

Molecular Evaluation of Enteric Viruses' Contamination of Raw Vegetables Grown in the Errachidia Region

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Abstract

The objective of this study was to determine the prevalence of contamination of fresh vegetables by enteric viruses in the Errachidia region. A total of 80 samples of four types of fresh vegetables (radish, lettuce, onion and parsley) were taken from different plots irrigated by water from WWTPs effluents or wells. The search for enteric viruses was carried out by real-time RT-PCR. Overall rate of enteric viruses' contamination was 69%. Depending of virus type, this rate was 66%, 59% and 8% for enteroviruses, Norovirus and Rotavirus respectively. No samples tested positive for hepatitis A virus. Vegetables from plots irrigated by WWTPs effluents were more contaminated than those irrigated by well water. Lettuce and parsley were often more contaminated than radish and onion. Our study shows that enteric viruses can contaminate fresh vegetables, requiring the implementation of measures to control this health risk.

Keywords: Enteric viruses, Norovirus, Enterovirus, hepatitis A virus, Er-rachidia, Morocco.

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INTRODUCTION

Microbial contamination of food is one of the main causes of disease in the world, which represents a real public health problem [1]. So far, more than 250 pathogens, including bacteria, viruses, prions, protozoa and fungi have been identified [2]. Among these food-borne pathogens the importance of viruses is often underestimated due to the difficulty of their detection compared to bacteria for example. However, several studies show that viruses are responsible for nearly 40% of foodborne infections in France [3] and more than 58% in the United States [4]. The majority of viruses involved in this type of disease are enteric viruses which infect the human gastrointestinal system and which are subsequently excreted into the external environment and transmitted by direct or indirect faecal-oral route.

Human enteroviruses (HE), rotaviruses (RV), noroviruses (NoV) and hepatitis A virus (HAV) are among the viruses most frequently found in raw and treated wastewater [6, 7]. These are very resistant viruses in the environment and can persist after purification treatments and then end up in surface

waters, coastal waters or food [8]. In a previous study in the Er-Rachidia region, we detected the persistence of contamination by enteric viruses in the local wastewater treatment plant (WWTP) effluents with also contamination of groundwater near the discharge sites [9].

The objective of this study was to determine the prevalence of enteric viruses' contamination of fresh vegetables produced in the Er-Rachidia region.

MATERIALS AND METHODS

Study Region

The Errachidia region is located in the center-east of the country with an arid climate and limited water resources. The region is crossed by the Ziz wadi which forms a valley of more than 282 km where oasis-type agriculture is practiced (Fig 1). The scarcity of water in the region has led some farmers to resort to effluent from the WWTPs for irrigation of their crops. These waters meet national bacteriological standards [10] but remain still remains largely contaminated by at least 3 families of enteric viruses [9].

Samples

In order to detect the presence of enteric viruses in fresh vegetables and to assess the degree of their contamination, a total of 08 plots of land planted with vegetable crops along the valley of the wadi Ziz were identified. Five plots (P1-P4) were irrigated by water from the WWTP effluents and the other five were irrigated exclusively by well water (P5-P8) (Figure 1).

During the months of March and April 2019, we collected a total of eighty (80) samples of fresh

vegetables from the ten previously selected plots. Group 1 is made up of samples taken from P1-P4 (n=40) and group 2 includes samples outcome from P5-P8 (n=40). The sampling plan consisted of carrying out 5 samples per month on the four types of vegetables selected (radish, lettuce, onion and parsley). To ensure good representativeness, we first collected a pre-sample from a vegetable harvesting crate that could contain between 2 and 6 kg of product. after homogenization, around 500 g are sent directly to the laboratory for analysis in coolers in less than 2 hours.

