites of Fnideq and Oued Negro.

2.2 Analysis of *Salmonella*

Preparation of shellfish and detection of *Salmonella* were done according to techniques recommended by the Standard ISO 6579 [4]. It includes four stages of the detection process:

- Pre-enrichment in non-selective liquid medium,
- Selective enrichment in liquid media,
- Plating on selective media,
- Serological and biochemical identification of suspected colonies.

Briefly, a dozen of each shellfish were shucked aseptically and 25 g of flesh were homogenized with 225 ml of Buffered Peptone Water (BPW) and incubated at 37ºC for 16 to 20 h. For enrichment 0.1 ml of BPW were transfered to 10 ml of Rappaport-Vassiliadis Soy(RVS) broth and 1 ml of BPW were inoculated to 10 ml of Muller Kauffmann Tetrathionate Novobiocin (MKTTn) broth and incubated at 41.5ºC and 37ºC for 24 h respectively. 10 µl loop full from RVS and MKTTn were spread on Xylose Lysine Deoxycholate agar (XLD) and Brillant Green Phenol Red Agar (BGPRA) and incubated at 37ºC for 24 h. Typical colonies on XLD and BGPRA, were plated onto nutrient agar plates for biochemical confirmation and serotyping.

Salmonella isolates were tested negative for oxidase and negative for urease. All biochemically typical *Salmonella* isolates were serotyped based on somatic (O), flagellar (H) and capsular (Vi) antisera (Bio Rad) according to Kauffmann-White scheme [5].

2.2.1 Extraction of isolated *Salmonella*

Salmonella strains were cultured on nutrient agar for 24 h at 37ºC. Extraction of DNA was performed by boiling for 8 min and centrifuged at 12000 rpm for 15 min. Supernatant were used for

amplification by conventional PCR with *Salmonella* specific primers.

2.2.2 Primers set and PCR amplification program

Salmonella specific primers S139 and S141 [6] have respectively the following nucleotide sequence based on the *invA* gene of *Salmonella* F- 5´*tatcgccacgttcgggcaa* 3´ and R- 5´ *tcgcaccgtcaaaggaacc*3' carried out in a 25 µl PCR mixture. It consisted of 2.5 µl 10X (10 mM), 1.6 µl $MgCl_2$, 0.5 µl of each primer, 0.5 µl of Taq DNA polymerase and 1.5 µl DNA template of each isolate were used in the reaction. Amplification was conducted in Master-gradient Thermocycler (Eppendorf). The cycle conditions were included: An initial incubation at 95ºC for 60 s, 35 cycles of 45 s at 95ºC, annealing at 62ºC for 30 s and elongation at 72ºC for 45 s, 10 min final extension period at 72ºC. The amplified DNA products from *Salmonella* specific-PCR were analysed with electrophoresis on 1.5% agarose w/v gels stained with ethidium bromide and visualized by UV illumination.

2.2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested according to the guidelines of the National Committee for Clinical Laboratory Standard [7] using the disk diffusion technique with commercially available discs (Oxoid). The antimicrobials tested were : Bacitracin 130 µg (B), Chloramphenicol 30 µg (C), Sulfamides 300 µg (SSS), Nalidixic Acid 30 µg (NAL), Ciprofloxacin 5 µg (CIP), Amoxicillin 25 µg (AMX), Clavulanic Acid + Amoxcille (20 + 10) µg (AMC), Cephalotin 30 µg (CEF), Streptomycin 10 µg (S), Cefatoxine 30 µg (CTX), Kanamycine 30 µg (K), Ceftrixane 30 µg (CTO).

