brief report

Acid resistance of *Salmonella* isolated from animals, food and wastewater in Tunisia

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Salmonella are important foodborne pathogens that infect a variety of hosts and cause a broad spectrum of diseases. During the past few decades, there has been an increase in salmonellosis worldwide. This can largely be attributed to an increase in transmission of broad host-range organisms to humans from infected food and water.[1] However, it is well demonstrated that *Salmonella* can disseminate and survive in various environmental niches for long periods of time. They are pervasive in nature and may contaminate animals, vegetables, water and especially food during its production and distribution.[2]

The spread and dissemination of these enteric pathogens were accompanied by an important increase in resistance to antibiotics all over the world.[3,4] Therefore, these bacteria are developing a cross-resistance to antibiotics often used in therapy or as additives.[5-7] The occurrence of multiple-antibiotic-resistant *Salmonella* was reported in many developed and non-developed countries and continues to cause major health problems.[8-12] Therefore, the use of antibiotics must be restricted to reduce the selection and spread of multi-resistant strains. This urgent need to replace antibiotics has prompted researchers to try to find other alternatives. Consequently, many compounds and chemicals, such as organic acids, were tested for their antimicrobial activities.

Organic acids have long been used both for preserving foods and in livestock production for disease prevention or as growth-promoting feed additives.[13-19] It has thus been demonstrated that organic acids have an antimicrobial activity which is pH dependent.[13] *Salmonella*, as enteric pathogens, encounter a low pH value in the environment, especially during its transit in the host. These bacteria must face this barrier to replicate and proliferate in the hosts. The study of acid resistance in previous research showed that some strains of *E. coli* are able to survive at very low pH values, but stop growing at pH values less than 4.4.[20-22]

This study was undertaken to determine if ten isolates of *Salmonella* could grow on nutrient broth acidified by acids commonly present in foods or used as acidulants. It was also of interest to see whether acid adaptation enhanced the survival and growth of these strains upon subsequent exposure to a low pH environment.

MATERIAL AND METHODS

Strains of *Salmonella* used in this study are described in Table 1. These strains were isolated from foods, wastewater and animals. Bacteria were routinely incubated in nutrient broth (NB) (Pronadisa, Spain) at 37°C overnight with shaking.

Salmonella isolates were assayed for susceptibility to eight different antibacterial agents by the disc diffusion method. All antibiotic discs used were supplied by Biomérieux (France). The respective quantities (μg/disc) of these compounds were as follows: ampicillin (AMP) (10); tetracycline (TET) (30); gentamicin (GEN) (10); nalidixic acid (NAL) (30); chloramphenicol (CHL) (30); amikacin (AMK) (30); neomycin (NEO) (30); streptomycin (STR) (10). The results were interpreted in accordance with the Clinical Laboratory and Standard Institute (CLSI).

All isolates of *Salmonella* were tested for acid resistance. A fresh overnight culture was used to inoculate 50 mL (OD_{600}=0.05) of the NB medium preadjusted with the appropriate acid to the desired pH, then incubated at 37°C with shaking. The optical density and pH of the medium were periodically measured. The pH of the samples were determined with a pH meter (*HANNA* Instruments pH 209) before and after inoculation and during the measurement time (pH 2.5, 3, 3.5, 4, 4.5, 5 and 7 were tested). For all of the acids, concentrations of both 1% and 10% were tested. The bacterial population was determined by plating on NB agar plates 0.1 mL of appropriately diluted cultures which were then incubated at 37°C for 24 hours.

Table 1. Resistance profiles of *Salmonella* isolates

Salmonella serovars	Source	Resistance profile
S. enteritidis	Poultry	TET, STR
S. mbandaka	Meat	TET, AMP, STR
S. lindenburg	Milk	TET, AMP, STR
S. nikolaifleet	Wastewater	TET, AMP, STR
S. cerro	Merguez*	TET, STR
S. montevideo	Wastewater	TET, AMP, NEO
S. hadar	Poultry	TET, AMP, NEO, GEN, STR
S. braenderup	Milk	TET, AMP, STR
S. zanzibar	Wastewater	TET, NAL
S. newport	Wastewater	TET

* A Tunisian food prepared with meat.