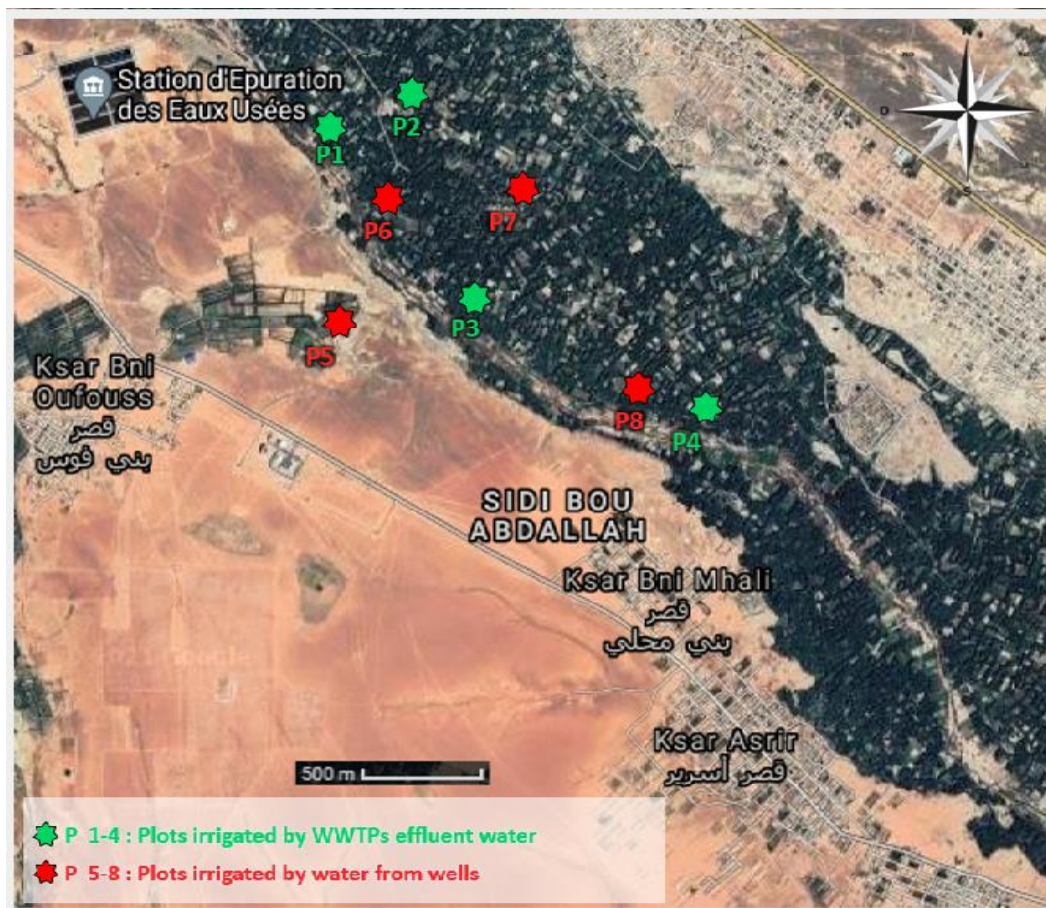


Figure 1: Location and distribution of sample collection sites

The Concentration of Viruses

Concentration of viruses from samples consists of extraction of enteric viruses from the food matrix. The protocol used in our study is an adaptation of that previously described by Dubois *et al.*, (2006) [11] and modified by Summa *et al.*, (2012) [12]. Briefly, 25 g of the sample to be analyzed are suspended and homogenized with 100 ml of virus extraction buffer TGBE (1% beef extract, pH 9.5, 100 mM Tris - 50 mM glycine) in the presence of the internal control. The mixture is stirred at 60 rpm for 20 min. After adjusting the pH between 9 and 9.2, the mixture is centrifuged at 10,000 rpm for 10 min at + 4° C. Subsequently, the supernatant is recovered and the pH is adjusted to 7.2. Then, end-point filtration is carried out using a cellulose

nitrate filter with 0.2 µm pores on a Sartolab® vacuum filtration unit from Sartorius®. The filters were then stored at -80 ° C until analysis. The internal control used in our study was Newcastle disease virus (NDV) (Lasota strain) added to each sample at a rate of 10³ TCID₅₀ per sample in order to assess the presence of PCR inhibitor and to validate the tests.

