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Removal of Antibiotic-Resistant Salmonella in Sewage Water from Wastewater Treatment Plants in Settat and Soualem, Morocco

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Abstract

The emergence of antibiotics resistant bacteria in the water environment is becoming an increasing worldwide concern. This study was conducted to highlight the importance of the Wastewater Treatment Plants in the purification of raw water and the elimination of resistant bacteria such as *Salmonella* circulating in the environment. The results showed the presence of *Salmonella* in sewage only and total absence in the treated water; a total of forty two strains of *Salmonella* were isolated, serotyping of these strains showed six different serotypes of *Salmonella* spp which 57.1% were *Salmonella* typhimurium, antibiotic resistance was studied and different antibiotics were tested by the method of Mueller Hinton agar diffusion. The antimicrobials and concentrations in µg tested were: Amikacin 30 (Ak), Ampicillin 10 (Amp), Choramphenicol 10 (Chl), Cephalotine 30 (Cef), Ceftazidime 30 (Caz), Trimethoprim /Sulfametoxazole 1.25/23.75(Sxt), Cefotaxime 30 (Ctx), Gentamicin 10 (Gen), Imipenem 10 (Ip), Kanamycin 30 (Kan), Streptomycin 10 (Str), Tetracycline 30 (Tet), Sulfonamides 200 (Su), Ciprofloxacin 5 (Cip); The results showed the presence of multi-resistant strains.

Keywords: Wastewater Treatment Plants, *Salmonella*, serotyping, antibiotic resistance

1. Introduction

Salmonellae naturally live in the intestines of humans and other animals; therefore, fecal material is the ultimate source of these bacteria; *Salmonella* spp. has been recognized as human and animal pathogen for over a century. Numerous serotypes have been described such as *S. Enteritidis* and *S. Typhimurium* which are frequently detected in farm animals, infection with *S. Typhi* or *S. Paratyphi*, which are exclusively human pathogens, results in enteric fever (Ohl and Miller, 2001). Clinical symptoms of enteric fever include diarrhea, abdominal pain, fever, and sometimes a maculopapular rash, the pathological sign of enteric fever is mononuclear cell infiltration and hypertrophy of the reticulo endothelial system, mortality from *S. Typhi* and *S. Paratyphi* is estimated to be between 10 to 15 percent without treatment (Ohl and Miller, 2001); Increasing numbers of *Salmonella* isolates are resistant to one or more antibiotics (Schlüter et al., 2007), the use of antibiotics are widely used to protect the health of human and animals or to increase growth rate of animals as food additive but may also accelerate the development of antibiotic resistance bacteria, which shade health risks to humans and animals (Kemper, 2008), the majority of antibiotics are excreted unchanged into the environment, these bacteria might be transmitted from environment to human via direct or indirect contact (Iversen et al., 2004; Kim et al., 2005; Rodriguez et al., 2006).

Sewage contains a high content of both organic and inorganic matter, as well as high densities of living organisms, including pathogenic, commensal and environmental bacteria; this characteristic composition makes sewage a particularly suitable ecological niche for the growth and spread of antibiotic resistance (Guardabassi and Dalsgaard, 2002).

The first objective is research and serotyping of *Salmonella* in raw and treated waters samples from Wastewater Treatment Plants Settât and Soualem. The second objective was to determine the antibiotic susceptibility profile of the *Salmonella* serovars by internationally standardized methods.

2. Material and Methods

2.1. Bacterial Strains

A total of (n= 72) raw water, (n=72) treated water taken monthly from both Wastewater Treatment Plants (WWTP) Settât and Soualem from July 2007 to August 2009; Forty two *Salmonella* strains was isolated in Wastewater Plants Settât (33°00'N, 7°37'W) (Tageo.com), and Soualem (34°26'N, 5°53'W) (GeoHack-Soualem) in Morocco.

2.2. Microbiological Analyses

The detection of *Salmonella* was done in three successive phases; For the first phase, buffered Peptone Water -BPW- (Oxoid) is used as a presumptive medium with incubation at 37 ° C for 24 h; For selective growth, a 0.1ml aliquot of culture was transferred to 10 ml Rappaport Vassiliadis (Oxoid) and incubated for 24 h at 41.5°C; The broth was then streaked onto XLD Agar (Merck) plates and Edel Kampel Macher Agar (Difco) for 24 h at 37°C. The isolates were confirmed *Salmonella* using the *Enterobacteriaceae* API 20E commercial kit (Biomérieux, Marcy l'Etoile, France).

2.3. Serotyping *Salmonella* spp

Serotyping of *Salmonella* is used to identify the isolates at subspecies level; A series of antibodies is used to identify the O (somatic) and H (flagellar) antigens on the bacterial cell surface; In those isolates that possess a capsule or a cell envelope, a third antigen Vi, is also identified; The Kauffman-White scheme is then used to identify the serovar based on the antigens present (Grimont and Weill, 2007).

