

Effect of environmental strains of *Salmonella typhimurium* on transfer of water and electrolytes in rat's intestine

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Received 2 September 2005, accepted 20 November 2005.

Abstract

Use of treated waste water in agriculture can increase the risk of enteric infection by direct contact or by consumption of products like fruits and vegetables which are irrigated by treated waste water. The major aim of this study was to investigate the effect of environmental strains of *Salmonella enterica* serovar *typhimurium* (a strain which was isolated from treated waste water and a strain which was starved in sea water during half and six months) on transfer of water and electrolytes (sodium, chloride, calcium and potassium) in different parts of rat's intestine. We have used Everted Gut Sac according of the technique of Wilson and Wiseman. Water flux was evaluated by weighing. Electrolytes were dosed by ISE COBAS INTEGRA 400 plus analyzer. We obtained variations of water and electrolytes secretions according to intestine's part variations. The highest secretion of water was noticed in ileum in the group which was infected with the reference strain S1 (-0.681 mg/g/h) ($P < 0.05$). Variations of electrolyte secretions between jejunum, ileum and colon were significant ($P < 0.05$) for sodium, chloride and calcium but not for potassium. The highest secretions of sodium (90 mmol/l/h), chloride (37.4 mmol/l/h) and potassium (1.48 mmol/l/h) and the highest absorption of calcium (0.38 mmol/l/h) were registered in colon and in group which was infected with the reference strain S1. The S2 and S3 strains were in a viable but non-culturable state inside intestine and caused less water and electrolyte secretions than strain S1.

Key words: *Salmonella*, environmental strains, Everted Gut Sac, water secretions, electrolytes secretions, viable but non-culturable state.

Introduction

The use of treated waste water in agriculture can increase the risk of enteric infection by direct contact or by consumption of products like fruits and vegetables which are irrigated by treated waste water. *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) is an enteropathogenic agent not normally belonging to the intestinal microflora but it can be present in human and animal digestive tube and so contaminate the environment by their excreta¹. These pathogenic bacteria cause consecutive diarrhea to contaminated water and food absorption¹. Facing stress several enteric pathogens go in a viable but non-culturable (VBNC) state^{2,3}. Many studies showed that the VBNC hypothesis constitutes the basis of questions about the threat of bacteria, which cannot be detected by standard microbiological testing methods of public health⁴. Several studies showed that VBNC bacteria were capable of retaining virulence^{5,6}. The entry of an enteropathogenic agent like *S. typhimurium* causes irritation and increase of intestinal motility, this allows to great quantity of liquids and sweeps the infectious agent to the anus⁷. The intestine exercises a protective function facing pathogenic agents⁸. The objective of this study was to explore the effect of environmental stains of *Salmonella* on water and electrolytes transfer in rat intestine.

Materials and Methods

Infection method: Four groups of Wistar male rats (each group was composed of 3 rats weighing 200-300 g) were kept on sterile

conditions (water drink, food and cages) at $20 \pm 2^\circ\text{C}$. Rats were treated in orally by 2 ml of nalidixic acid solution [(0.1 g/ml)/100 g body wt] during a week to eliminate a contamination by *Salmonella*⁹. To prove that there is no *Salmonella* in tractus digestif rat, 2 g of rat's saddles were analyzed according to the operating mode of ISO 6579 Norm¹⁰. Two weeks after the antibiotic treatment, only three from the four rats groups were infected by three bacterial suspensions (0.5 ml with 10^8 cfu/ml). The first group was infected by *S. typhimurium* ATCC14028s (S1), the second group by a *S. typhimurium* strain which was isolated from waste water stemming from secondly treatment (S2) and the third group by *S. typhimurium* strain which was starved in sea water during half and six months (S3).

Treatment of animals: Rats had free access water but food was withdrawn 24 h prior to the experiment. They were anesthetized with intraperitoneal pentobarbital (4.2 mg/100 g body wt)¹¹. Different intestinal parts (jejunum, ileum and colon) were immediately extracted, stripped of adhering tissue and cleaned with a ringer solution (pH 7.4) (0.154 mmol NaCl, 0.0034 mmol KCl, 0.0024 mmol NaNO_3 , 0.021 mmol CaCl_2 and 0.5 l distilled water) then taken and divided into different segments (medium length 4 cm).

Everted Gut Sacs (EGS) were prepared according to the technique of Wilson and Wiseman¹². A canule was fixed in a cote of each intestinal segment to inject and take tested liquids and

the free cote was ligatured. The sacs were hanged in an incubatory medium containing half diluted ringer solution. The EGS were filled (400 µl) with a ringer solution (pH 7.4) maintained at 37°C and constantly oxygenated with air. Incubation time was 2 hours.