Extraction of Nucleic Acids

Each filter was aseptically divided into 2 sections. One of the two parts was then inserted into a 2 ml screw cap polypropylene tube containing 200 mg of 106 µm glass beads and 1 ml of diluent buffer (Dulbecco's PBS with 0.01% Tween 80 and 0.001 % Antifoam A). The tube is vortexed for 5 min at high

speed and the sample is centrifuged at 5,000 g for 2 minutes at 4°C. The supernatant is used for extraction. Extraction of viral nucleic acids was performed by manual technique using the GeneJET Viral DNA / RNA purification kit (Thermo Fisher Scientific™, Massachusetts, USA) according to the manufacturer's recommendations.

Virus Detection

Enteric virus detection was performed using a one-step real-time RT-PCR assay on the Applied Biosystems™ 7500 thermal cycler (Thermo Fisher Scientific, Massachusetts, USA) at the virology laboratory of the Hassan II Agronomic and Veterinary Institute. The kit used for detection was the SensiFAST™ Probe Lo-ROX One-Step (Bioline, Cincinnati, USA) with previously reported primers and

probes for HEV, NoV, HAV and NDV (Table 1). Each virus was tested in duplex with the internal control (NDV) in the same tube. Thus, in a final volume of 20 µl, each reaction mixture contained: 10 µl of 2X SensiFAST Probe Lo-ROX One-Step Mix, final concentrations of 0.4 µl of each primer; 0.2 µl of each TaqMan probe; 0.2 µl of the reverse transcriptase enzyme; 0.4 µl of riboSafe RNase inhibitor; 1.8 µl of molecular biology quality water and 4 µl of matrix (sample extract, positive/negative template). The thermal profile adopted for this real-time RT-PCR was as follows: 10 min at 45 °C; 2 min at 95 °C followed by 40 cycles of 5 s at 95°C and 20 s at 60°C. Positive and negative controls were included in each run. All samples where Internal Control (NDV) was not amplified were retested at a 1/10 and 1/100 dilution.

Table 1: List of primers and probe used in virus detection RT-PCRs

Virus	Primers and probes	SEQUENCE (5'-3')	Reference
EV	EV-UTR-F	ACATGGGTGCGAAGAGTCTATTGAGCT	13
	EV-UTR-R	CGACTACTTTGGGTGTCCGTGTTTC	
	Probe	FAM-TCCGGCCCTGAATGCGGCTAAT-BHQ1	
HAV	HAV68 F	TCACCGCCGTTTGCCTAG	14
	HAV240 R	GGAGAGCCCTGGAAGAAAG	
	Probe	FAM-CCTGAACCTGCAGGAATTAA-BHQ1	
NoV G I	QNIF4 F	CGCTGGATGCGNTTCCAT	15
	NV1LCR R	CCTTAGACGCCATCATCATTTAC	
	Probe	FAM-TGGACAGGAGAYCGCRATCT-BHQ1	
NoV GII	QNIF2 F	ATGTTTCAGRTGGATGAGRITTCWGA	15
	COG2R R	TCGACGCCATCTTCATTACA	
	Probe	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	
RV	JVK F	CGATGGTTGATGCTCAAGATGGA	16
	JVK R	TCATTGTAATCATATTGAATACCCA	
	Probe	FAM-ACAACCTGCAGCTTCAAAGAAGWGT-BHQ1	
NDV	NDVM F	AGTGATGTGCTCGGACCTTC	17
	NDVM R	CCTGAGGAGAGGCATTTGCTA	
	Probe	VIC-TTCTCTAGCAGTGGGACAGCCTGC-BHQ1	

