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Assessment of enterovirus contamination in vegetable samples in Morocco by reverse transcription Nested-PCR

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Abstract

Vegetables are often implicated in the transmission for food-borne diseases, but local research on enteric viruses in vegetables is scarce despite its importance for public health. In this paper we report the results of an investigation on the presence of enteroviruses in vegetables irrigated by wastewater in Morocco. Viruses were concentrated from vegetable tissues by direct glycine elution and PEG 8000 precipitation. Total RNA was then extracted from the PEG precipitate by the guanidine thiocyanate method and transcribed into cDNA followed by "nested-PCR". Enterovirus genomes were detected in 16% of analysed samples. The range of positive samples is: eggplant, broad bean, tomato, pepper, potato, garden pea and radish. The Enterovirus contamination observed in this study should be considered as a serious risk to public health.

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Introduction

Foodborne viral infections are now recognized as a major cause of illness in humans. Indeed Each year, enteric viruses are responsible for the majority of non-bacterial gastro-enteritis or infectious hepatitis (Koopmans *et al.*, 2003). These viruses are transmitted to humans via the faecal-oral route, typically from contaminated drinking water or contaminated foods such as; raw shellfish, fresh fruits and vegetables and ready-to-eat foods (Sair *et al.*, 2002). Considering the low infectious dose of these viruses, only a small amount, present in the contaminated food, is habitually sufficient to infect a human host, which in turn will shed a large number of viral particles in the acute phase of the disease, thus increasing the risk of contaminating the environment through faecal contamination (Brassard *et al.*, 2005). For that, several studies were interested in viral contamination of water and shellfish. However, environmental contamination may concern other types of food such as vegetables which can be contaminated by using wastewater in the irrigation process (Croci *et al.*, 2002). Few studies have been performed to evaluate viral contamination of vegetable products in Morocco. Nevertheless, a wide range of these products may be contaminated by enteric viruses, including lettuce, celery, onions, potatoes and coleslaw (Dubois *et al.*, 2002). Indeed, viral infections associated with the consumption of vegetables are a growing concern for consumers and public health organizations (Lukasik *et al.*, 2001).

In Morocco, wastewater is increasingly being used in agriculture. In few cases irrigated vegetables do not undergo any control before being exposed in the markets, after which they may be eaten cooked or raw. This can cause foodborne viral diseases.

Our aim is to evaluate the contamination of vegetables irrigated by wastewater in the region by enteric viruses. In this purpose, direct glycine elution was used followed by PEG 8000 precipitation for concentration of viral particles before RT-nested PCR was carried out for the enterovirus detections in considered samples.

Materiel and methods

Samples

A total number of 50 vegetable samples, various types of vegetables were obtained from several wastewater-irrigated agricultural regions in Morocco. The vegetable samples were collected in sterile polyethylene bags and steps were taken to avoid vegetables contamination by soil and other contamination sources. Each sample was collected in triplicate to avoid sampling errors. The vegetables were eggplant, broad bean, tomato, pepper, zucchini, artichoke, beets, potato, garden pea, turnip, radish and carrot. All the samples were transported to the laboratory under low temperature (<7 degrees Celsius) and stored at 4 °C until testing. They were analyzed within 20 hours of sampling. Each sample is rinsed several times with sterile distilled water to eliminate the soil. Vegetables produce was directly used for the concentration process.

Viral concentration from samples

Viruses were concentrated from vegetables by direct glycine elution according to Dubois *et al.*, (2002) with minor differences. 60 g of each sample was homogenized for 2 min with 100 ml of elution buffer (Glycine / NaCl [0.1M / 0.3M] pH 9.5) using a mixer at maximum speed for 2 min. The pH of homogenate was maintained at 9.5. This homogenate was agitated for 20 min at room temperature. After, the homogenate was centrifuged at 10000 xg for 15 min at 4°C and the aqueous phase was recovered and its pH was adjusted to 7.2 with 5 N HCl under constant agitation. We added an equal volume of PEG 8000/NaCl (16% /0.6 N) and the solution was incubated overnight at 4°C. Viruses were concentrated by centrifugation at 10000 xg for 2 h at 4°C. The pellet was suspended in 2 ml of 0.15 M Na_2HPO_4 buffer (pH 9) and mixed with one volume of chloroform/butanol (1:1 solution). The suspension was allowed to stand for 5 min at room temperature and then centrifuged at 12000 xg for 15 min at 4°C. The upper aqueous phase containing viruses was recovered and used for analysis or kept frozen at -20°C until use.

Total RNA extraction

Total RNA was extracted by a guanidine thiocyanate method (Chomzinsky and Sacchi, 1987). Briefly, each sample aliquot (100 µl) was treated with 500 µl of lysis solution (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7], 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol). About 60 µl of 2 M sodium acetate (pH 5.2) was added followed by 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1). The tube was then incubated 15 min on ice. The RNA in the aqueous phase after centrifugation (12000 xg for 20 min at 4 °C) was precipitated with one volume of isopropanol overnight at -20 °C followed by centrifugation at 12000 xg during 30 min at 4°C. The pellet was washed with 1 ml of 70% ethanol and dried. The resulting RNA precipitate was dissolved in 15 µl of RNAse-free water (Promega).

Oligonucleotides

Primers used in RT-PCR have been designed previously by Schvoerer *et al.*, (2000) on the basis highly conserved 5' noncoding region to give a predicted product size of 435 bp.