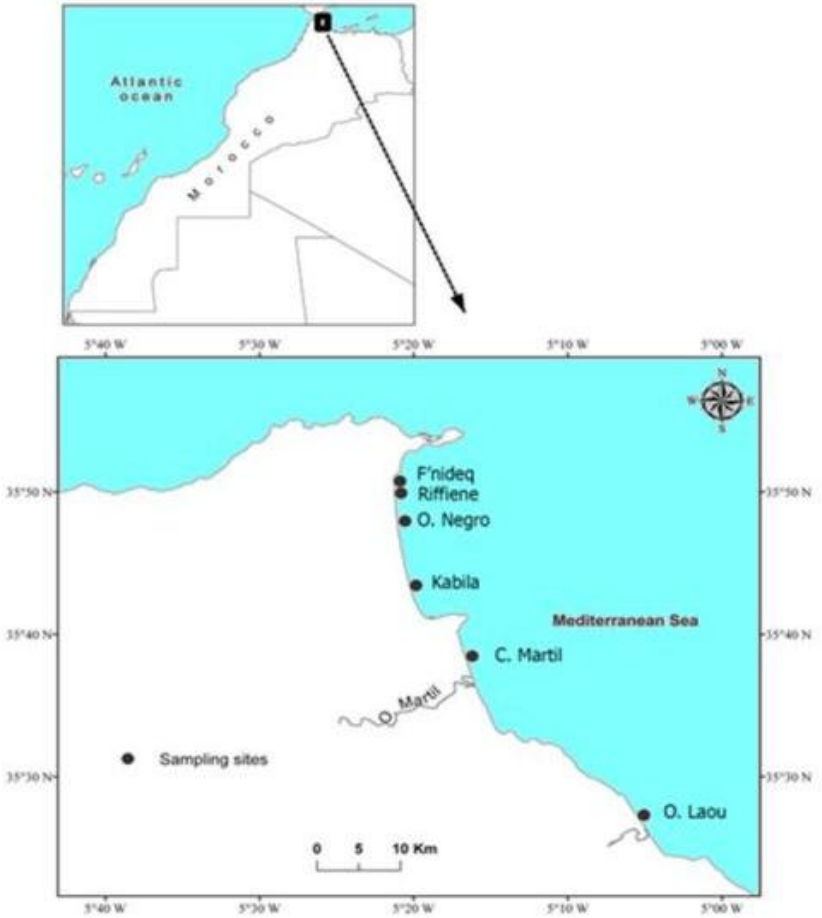

Fig. 1. Map of northwest coast of Morocco showing sampling sites

3. RESULTS AND DISCUSSION

During years of 2008 and 2012, as many as 272 samples of shellfish consisted of clams, cockles, mussels were analysed for presence of *Salmonella*. Up to 21 samples (7.7%) were positive and a total of 90 strains were recovered. Serolygical analyses displayed four serovars: Kentucky (n=39), Reading (n=27), which were dominant and Glostrup (n= 14) and Newport (n=10) as mentioned in Table 1.

In 2008, three serotypes of *Salmonella* were identified: Kentucky, Glostrup and Newport. Wheras in 2012, only two serotypes were identified: Kentucky and Reading. Exception of *S.* Newport, which was identified only in cockles in 2008, the others serotypes were identified as well as in clams than in cockles. Due to the limited number of mussels samples (n =24) in this study, it is difficult to assess evidently prevalence of *Salmonella*.

Concerning sites, Oued Laou was free of *Salmonella* in years 2008 and 2012. By contrast, Fnideq registred the most prevalence of *Salmonella*, followed by Corniche Martil. Prevalence of *Salmonella* was higher in cockles than in clams in general.

Comparison between 2008 and 2012 showed certain prevalence of *Salmonella* in the first year than in the second. Prevalence of *Salmonella* was 8.7% and 7.2% respectively in 2008 and 2012. Detection of *Salmonella* was essentially observed in wet period between october and april.

All isolated strains were serotyped as *Salmonella* strains. To confirm this, they were analyzed by conventional PCR using primers previously described as *invA* gene specific. PCR results indicated all *Salmonella* strains tested were positive for presence of a 275 bp fragment of *invA* gene.

Concerning antibiogram results, serotype *S.* Newport were almost fully susceptible to all antibiotics tested. Whilst, many resistant isolates were detected with 75.8% of isolates resistant to at least one antimicrobial agent. Isolates were resistant to streptomicin, chloramphenicol, nalidixic acid, ciprofloxacin, amoxicillin, respectively with (90.6%; 51.6%; 31.6%; 30.5% and 19%). The most common pattern of multidrug resistance included resistance to chloramphenicol, ciprofloxacin, nalidixic acid. Thus, *S.* Glostrup, showed resistance to: amoxicilin, nalidixic acid and streptomycin. By against, *S.* Reading showed resistance profil for: chloramphenicol, streptomycin and sulfamides. Whereas, *S.* Kentucky showed high resistance to quinolones nalidixic acid and ciprofloxacine (Table 2).

Table 1. Incidence of *Salmonella* spp at each sampling site and per shellfish species

Sampling site	Shellfish species	No. of samples per year (% *Salmonella* positive)		
		2008	2012	Total
Fnideq	Cockles	13 (3.8)	12 (1.2)	25 (2.2)
	Clams	13 (2.9)	12 (0.6)	25 (1.5)
	Mussels	0	12 (0.6)	12 (0.6)
Riffiene	Cockles	0	12 (0.6)	12 (0.6)
	Clams	0	12 (0.6)	12 (0.6)
Oued Negro	Cockles	0	12 (0.6)	12 (0.6)
	Clams	0	12 (0.0)	12 (0.0)
	Mussels	0	12 (0.0)	12 (0.0)
Kabila	Cockles	13 (0.0)	12 (0.6)	12 (0.6)
	Clams	13 (0.0)	12 (0.6)	12 (0.6)
Corniche Martil	Cockles	13 (1.9)	12 (1.2)	12 (1.1)
	Clams	13 (0.0)	12 (0.6)	12 (0.4)
Oued Laou	Cockles	13 (0.0)	12 (0.0)	12 (0.0)
	Clams	13 (0.0)	12 (0.0)	12 (0.0)
Total	Cockles	52 (5.8)	72 (4.2)	124 (4.8)
	Clams	52 (2.9)	72 (2.4)	124 (2.6)
	Mussels	0	24 (0.6)	24 (0.6)