RESULTS

Specific real-time RT-PCR tests have shown that the rate of raw vegetables contamination detection varies depending on the virus, the vegetable and the water used for the irrigation (Table 2). During our study we never detected the presence of the HAV virus. However, the overall contamination of vegetables by enteric viruses regardless of the origin of the irrigation water was 66%, 59% and 8% for HEV, NoV and RV respectively. HEV were the most enteric viruses present in the samples studied with an average contamination prevalence of 70% (60%-80%) of Group 1 fresh vegetables and 63% (50%-70%) of those from Group 2 but this difference is not statistically significant. There was also no significant difference in HEV contamination depending on the type of vegetable although the lettuce was slightly more contaminated. The NoV were in second rank of contamination with prevalence rates of 68% for Group1 vegetables and

50% for those from Group 2. Radish 90% and parsley 80% were the most contaminated in plots irrigated by WWTP effluents while lettuce 60% was the most contaminated in plots irrigated by well water. RV contamination was lowest with rates of 20% for parsley collected in Group 1 and 10 % for radish and lettuce regardless of the origin of the sample. Comparison of the contamination rate according to the irrigation water reveals that vegetables produced in plots irrigated by the WWTP effluent water (P1-4) appears to be more contaminated than those from plots irrigated by well water (P5-8). Thus, in the first plots, the rates were 70%, 68% and 10% respectively for HEV, NoV and RV; while these rates were 63%, 50% and 5% respectively for HEV, NoV and RV.

Regarding the genotyping of NoVs, 47 samples 89% were positive for Genotypes II, against only 06 11% for genotype I.

Table 2: RT-PCR test results depending on the virus, the vegetable and the nature of the irrigation water

	Vegetables	Number of samples	HEV	NoV	RV	HAV
Group 1	Radish	10	7 (70%)	9 (90%)	1 (10%)	0 (0%)
	Lettuce	10	8 (80%)	7 (70%)	1 (10%)	0 (0%)
	Onion	10	6 (60%)	3 (30%)	0 (0%)	0 (0%)
	Parsley	10	7(70%)	8 (80%)	2 (20%)	0 (0%)
	Total	40	28 (70%)	27 (68%)	4 (10%)	0 (0%)
Group 2	Radish	10	6 (60%)	4 (40%)	1 (10%)	0 (0%)
	Lettuce	10	7 (70%)	6 (60%)	1 (10%)	0 (0%)
	Onion	10	5 (50%)	5 (50%)	0 (0%)	0 (0%)
	Parsley	10	7 (70%)	5 (50%)	0 (0%)	0 (0%)
	Total	40	25 (63%)	20 (50%)	2 (5%)	0 (0%)
Total of samples	80	53 (66%)	47 (59%)	6 (8%)	0 (0%)	

DISCUSSION

This is the first study carried out in the Errachidia region for the evaluation of the contamination of raw vegetables by enteric viruses. The choice of viruses included in our study was motivated by a hazard analysis and a risk assessment. Indeed, HEV, NoV, HAV and RV are the enteric viruses most implicated in foodborne infections worldwide [4,18]. In addition, in a previous study we detected the persistence of contamination by enteric viruses in the local WWTP effluent water as well as their presence in groundwater [9]. The choice of vegetables studied (lettuce, radish, parsley and onions) was motivated by the fact that these are foods that can be eaten raw in salads or appetizers which does not allow sanitizing treatment. Even if it remains difficult to compare the contamination rates obtained with those reported in the literature due to differences in detection and sampling techniques. The contamination rate obtained for HEV 66% is higher than the rate reported in Sicily 2.9% [19] but remains lower than the contamination rate detected in Argentina 79% [20]. For NoVs, the results obtained 59% is higher than that reported by several European studies concerning contamination by NoV with rates ranging from 2.9% in Sicily [19] to 7.4% in Greece, Serbia and Poland [21] and 11.9 to 15.5% in France [22]. High rates of contamination found in our study compared to European rates are very probably related to the nature of the samples in our study. In fact, our samples were taken from cultivation area irrigated by water known to be contaminated by enteric viruses, whereas for the European studies it is an all-round sample taken at market. The contamination rates are rather close to levels reported in Egypt 33% [23] and in Argentina 58% [20]. The RV 8% prevalence rate remains relatively low and remains comparable to that reported in Argentina 5% [20] and well below the rate reported in Egypt 20% [23].