2.4. Antimicrobial Susceptibility Testing

The antimicrobial susceptibilities of *Salmonella* isolates were determined by the disk diffusion method of the Clinical and Laboratory Standards Institute on Mueller–Hinton agar (Biorad). The antimicrobials and concentrations in µg tested were: Amikacin 30 (Ak), Ampicillin 10 (Amp), Chloramphenicol 10 (Chl), Cephalotine 30 (Cef), Ceftazidime 30 (Caz), Trimethoprim /Sulfamethoxazole 1.25/23.75(Sxt), Cefotaxime 30 (Ctx), Gentamicin 10 (Gen), Imipenem 10 (Ip), Kanamycin 30 (Kan), Streptomycin 10 (Str), Tetracycline 30 (Tet), Sulfonamides 200 (Su), Ciprofloxacin 5 (Cip).

3. Results

Microbiological research has shown that *Salmonella* is detected in raw wastewater throughout the period of collection, while in the treated water they are absent for both Wastewater Plants (WWTPs) Sett and Soualem.

3.1. Serotyping Results

Considering all isolations made, a total of forty two strains of the 144 samples selected for investigation were found to be *Salmonella* positive. Six different serovars of *Salmonella* spp were identified, 57.14% are identified *Salmonella* typhimurium, 21.42% *Salmonella* hadar, 7.14% *Salmonella* senftenberg and 4.76% for *Salmonella* Newport, *Salmonella* give and *Salmonella* anatum respectively. The distribution of the serotypes was investigated as presented in Table1.

Table 1: Characteristics of the *Salmonella* spp. Isolates

Serovar	No. of isolates (%)	Origin (No. of isolates)	Antigenic formula		
			Ag O	Ag H ₁	Ag H ₂
S. typhimurium	24 (57.14)	Sett(10), So (14)	1, 4, [5], 12	i	1,2
S.hadar	9(21.42)	Sett(6), So (3)	6, 8	z10	e,n,x
S.senftenberg	3(7.14)	Sett(3)	1, 3, 19	g,[s],t	-
S.give	2(4.76)	Sett(2)	3, 10, [15]	l,v	1,7
S.newport	2(4.76)	Sett(2)	6, 8	e,h	1,2
S.anatum	2(4.76)	Sett(2)	3, 10	e,h	1,6

Ag O, flagellar antigen; Ag H₁ and H₂, somatic antigens; Sett, Wastewater Treatment Plant Sett; So, Wastewater Treatment Plant Soualem.

3.2. Antimicrobial Resistance

Only serotypes S. Typhimurium, S. Hadar and S. Senftenberg exhibited resistance to two or more antibiotics, none of the isolates from the other serovars S. Give, S. Newport and S. Anatum were resistant to any of the fourteen antibiotics tested; The table 2 summarizes the results of resistance to antibiotics tested for serotypes detected, all *Salmonella* isolates were susceptible to Amikacin, Cephalotine, Ceftazidime, Cefotaxime, Gentamycin, Imipenem, Sulfonamides and Ciprofloxacin.

Table 2: Antimicrobial resistance in *Salmonella* serovars isolated from raw water

Antibiotic	Antibiotic sensitivity of serovars (No of isolates)								
	S. Typhimurium (n=24)			S. Hadar (n=9)			S. Senftenberg (n=3)		
	R	I	S	R	I	S	R	I	S
Amp	17	4	3	5	3	1	0	0	3
Chl	12	7	5	6	3	0	0	0	3
Str	12	9	3	5	2	2	0	0	3
Sxt	22	2	0	0	2	7	0	0	3

Table 2: Antimicrobial resistance in *Salmonella* serovars isolated from raw water - continued

Tet	14	8	2	6	2	1	2	1	0
Kan	0	1	23	0	2	7	2	1	0

R, resistant; I, Intermediate; S, Susceptible; Amp, Ampicillin; Chl, Chloramphenicol; Str, Streptomycin; Sxt, sulphamethoxazole/ trimethoprim; Tet, Tetracycline; Kan, Kanamycine.