Table 2. Pattern of resistance to antibiotics in *Salmonella* isolates per shellfish species and per site

Serotype	Nº. of isolates	Origin	Antibiogram	Period
S. Kentucky	1	Cockles/C. Martil	Amx, Na, Cip	Feb/2008
	7	Cockles/Fnideq	Amx, Na, Cip	Mar/2008
	6	Cockles/Fnideq	Amx, Na, Cip	Oct/2008
	5	Cockles/Fnideq	Amx, Na, Cip	Dec/2008
	4	Clams/Fnideq	Amx, Na, Cip	Dec/2008
	3	Cockles/Kabila	C, S, SSS, Na, Cip	Apr 2012
	3	Clams/Riffiene	C, S, SSS, Na, Cip	Oct/2012
	2	Mussels/Fnideq	C, S, SSS, Na, Cip	Nov/2012
	8	Cockles/Fnideq	C, S, SSS, Na, Cip	Nov/2012
S. Glostrup	11	Clams/Fnideq	Amx, Na	Feb/2008
	2	Clams/Fnideq	Amx, S, Na	Mar/2008
	1	Cockles/Fnideq	Amx, S, Na	Oct/2008
S. Newport	8	Cockles/C. Martil	Sensibles	Jan/2008
	1	Cockles/Fnideq	Amx, C	Oct/2008
	1	Cockles/Fnideq	Sensibles	Dec/2008
S. Reading	3	Cockles/C. Martil	C, S, SSS	Apr/2012
	2	Clams/C. Martil	C, S, SSS	Apr/2012
	2	Clams/Kabila	C, S, SSS	Apr/2012
	5	Cockles/Fnideq	C, S, SSS	Oct/2012
	4	Clams/Fnideq	C, S, SSS	Oct/2012
	4	Cockles/O. Negro	C, S, SSS	Oct/2012
	4	Cockles/Riffiene	C, S, SSS	Oct/2012
	3	Cockles/C. Martil	C, S, SSS	Oct/2012

In our study, prevalence of *Salmonella* in shellfish harvested from mediterranen coast of Morocco was 7.7%. This finding is in agreement with previous studies taken elsewhere.Thus in Morocco, it found 7.1% of *Salmonella* in mussels (279 samples) [2] and in USA, it found 7.4% in oysters (1296 samples) [8]. But in Spain, it reported that nearly 67% of total *Salmonella* isolated were from shellfish [9]. In Egypt a prevalence of 8% in shellfish sold in markets was detected [10]. However, other previous study carried in Morocco, displayed a prevalence of 15% of *Salmonella* in mussels [11].

Majority of *Salmonella* detection in samples were in wet period between october and april. Probably , it was due to runoff in this period of year which carry bacterial loads to coastline. Additonally, all sites of sampling were located opposite opening of rivers at 300 m away.

Four serovars of *Salmonella* were identified in shllefish samples: *S.* Newport, *S.* Glostrup, *S.* Reading and *S.* Kentucky. In other study, two servars were detected: *S.* Blockley and *S.* Kentuky were found in mussels form Morocco [2]. In USA, ten servars with *S.* Newport, the main servoar was identified (77.3%) in oysters [8].

Only serovar *S.* Kentucky was identified in the year 2008 and in the year 2012. It seems it was spread in the environment more than the others servoars identified. S. Kentucky had been isolated from seafood, cattle and poultry [12] although less frequently than from infected humans in Europe.

If we compare serovars isolated on resistance to antibiotics, we found *S.* Newport isolates were more susceptibles than the others. Followed by *S.* Glostrup isolates which were resistant to nalidixic acid, streptomycin and amoxicillin. *S.* Reading isolates were resisitant more to chloramphenicol, streptomycin and sulfamides. When *S.* Kentucky isolates were more resistant to nalidixic acid and ciprofloxacin. In others studies, *S.* Kentucky was always resistant to some antibiotics and especially to quinolones ciprofloxacin and nalidixic acid [2,13,14,15].

Presence of *Salmonella* contamination in shellfish was detected in wet period of the year with most of the contamination events coinciding with periods of rainfall. There were some serovars identified, but the most important and resistant to ciprofloxacin were *S.* Kentucky. Distribution of *Salmonella* on shellfish species was slightly for cockles than in clams. More,

prevalence of *Salmonella* was very increased in sites which received water discharges from rivers like Fnideq and Corniche Martil in this study than the others.

We think implementation of two treatment plants waste waters in the region recently (2011), has influenced positively on reduction of prevalence of *Salmonella* in shellfish. By contrast, resistance of serovars to antibiotics has increased from 2008 to 2012, probably due to use abusively of antibiotics in treatment in breeding.

4. CONCLUSION

Results of this study illustrated the extent of antibiotic resistance in *Salmonella* food-borne pathogens in shellfish samples. It seemed that strains isolated of *Salmonella* were multidrug resistant and almost one third of strains were resistant to quinolone.

The results emphasize the need of a monitoring programme of bacterial pathogenes *Salmonella* on shellfish to protect human health.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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