Moreover, even if Morocco is considered as an endemic country for HAV by the World Health Organization, no positive sample was detected during our study. These results place Morocco more in line with European standards with contamination prevalence of 0% in Sicily [19] and from 0% to 1.32% in Greece-

Serbia and Poland [21] and far behind countries like Egypt with 55% of contaminated samples [23]. The absence of HAV detection is probably related to the absence of HAV in irrigation water [9]. Indeed, the detection of HAV in water is quite rare if the samples are not taken during or immediately after hepatitis A epidemics [24, 25].

Our results show that the vegetables of group 1 irrigated by the effluent from the WWTPs were more contaminated than those irrigated by the water from wells. Thus, the contamination rate for group 1 was 70%, 68% and 10% for HEV, NoV and RV against 63%, 50% and 5% for group 2. This result is consistent with our previous study which demonstrated that effluents WWTPs water was more contaminated than groundwater. The higher contamination rate of lettuce and parsley compared to radishes and onions is most likely related to the cultivation method. In fact, irrigation is much more frequent for these two vegetables from planting until harvest.

CONCLUSION

This study provides the first findings on molecular detection of several enteric viruses contaminated raw vegetables in Er-rachidia region which could be a potential risk for human infection if not being disinfected appropriately or cooked before consumption. It also highlights the risk of enteric virus transmission following the use of contaminated water in irrigation.

RÉFÉRENCES

1. NIH. (2007). Understanding Emerging and Re-emerging Infectious Diseases. MD, USA.
2. Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases (DFWED). Dernière mise à jour: May 3, 2019; <https://www.cdc.gov/foodsafety/diseases/index.html>.