EVB antisens primer 5'-ATTGTCACCATAAGCAGCCA-3'

EVA sens primer 5'- CAAGCACTTCTGTTTCCCCGG -3'.

For nested RT-PCR, we have used the following primer:

EVC Sense 5'-CCTCCGGCCCCTGAATGCGGCTAAT-3'

EVD antisense 5'-GAAACACGGACACCCAAAGTA-3' (Rotbart 1990)

cDNA synthesis

cDNA was synthesised using extracted RNA with the specific antisens primer (EVB). A 25 µl reaction volume was used, containing 5 µl of 5X reverse transcriptase buffer [250mM Tris-HCl (pH 8.3 at 25°C) 375mM KCl 15mM MgCl2 50mM DTT], 20 U of rRNasin® RNase inhibitors (Promega), 0.5 mM each dNTP , 200 U of M-MLV reverse transcriptase (Promega), 0.5 µM of primer EVB and 4.8 µl of RNA extract. RNase free H2O (Promega) was used to adjust reaction volume. Reverse transcription was carried out at 40°C for 1 h, and then DNA-RNA hybrid denaturation was performed by heating at 70°C for 15 min.

Polymerase chain reaction

10 µl of cDNA was mixed with 40µl PCR mix. PCR was performed in a 50 µl reaction volume containing 5 µl of 10X *Taq* DNA polymerase buffer, 0.5 mM of each dNTP, 2mM $MgCl_2$, 0.5 µM each primer (EVA and EVB) and 2.5 U of *Taq* DNA polymerase.

Nested PCR

2 µl of PCR amplicon was added to 48 µl of nested PCR mix. The reaction was carried out in a 50µl volume with concentrations similar to first step PCR mix except that the primers EVC and EVD were used instead of EVA and EVB.

The same amplification programme was used in both PCR and nested PCR steps. It consisted of eight min preliminary denaturation followed by 35 cycles: 45s at 94°C for denaturation 45 s at 50 °C for hybridization and 60 s at 72 °C for extension. A final extension was done at 72 °C for 7 min. PCR was carried out on a Perkin Elmer® 2400 thermal cycler. A 435 bp and 118 bp amplicon size were expected respectively in PCR and nested PCR steps. Nested RT-PCR products were analysed on 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV transluminator.

Results and discussion

We detected enterovirus genomes in eight samples out of 50 samples analysed (Fig. 1). These positive results lead to possible contamination hazard for consumers. The RT-PCR method cannot distinguish between infectious and inactivated viral particles. Free viral RNA is known to survive briefly in the environment due to the presence of bacterial endonucleases (Tsai *et al.*, 1995). However, RT-PCR detection of enterovirus genome is essentially due to well-protected RNA in viral particles which is inactivated or not (Schvoerer *et al.*, 2000; Haramoto *et al.*, 2004). In addition, some studies

have shown a correlation between the detection of viral RNA by RT-PCR and the detection of infectious particles (Le Guyader *et al.*, 1994). Furthermore, enteric viruses have properties that make them very stable in environment where they may remind infectious after several months (Sair *et al.*, 2002; Griffin *et al.*, 2003).

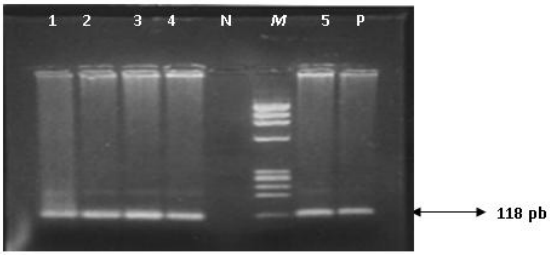

Fig. 1. 2% agarose gel electrophoresis stained with ethydium bromide. 1-5: positive samples; M" DNA molecular weight marker ADN ΦX 174 Hae III digest (Promega); N: negative control; P: positive control.

Table 1. Enterovirus detection and sample distribution.

Vegetables (n=50)	Number of samples	Number of positive samples
Eggplant	8	1/8
Broad bean	7	1/7
Tomato	6	1/6
Pepper	4	1/4
Zucchini	4	0/4
Artichoke	4	0/4
Beets	4	0/4
Potato	3	1/4
Garden pea	3	1/3
Turnip	3	0/3
Radish	2	2/2
Carrot	2	0/2

n: total number of samples

Enteroviruses have been proposed as an indicator of enteric virus contamination in the environment (Formiga-Cruz *et al.*, 2003). So detection of enterovirus in the samples analysed during our study may indicates the presence of other enteric viruses in this samples (Karamoko *et al.* , 2006). These analyzed products may be an important source of human foodborne viral disease, especially when they are consumed raw. The study of Croci *et al.*, (2002), showed that lettuce, fennel and carrot maintained a high quantity of HAV on their surface after being immersed in contaminated water. Furthermore, they demonstrated that washing apparently does not guarantee the elimination of the viruses such as HAV and poliovirus. These pathogens have the potential to persist on fresh fruits and vegetables for several days under commonly used household storage conditions (Kurdziel *et al.*, 2001; Cheong *et al.* 2009). The positive samples were represented by eggplant, broad bean, tomato, pepper, potato, garden pea and radish (Table 1). a generalized program of wastewater treatment according to agricultural reuse standards in the country should help to improve the the sanitary quality of these crops.

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