4. Discussion

Morocco is a country which the demands on this resource are growing as the country expands and the population increases. Urgent steps must be taken to protect the quality of water resource for the country to develop economically and meet the wide ranging needs for water. International organizations recommend to their member countries microbiological studies correlating the density of bacterial indicator organisms with the presence and density of pathogens such as *Salmonella* (Kathy P., 1996); However, surveillance of *Salmonella* is only performed by a few countries, the reliability of the indicator based standards to predict the presence of this pathogen is still a matter of debate (Campos et al., 2002). In this part of the study, the efficiency of WWTPs located in Settât and Soualem areas were assessed for their ability to remove *Salmonella*. The treated wastewater from WWTP of Settât will be used to irrigate some 300 hectares in the vicinity of that city, a population of around 175 000 people will benefit (RADEEC, 2003), and the treated water from WWTP of Soualem will irrigate 50 hectares; a population of around 25.000 people will benefit (RADEEC, 2005), hence the need to have a total absence of *Salmonella* as recommended by World Health Organization (MEDAWARE, 2005).

The cities of Settât and Soualem are part of a region called Chaouia-Ourdigha/grand Casablanca, the agricultural area represents about 56.7% of the total area of the region and a significant poultry industry; The installation of large projects poultry (turkey, packing eggs, poultry meat) is considered among the successful projects in this region, this business provides an important opportunity for the socio-economic development of the region, there's production of 31.6% of national production of poultry meat and 47.6% of the national egg consumption (Anonymous, 2008).

The presence of *Salmonella* only in raw water and their absence in the treated water shows that the type of treatment adopted in the two stations is effective in eliminating this bacteria, in fact the treatment of raw water adopted for the two stations is natural lagoons; The sojourn time for each station is between 40 to 46 days for WWTP Settât and 22 days for WWTP Soualem, the first step is screening, then the ponds are arranged and operated in series, with anaerobic ponds preceding the facultative ponds which then feed into several maturation ponds; Several studies have shown the effectiveness of the storage and prolonged solar radiation in decontamination, during daylight, lethal solar radiation greatly accelerates the bacterial die-off; the ultraviolet light is the most lethal (MED POL, 2004).

The *Salmonella* now enjoy a wide variety of phenotypic and genotypic markers for characterization, despite this diversity, the serotyping is still the technique used as first-line before any other characterization and allows a good discrimination with more than 2400 serotypes identified in the world according to Kauffmann–White taxonomic scheme; This scheme is based on a combination of biochemical reactions and serotyping of the somatic “O,” flagellar “H,” and capsular “Vi” antigens (Grimont and Weill, 2007). Some serotypes are strongly associated with a disease, as the case of *S. hadar* and *S. newport* that are associated with poultry production, while *S. typhimurium* can be isolated from all types of production sectors (Bertran, 2007; Brisabois, 2001); In our study, six different serovars of *Salmonella* spp. were identified: *Salmonella* Typhimurium, *Salmonella* Hadar, *Salmonella* Senftenberg, *Salmonella* Newport, *Salmonella* GIVE and *Salmonella* Anatum; According to the FAO (2010), *S. Weltevreden*, *S. Enteritidis*, *S. Newport*, *S. Anatum*, *S. Senftenberg*, *S. Typhimurium* and *S.*

Saintpaul were the most commonly isolated serovars worldwide; Serovar *S. Typhimurium* has been shown to be the most common clinically significant serovar isolated from environmental samples in many parts of the world (Martinez-Urtaza et al., 2004a; Simental and Martinez-Urtaza, 2008), which attests to its capacity of adaptation and survival in external environments (Baudart et al., 2000); *S. Senftenberg* has been recognized one of the major serotypes identified in marine environments and raw seafood worldwide, it has been one of the predominant serovars detected in the coastal waters of Portugal (Catalao Dionisio et al., 2000), in raw seafood imported into the United States especially from tropical countries (Heinitz et al., 2000) and in environmental samples in France and Brazil (Baudart et al., 2000; Tavechio et al., 2002).

In recent years, testing of *Salmonella* isolates from different environments has shown an increasing proportion of multidrug resistant *Salmonella* species (Cheng et al., 2004), information about antimicrobial resistance among *Salmonella* strains isolated from environmental sources and food showed a differentiated incidence rate of resistant strains among isolates obtained from developed and developing countries (FAO, 2010); Antimicrobial resistance was detected in 9 percent of the total of strains isolated from environmental sources and shellfish over different studies in Spain (Martinez-Urtaza et al., 2004b; Martinez-Urtaza and Liebana, 2005), conversely, the presence of antimicrobial resistant strains among strains isolated from the marine environment in Morocco reached 49.1 percent of the strains (Setti et al., 2009), whereas in Mexico, 50.4 percent of the strains recovered from water samples showed resistance to antimicrobials (FAO, 2010), in a study carried out in Cochin, India, 82 percent of the strains isolated from seafood products presented antimicrobial resistance (Rakesh et al., 2009), whereas in Vietnam, antimicrobial resistance was observed in 11.1 percent of strains (Van et al., 2007); However, these were observational studies and the methodology and spectrum of antibiotics used by different investigators vary and this may contribute to the high degree of variation observed; In our study, on forty two serovars isolated nineteen strains were resistant at least to two antibiotics, twelve *S. typhimurium*, five *S. Hadar* and two *S. Senftenberg*; which represent a percentage of 45.2 percent.