Determination of water and electrolytes fluxes: Water flux was determinate in the absence and the presence of *Salmonella* strains. Results were expressed in mg of water/g of fresh intestine/h. Electrolytes flux (sodium, chloride, calcium and potassium) was determined by COBAS INTEGRA 400 plus analyzer inside and outside sacs. Results were expressed in mmol/l/h.

Strains recovery from intestinal sacs: *Salmonella* strains which were not recovered were revived by inoculation in nutrient broth, incubated at 37°C and shaken in the Marie bath. Inoculation in nutrient medium and SS medium was done every day as far as *Salmonella* strains were recovered. They were identified by biochemical test (with Api 20 E gallery of bio Mérieux) and serologic test which was done in Pasteur Institute of Tunis.

Statistical analysis: Statistical significance of results was analyzed by variance analysis method (ANOVA). Results were considered significant when $P < 0.05$.

Results

Determination of water flux: During basal conditions, there was a water secretion in jejunum (N=12) and ileum (N=12), the respective values were -0.075 ± 0.08 and -0.389 ± 0.17 mg/g of fresh intestine/h. On the other hand an absorption of water was registered in colon (N=12) and the flux of water was 0.238 ± 0.07 mg/g of fresh intestine/h. A secretion of water was registered in the three intestinal parts of rats which were treated by the different tested strains (Fig. 1). In fact S1 increased water secretion in jejunum, ileum and colon ($P < 0.05$); S2 increased evenly secretion in jejunum, ileum and colon ($P < 0.05$) and a significantly increased secretion was also noted in jejunum, ileum and colon in the presence of S3 ($P < 0.05$). The environmental strains S2 and S3 caused less water fluxes than the reference strain S1. The respective values for S2 and S3 were in jejunum -0.75 ± 0.14 and

-0.43 ± 0.08 , in ileum -0.81 ± 0.2 and -0.64 ± 0.19 and in colon -0.13 ± 0.07 and -0.03 ± 0.01 mg/g of fresh intestine/h. The respective values of water fluxes registered in the EGS treated by S1 were in jejunum -0.85 ± 0.18 , in ileum -1.336 ± 0.0 and in colon -0.18 ± 0.05 mg/g of fresh intestine/h (Fig. 1).

Determination of electrolyte fluxes: *Salmonella* strains caused more secretion of sodium, chloride and potassium and absorption of calcium in jejunum than were found in basal conditions (Table 1). Similar results were obtained in ileum but were found not significant (Table 1). There was inversion of electrolyte transport between control and treated rats in colon. Thus, secretion of sodium, chloride and potassium and absorption of calcium were noticed in treated rats instead of absorption of sodium, chloride and potassium and secretion of calcium in control (Table 1). The secretion of sodium, chloride and potassium and absorption of calcium were highest in rats infected by S1 in colon and least in rats infected by S3 (Table 1). Variation of different electrolytes fluxes between jejunum, ileum and colon was significant ($P < 0.05$) for sodium, chloride and calcium but not for potassium.

Strains recovery from intestinal sacs: The reference strain S1 was recovered from the different intestinal sacs and it presented biochemical (Table 2) and serological characters (Table 3) of *S. typhimurium* which was inoculated for animals. S2 and S3 strains were not detected in recovered liquids after 48 h of reviviscence. They presented some biochemical and serological modifications, which may be consequences of stressed conditions¹³.

Discussion and Conclusions

The use of EGS showed that infection by *S. typhimurium* induced secretion of water even in colon where water is normally absorbed in humans and animals¹⁴. This effect can be induced also by *Shigella*¹⁵ and *Vibrio cholerae*¹⁶. The validity of the intestinal segments was verified by histological study (unpublished data). During control basal conditions, sodium ion was secreted in jejunum, ileum and absorbed in colon. *Salmonella* infections caused secretion of sodium in all tested intestinal parts. We noticed that these movements follow transport of water. Ratio