3. Van Cauteren, D., Le Strat, Y., Sommen, C., Bruyand, M., Tourdjman, M., Da Silva, N. J., ... & Desenclos, J. C. (2017). Estimated annual numbers of foodborne pathogen-associated illnesses, hospitalizations, and deaths, France, 2008–2013. *Emerging infectious diseases*, 23(9), 1486-1492.
4. Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., ... & Griffin, P. M. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging infectious diseases*, 17(1), 7-15.
5. Greening, G. E., & Cannon, J. L. (2016). Human and animal viruses in food (including taxonomy of enteric viruses). *Viruses in foods*. Springer, Cham. (pp. 5-57). <https://doi.org/10.1007/978-3-319-30723-7>.
6. Fong, T. T., & Lipp, E. K. (2005). Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiology and molecular biology reviews*, 69(2), 357-371.
7. Carducci, A., Morici, P., Pizzi, F., Battistini, R., Rovini, E., & Verani, M. (2008). Study of the viral removal efficiency in a urban wastewater treatment plant. *Water Science and Technology*, 58(4), 893-897.
8. Kotwal, A., Singh, H., Verma, A., Gupta, R., Jain, S., Sinha, S., ... & Ranjan, R. (2014). A study of Hepatitis A and E virus seropositivity profile amongst young healthy adults in India. *Medical journal armed forces india*, 70(3), 225-229.
9. Bounagua, R., El Rhaffouli, H., El Boukhrissi, F., Rahmatallah, N., Yassin, Z., El, H., ... & Fassi-Fihri, O. (2021). Molecular Detection of Enteric Viruses in Treated Wastewater, Surface and Groundwater in Errachidia Region, Morocco. *Saudi J Pathol Microbiol*, 6(1), 23-28.
10. Hamid, C., Elwatik, L., Ramchoun, Y., Fath-Allah, R., Ayyach, A., Fathallah, Z., ... & Hbaiz, E. M. (2014). Étude des performances épuratoires de la technique du lagunage aéré appliquée à la station d'épuration de la ville d'Errachidia-Maroc. *Afrique Science: Revue Internationale des Sciences et Technologie*, 10(2), 173-183.
11. Dubois, E., Hennechart, C., Deboosère, N., Merle, G., Legeay, O., Burger, C., ... & Traoré, O. (2006). Intra-laboratory validation of a concentration method adapted for the enumeration of infectious F-specific RNA coliphage, enterovirus, and hepatitis A virus from inoculated leaves of salad vegetables. *International Journal of Food Microbiology*, 108(2), 164-171.
12. Summa, M., von Bonsdorff, C. H., & Maunula, L. (2012). Evaluation of four virus recovery methods for detecting noroviruses on fresh lettuce, sliced ham, and frozen raspberries. *Journal of virological methods*, 183(2), 154-160.
13. Cabrerizo, M., Calvo, C., Rabella, N., Muñoz-Almagro, C., del Amo, E., Pérez-Ruiz, M., ... & Trallero, G. (2014). Design and validation of a real-time RT-PCR for the simultaneous detection of enteroviruses and parechoviruses in clinical samples. *Journal of virological methods*, 208, 125-128.
14. Costafreda, M. I., Bosch, A., & Pintó, R. M. (2006). Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and environmental microbiology*, 72(6), 3846-3855.
15. da Silva, A. K., Le Saux, J. C., Parnaudeau, S., Pommepuy, M., Elimelech, M., & Le Guyader, F. S. (2007). Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. *Applied and environmental microbiology*, 73(24), 7891-7897.
16. Jothikumar, N., Kang, G., & Hill, V. R. (2009). Broadly reactive TaqMan® assay for real-time RT-PCR detection of rotavirus in clinical and environmental samples. *Journal of virological methods*, 155(2), 126-131.
17. Wise, M. G., Suarez, D. L., Seal, B. S., Pedersen, J. C., Senne, D. A., King, D. J., ... & Spackman, E. (2004). Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. *Journal of Clinical Microbiology*, 42(1), 329-338.
18. Le Guyader, F. S., Le Saux, J. C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., ... & Atmar, R. L. (2008). Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *Journal of clinical microbiology*, 46(12), 4011-4017.
19. Purpari, G., Macaluso, G., Di Bella, S., Gucciardi, F., Mira, F., Di Marco, P., ... & Guercio, A. (2019). Molecular characterization of human enteric viruses in food, water samples, and surface swabs in Sicily. *International Journal of Infectious Diseases*, 80, 66-72.
20. Prez, V. E., Martínez, L. C., Victoria, M., Giordano, M. O., Masachessi, G., Ré, V. E., ... & Nates, S. V. (2018). Tracking enteric viruses in green vegetables from central Argentina: potential association with viral contamination of irrigation waters. *Science of the Total Environment*, 637, 665-671.
21. Kokkinos, P., Kozyra, I., Lazic, S., Bouwknecht, M., Rutjes, S., Willems, K., ... & Vantarakis, A. (2012). Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries. *Food and Environmental Virology*, 4(4), 179-191.

22. Loutreul, J., Cazeaux, C., Levert, D., Nicolas, A., Vautier, S., Le Sauvage, A. L., ... & Morin, T. (2014). Prevalence of human noroviruses in frozen marketed shellfish, red fruits and fresh vegetables. *Food and environmental virology*, 6(3), 157-168.
23. Shaheen, M. N., Elmahdy, E. M., & Chawla-Sarkar, M. (2019). Quantitative PCR-based identification of enteric viruses contaminating fresh produce and surface water used for irrigation in Egypt. *Environmental Science and Pollution Research*, 26(21), 21619-21628.
24. Souza, F. G. D., Silva, F. P. D., Staggemeier, R., Rigotto, C., & Spilki, F. R. (2018). Low occurrence of Hepatitis A virus in water samples from an urban area of Southern Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*, 60:e69.
25. Schlindwein, A. D., Rigotto, C., Simões, C. M. O., & Barardi, C. R. M. (2010). Detection of enteric viruses in sewage sludge and treated wastewater effluent. *Water Science and Technology*, 61(2), 537-544.