Multidrug resistance, with rates of resistance to Ampicillin, Chloramphenicol, and Trimethoprim-Sulfamethoxazole of more than 50%, has been reported in many areas of the world. Extended-spectrum Cephalosporins and Fluoroquinolones have been suggested as alternative agents in the treatment of infections caused by multidrug resistant *Salmonella* serotypes (Cheng et al., 2004), these data correspond to results obtained in this study showing that the serotype isolated *S. typhimurium* is resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole/trimethoprim and tetracycline. Resistance to antimicrobial agents in bacteria is mediated by several mechanisms including changes in bacterial cell wall permeability, energy dependant removal of antimicrobial agents via membrane-bound efflux pumps, modification of the site of drug action, and destruction or inactivation of antimicrobial agents (Cheng et al., 2004).

Bacteria have evolved different strategies to cope with severe effects caused by antimicrobial drugs; intrinsically resistant species for example render their cell walls impermeable for antibiotics or express efflux systems facilitating transport of these compounds out of the cell (Schlüter et al., 2007). Moreover, bacteria are able to horizontally acquire resistance via uptake of foreign DNA by means of conjugation, transduction or transformation (Thomas and Nielsen, 2005); In this context, mobile genetic elements such as plasmids, transposable elements or integron-specific gene cassettes play an important role (Toussaint and Merlin, 2002; Frost et al., 2005), these elements mainly encode enzymes for modification or inactivation of antibiotics, efflux systems, or enzymes catalyzing target-site modifications (Schlüter et al., 2007); And some examples include such as β -lactamases that cleave the amide bond of the β -lactam ring thereby inactivating the drug (Schlüter et al., 2007), there's also resistance against tetracyclines that include active efflux, ribosome protection, target-site alterations and reduction of cell permeability (Roberts MC., 2005; Chopra and Roberts, 2001). Release of bacteria carrying resistance plasmids with the final effluents of the WWTP into the environment promotes dissemination and recombination of resistance determinants among environmental bacteria (Schlüter et

al., 2007). In our study, *Salmonella* is detected only in raw wastewater throughout the period of collection, while in the treated water they are absent for both WWTPs Settât and Soualem, and reuse in irrigation applies only to treated water, not raw water, that is why our study was limited to the antibiotic susceptibility for each serotype of *Salmonella*, without the study of intrinsic factors and the search for resistance plasmids. Likewise, it would be informative to compare resistant bacteria in raw water obtained from WWTPs that receive effluents from hospitals with those that are not connected to any medical facilities.

Moreover, environmental conditions of activated sludge or biofilms facilitate horizontal transfer of the antibiotic resistance genes (ARGs) from one host to another because of the nutritional richness and high bacterial density and diversity (Tennstedt et al., 2003; Schlüter et al., 2007); Many ARGs, for example, *van A* (Vancomycin resistance gene of *Enterococcus* and *Staphylococci*) and *van B* (Vancomycin resistance gene of *Enterococcus*), cannot be effectively removed by activated sludge process widely used in sewage treatment plants, the genes being found in both influent and effluent water (Iversen et al. 2002; Caplin et al. 2008), another example is that of the gene *tet* (Tetracycline resistance gene of *Salmonella*) preferentially migrate from wastewater to biofilm (Engemann et al. 2008).

The wastewater generated in addition to water purified water, by-products known as sewage sludge, Moroccan law adopted five draft standards developed by the Technical Committee for Standardization of wastewater which on good practice valuation, removal, drying, incineration and landfilling of sludge and risk assessment (PNM 03.7.260, PNM 03.7.262, PNM 03.7.263, PNM 03.7.264 and PNM 03.7.265) (Anonymous, 2010).

Some efforts have to be made to reduce the possibility of resistant bacteria entering into and spread in the environments, the most effective and direct approach is thought to be the reasonable use of antibiotics in health protection and agriculture production. The discharge of wastewater containing resistant bacteria in the environment poses a real public health problem; hence the importance of the installation and operation of treatment plants to reduce the rate of diseases of waterborne diseases. The efficiency of the two Wastewater Treatment Plants Settât and Soualem for the removal of *Salmonella* from their final effluents had a positive effect on the quality of treated water to be reused mainly for irrigation of agricultural land.

5. Conclusion

All aspects of the control of microbial quality in water and wastewater, general environmental health protection are critical; this will have impacts on the quality of wastewater reused. With the use of traditional contamination indicators the appearance of emergent microorganisms generates new questions about the forecast in the occurrence of these microorganisms.

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