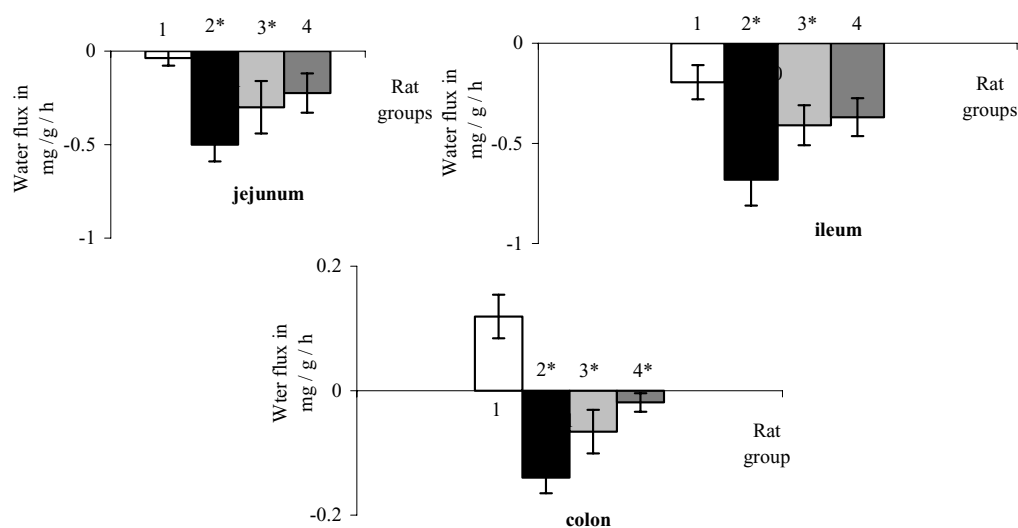


Figure 1. Effect of *S. typhimurium* on variations of water fluxes in rat jejunum, ileum and colon. (1) Control group. (2) Group infected by S1. (3) Group infected by S2. (4) Group infected by S3. * Significant variation (ANOVA). Error bars represent standard deviation between three treated rats.

between water and sodium transport was considered in some other studies¹⁷⁻¹⁹. The movement of chloride was comparable to the movement of sodium in the control basal conditions and in the conditions of *Salmonella* infections. Therefore, absorption of sodium and chloride ions appears like being two coupled processes²⁰. Potassium flux had the same trend as sodium and chloride fluxes. This can be justified by the fact that $3\text{Na}^+-2\text{K}^+$ pump in lateral membrane of small intestine supplies energy to water and ion transport through intestinal epithelium. Diffusible ions circulate by $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter in serosa²¹. We observed that calcium transport performs opposite trend

compared to sodium, chloride and potassium transport. In fact, calcium exit is assured by ionic exchanger $\text{Na}^+/\text{Ca}^{2+}$ ²². Thus, *S. typhimurium* causes sodium and chloride secretion and varies consequently osmotic pressure so to displace water at the same time. This can cause diarrhea to human. The enterocyte capacity to secrete water and electrolytes in intestinal lumen constitutes a mucosal protection facing pathogenic agents²¹. This allows diluting infectious agents and causes fast movements of faeces to the anus²³. The local immunity can justify the passage of stressed environmental *Salmonella* into a VBNC state in the intestine. Gastroenteritis induces a release of cytokines stunts

like tumor necrosis factor- α (TFN- α), interferon- γ (IFN- γ) and interleukin-4 (IL-4); this influxes on the adherence of bacteria as *S. typhimurium*, *Listeria monocytogenes*, *Escherichia coli* and *Proteus mirabilis*. IFN- γ may increase the bacteria adherence to the intestinal epithelium and IL-4 may decrease the bacteria migration through the intestinal epithelium²⁴. The defenses of the intestinal local immunity and the presence of some toxic metabolites as volatile fatty acids which were secreted by endogen microflora²⁵ may constitute a stress and induce passage of S2 and S3 to a VBNC state inside the

Table 1. Variation of electrolytes fluxes.

Bacteria strains	Part of intestine	Electrolytes outside sac (mmol/l/h)			
		Sodium	Chloride	Potassium	Calcium
-	Incubatory solution	85.5	85.54	1.95	1.32
Control	Jejunum	120±0.12	100.12±0.11	2.38±0.23	0.90±0.14
	Ileum	108.2±0.1	87±0.09	3.00±0.12	1.20±0.19
	Colon	78±0.20	81±0.20	1.90±0.14	1.43±0.21
S1	Jejunum	122±0.22	116±0.14	2.80±0.16	0.52±0.17
	Ileum	118±0.10	110±0.10	3.40±0.07	0.99±0.26
	Colon	168±0.25	118.4±0.19	3.38±0.20	1.24±0.09
S2	Jejunum	118±0.09	110.32±0.08	2.65±0.31	0.65±0.12
	Ileum	115±0.11	100±0.17	3.20±0.23	1.05±0.10
	Colon	165±0.06	118.4±0.13	3.20±0.14	1.27±0.17
S3	Jejunum	115±0.08	108±0.21	2.50±0.24	1.08±0.09
	Ileum	110±0.14	92±0.10	3.10±0.27	1.50±0.10
	Colon	160±0.12	98.2±0.14	2.80±0.18	1.10±0.14

(S1) Reference strain. (S2) Strain isolated from treated waste water. (S3) Strain starved in sea water.