RESULTS

In this study, eight antibiotics were tested: ampicillin, tetracycline, gentamicin, nalidixic acid, chloramphenicol, amikacin, neomycin and streptomycin. The findings obtained showed a greater resistance of all non-typhoidic *Salmonella* serotypes isolated from food, animals and water. Tetracycline was the only antibiotic that had no effect on any of the isolates tested. Ampicillin and streptomycin followed in lack of effect. On the other hand, amikacin, chloramphenicol, gentamicin and nalidixic acid were the most active against these isolates. Therefore, in the *Salmonella* population tested, *Salmonella hadar* (isolated from poultry) showed the highest rates of resistance (five resistant) and *Salmonella newport* the lowest (one resistant) (Table 1).

The results showed that the antimicrobial activity of all acids increase as their concentrations increase. For periodic and acetic acids, a concentration of 1% was capable of reducing the number of colony-forming units by a factor of ten, whereas the same concentration of hydrochloric and citric acid reduced this number only by a factor of five. Figure 1 shows the growth kinetics of *Salmonella hadar* when incubated in an NB medium adjusted with the appropriate acid as an example of the responses of the other isolates tested.

However, the results also showed that the minimum pH for growing of all *Salmonella* tested was dependant on the type of acid used. Therefore, the pH was 3 when using citric acid or hydrochloric acid as acidulants and 4.3 when using periodic or acetic acids. Lag time was similar as well for citric and hydrochloric acids. Moreover, acetic and periodic acids were significantly more inhibitory than both citric acid and hydrochloric acid towards all of the isolates tested.

Figure 2 illustrates the growth kinetics of the ten isolates of *Salmonella* in an NB medium with citric acid added at a concentration of 10%. Therefore, except for *Salmonella hadar* which has a pH minimum for growth of about 3, all other isolates have a minimum of about 3.5. In addition, according to Figure 1, an increase in pH from 3.5 to 7 decreases the lag time of all *Salmonella* isolates. This decrease was serotype dependant; however, *S. hadar* showed the least lag time. For *S. enteritidis*, its lag time at pH 3.5 was different from all other isolates.

DISCUSSION

For years, antibiotics have been used in different fields such as medicine, agriculture and the food industry. However, the situation has changed in recent years due to the emergence of bacterial resistance and the decreased effectiveness of antibiotics.[8,9,10,11,23] In Tunisia, few studies have been addressed the antibiotic resistance of *Salmonella* and its evolution. Our results show that the resistance profiles of all serotypes tested are very similar (Table 1). Tetracycline and ampicillin do not show any effect on the isolates. These antibiotics have been the most commonly used and therefore the very frequent occurrence of resistance among *Salmonella* is probably a consequence of this. Although this problem is well documented worldwide, the situation in Tunisia will become more dangerous in the near future.[10,12,23] Abusive use of these molecules in fields such as agriculture, medicine and industry must be limited. Antibiotics must be used only when necessary, and

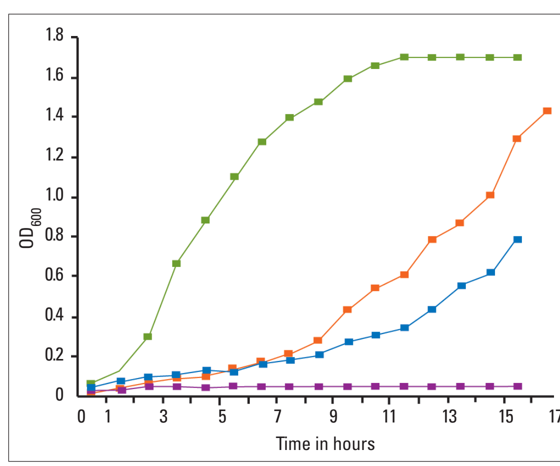

Figure 1. Response of *Salmonella hadar*, incubated in an NB medium with acetic acid (■), hydrochloric acid (■), citric acid (■) at the pH 3.5. All acids were tested at a concentration of 1%. For a control growth curve, *Salmonella hadar* was incubated in an NB medium pH 7 (■) (OD = optical density).

the consumption of antibiotics for purposes other than the treatment of infectious diseases must be avoided as much as possible.[24] Alternatives that are cheap, readily available and have few effects must be substituted.