Table 2. Chemical profiles of tested strains before animal inoculations and after recovery from intestinal sacs.

Strains	ONPG	ADH	LDC	ODC	CIT	H ₂ S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO ₃	NO ₂
S1	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	-	+
S2	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	-	+
S3	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	+	-	+	-	-	+
S1'	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	-	+
S2'	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
S3'	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+

(S1) Reference strain. (S2) Strain isolated from treated waste water. (S3) Strain starved in sea water recovered from intestinal sacs. (+) Positive character. (-) Negative character. (ONPG..., NO₂) Gallery Api substrates.

Table 3. Serological characters of tested strains before inoculations to animals and after recovery from intestinal sacs.

Strain	Auto agglutination	Ag O OMA	Ag H						Serotype
			HMA	Hi	HMB	HMC	H1	H2	
S1	-	4, 5, 12	+	+	-	-	-	-	(4;5;I;1,2)
S2	-	4,5,12,27	+	+	-	-	+	+	(4;5;i;1,2) with phase inversion
S3	-	4, 5,12	+	+	-	-	+	+	(4;5;i;1,2) with phase inversion
S1'	-	4, 5, 12	+	+	-	-	+	+	(4;5;i;1,2) with phase inversion
S2'	-	4, 12	+	+	-	-	-	-	(4;i;1,2)
S3'	-	4, 12	+	+	-	-	-	-	(4;i;1,2)

(S1) Reference strain. (S2) Strain isolated from treated waste water. (S3) Strain starved in sea water. (') Strains recovered from intestinal sacs. (+) Positive character. (-) Negative character. (OMA) Somatic antigen for A group. (HMA) Flagella antigen for A group. (HMB) Flagella antigen for B group. (HMC) Flagella antigen for C group. (H1) First flagella base. (H2) Second flagella as phase (4, 5, 1, 12, 27) Monovalent antigens.

intestine. This passage is produced by progressive cultural and biochemical modifications²⁶.

The findings may establish that environmental strains of *Salmonella* cause water and electrolytes losses in the rat intestine without detecting germs which cause these disorders. Environmental strains pass into a VBNC state inside intestine and they are detected after reviviscence. Identification by DNA analysis using RAPD (Random Amplified Polymorphic DNA) method of recovered strain will be viewed.

Acknowledgements

We thank Prof. K. Mehdouani and the technicians of bacteriological laboratory in Ibn El Jazzar hospital (Kairouan, Tunisia) for facilitating electrolytes analysis, Prof. M. Gauthier (Inserm Nice, France) for supplying the referenced strain *S. typhimurium* ATCC14028s and M^s S. Saidi for supplying the strain which was incubated in sea water.

References

- ¹Dhiaf, A. and Bakhrouf, A. 2004. Recovery in embryonated chicken eggs of viable but non-culturable *Salmonella*. J. Food Agric. Environ. **2**(2):104-107.
- ²Xu, H.S., Robert, N., Singleton, F.L., Attwell, R.W., Grimes, D.J. and Colwell, R.R. 1982. Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microbiol. Ecol. **8**:313-323.
- ³Rollins, D.M. and Colwell, R.R. 1986. Viable but non-culturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. **52**:531-58.
- ⁴Barer, M.R., Gribbon, C., Harwood, R. and Nwoguh, C.E. 1993. The viable but non-culturable hypothesis and medical bacteriology. Rev. Med. Microbiol. **4**:183-193.
- ⁵Colwell, R.R., Tamplin, M.L., Brayton, P.R., Gauzens, A.L., Tall, B.D., Herrington, D., Levine, M.M., Hall, S., Huq, A. and Sack, D.A. 1990. Environmental aspects of *Vibrio cholerae* in transmission of cholera. In Sack, R.B. and Zinnaka, Y. (eds). Advances of Cholera and Related Diarrheas. Vol. 7. KTK Scientific, Tokyo Japan, pp. 327-343.
- ⁶Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A. and Levine, M.M. 1996. Viable but non-culturable *Vibrio cholerae* O1 revert to a culturable state in the human intestine. World J. Microbiol. Biotechnol. **9**:210-222.
- ⁷Don, W., Fawcett, P. and Ronald J.T. 2002. Digestive functions. In Maloine (ed.). Essential of Histology. Scientific Press, New York, pp. 315-325.
- ⁸Arthur, C. and Guyton, M.D. 1982. Principes généraux de la fonction gastrointestinale- Motilité, contrôle nerveux et circulation sanguine: Précis de Physiologie Médicale. Médicale (eds.). Paris Press, Paris, pp. 680-738.
- ⁹Piddock, L.J.V., Ricci, V., McLaren, I. and Griggs, D.J. 1998. Role of the mutations in the gur A and par C gene of nalidixic acid resistant *Salmonella* serotypes isolated from animals in the United Kingdom. J. Antimicrob. Chemother. **41**:635-641.
- ¹⁰ISO 1993. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. ISO 6579/TC34/SC9. Microbiology - General guidance on methods for the detection of *Salmonella*. International Organization for Standardization, 16 p.
- ¹¹Charpin, G., Abdul, R., Chikh, I., Guignard, H., Jordan, G., Dumas, C., Pansu, D. and Descroix-Vagne, M. 1992. Effect of sorbin on duodenal absorption of water and electrolytes in the rat. Gastroenterol. **103**:1568-1573.
- ¹²Wilson, T.H. and Wiseman, G. 1954. The use of sacs reverted of small intestine for study of transference of substance from mucosal to serol surface. J. Physiol. London **123**:116-125.
- ¹³Baudart, J., Lemarchand, K., Brisabois, A. and Lebaron, P. 2000. Diversity of *Salmonella* strains isolated from the aquatic environment as determined by serotyping and amplification of the ribosomal DNA spacer region. Appl. Environ. Microbiol. **66**:1544-1552.
- ¹⁴Curran, P. and Macintosh, J. 1962. A model system for biological water transport. Nat. **193**:341-348.
- ¹⁵Fiorito, P., Burgos, J.M., Miyakawa, M.F., Rivas, M., Chillemi, G., Berkowski, D., Zotta, E., Silberstein, C. and Ibarra, C. 2000. Effect of *Schigella* into human water transport. Dig. Dis. Sci. **45**:480-48.
- ¹⁶Rabbani, G.H., Albert, M.J., Rahman, H. et al. 2001. Cholera toxin in proximal colon of rabbit *in vivo*. J. Membr. Biol. **171**:177-182.
- ¹⁷Khan, J.M., Wingertzahn, M.A., Teichberg, S., Vancurova, I., Harper, R.G. and Wapnir, R.A. 2000. Development of the intestinal SGLT1 transporter in rats. Mol. Gent. Meta. **69**:233-239.
- ¹⁸Hines, O.J., Whang, E.E., Bilchik, A.J., Zinner, M.J., Welton, M.L., Lane, J., Mcfadden, D.W. and Ashley, S.W. 2000. Role of Na⁺ - glucose cotransport in jejunal meal - induced absorption. Dig. Dis. Sci. **45**:1-6.
- ¹⁹Loo, D.D., Wright, E.M. and Zeuthen, T. 2002. Water pumps. J. Physiol. **542**:53-230.
- ²⁰Darryl, W., Burgess, M. D., Miarczynski, M. J. and O'Donnell, C. M. 2000. Na⁺ and Cl⁻ transport by the urinary bladder of the freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Zool. **287**:1-14.
- ²¹Larsen, E.H., Sorensen, J.B. and Sorensen, J.N. 2002. Analysis of the sodium recirculation theory of solute-coupled water transport in small intestine. J. Physiol. **542**:33-50.
- ²²Silbernagl, S. and Despopoulos, A. 1983. Atlas de Poche de Physiologie. 4th ed. Paris Press, Paris, 123 p.
- ²³Abraham, L. and Kierszenbaum, M.D. 2002. Histology and Cell Biology - An Introduction to Pathology. Mosby (ed.). Scientific Press, New York, 444 p.
- ²⁴Hess, D.J., Henry-Stanley, M.J., Erickson, E.A. and Wells, C.L. 2003. Effect of tumor necrosis factor alpha, interferon gamma, and interleukin-4 on bacteria-enterocyte interactions. J. Exp. Zool. **380**:12-16.
- ²⁵Armelle, R. 2001. Les probiotiques: Prévention et santé. Ann. Bio. Clin. **10**:195-187.
- ²⁶Besnard, B., Federighi, M., Declerq, E., Jugiau, F. and Cappelier, J.M. 2002. Environnemental and physicochemical factors induce VNC state in *Listeria monocytogenes*. Vet. Res. **33**:359-370.