The majority of treatments used in Tunisia to eradicate pathogens from poultry contain chlorine and present a certain number of disadvantages. However, unlike chlorine, organic acids were recognized as safe for the environment and are generally used as preservatives in food. In this study, the efficiency of organic acids towards these pathogens was tested. Acetic and periodic acids showed great effectiveness on *Salmonella* tested at a concentration of 1% and pH 4. It is well known that organic acids have important antibacterial capacity and no negative effect on the environment.[13] These molecules are thus generally used as food preserving additives.[12,14,15,17,18] At the same concentration and pH, citric acid and hydrochloric acid increase the doubling time of all serotypes tested without affecting their growth and survival. The killing effect of the acids used increased in the following way: citric acid < hydrochloric acid < acetic acid < periodic acid. The results showed that the situation would be clinically worse for the following reasons: First, acid tolerance is a contributing factor in the intracellular survival and virulence of *Salmonella*. Secondly, some isolates have also demonstrated high levels of resistance. In fact, Wain et al have suggested the existence of a relationship between virulence and drug resistance.[25] Therefore, it should be stressed that pathogens have shown both high levels of drug resistance and also acid resistance, especially *Salmonella hadar*, which has the highest antibiotic and acid resistance. This link between acid and antibiotic resistance could have significant implications for the food industry and needs to be further investigated in foodborne pathogens in a real food-processing situation. The acid tolerance response provides cross-protection to heat, osmotic, and oxidative challenges.[26] This work could help to assess the risk of stressed-adapted bacteria and alert public health professionals and inspection authorities.

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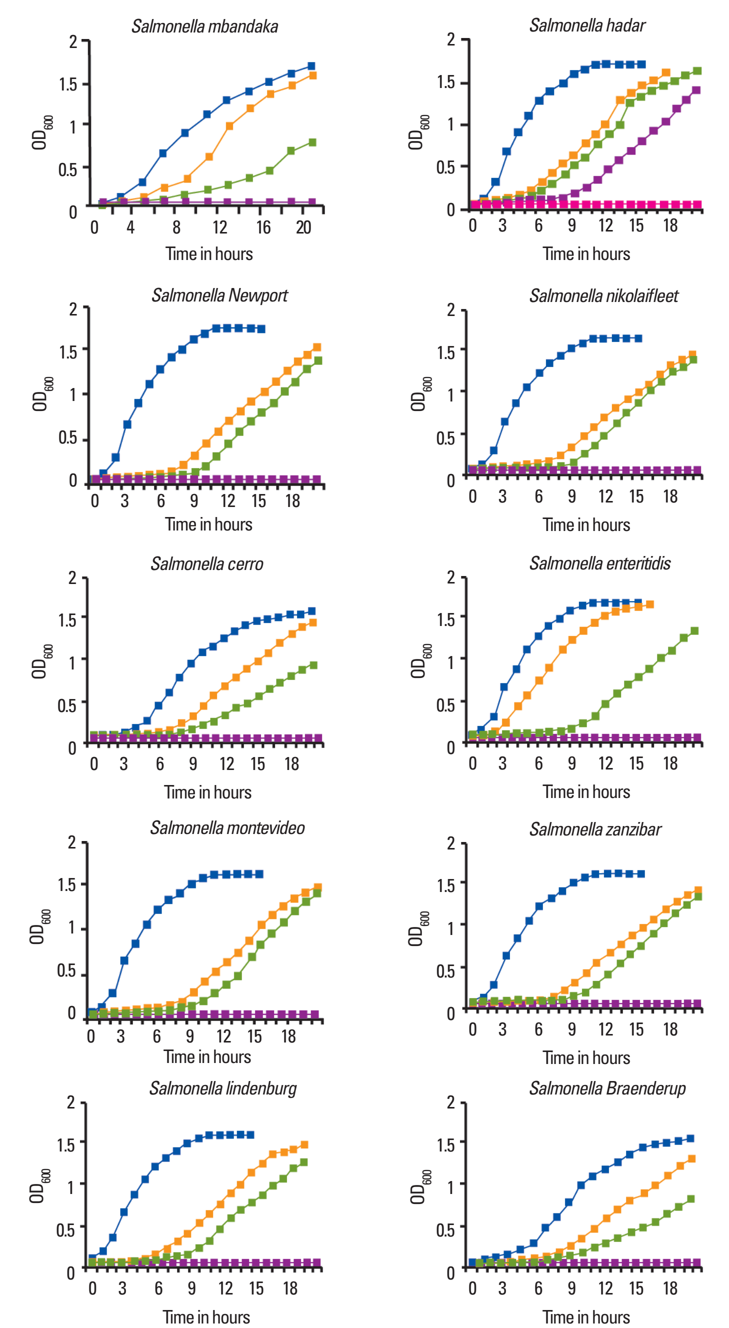

Figure 2. *Salmonella* isolates incubated in an NB medium adjusted by citric acid (1%) at range of pH 7 ( ), pH 4 ( ), pH3.5 ( ), pH 3 ( ) and pH 2.5 ( ). (OD = optical density).

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