Egypt. J. Microbiol. **38**, No. 2, pp. 183 - 194 (2003)

Propagation of Small Round Structured Viruses *in Vitro* and Detection by RT-PCR in Wastewater

M.A. Ali,[1]* and M. El-Gendy [2]

Environmental Virology Lab, Department of Water Pollution Researches[1], Pharmacognosy Lab., Department of Pharmaceutical Industry[2], National Research Centre, Cairo, Egypt.

OUTBREAKS of small round-structured viruses have been associated with contaminated drinking water and food. Accordingly, wastewater is considered the main indicator for the incidence of these viruses between populations. In this study 72 raw wastewater and treated effluent samples were collected from three wastewater treatment plants in Cairo. The collected samples were concentrated by filtration through nitrocellulose membranes and detoxified by extraction with freon followed by reconcentration by PEG 6000 precipitation method. All concentrated samples were inoculated in three cell culture types, Vero, BS-C-1, and Huh-7 cells. To enhance virus isolation and propagation, the cells were treated for 2hr at 37°C with 40 µg of the water extract of *Aerva Javanica* (Burm.F.),Juss.Ex Schult plant and then the wastewater concentrated samples were inoculated for three successive passages, 4 days each. By RT-PCR, SRSVs were detected in 11 wastewater samples, and only one sample for untreated Huh-7 cells. In the treated BS-C-1 and Huh-7 cells, 12 SRSV positive RT-PCR samples were detected but for treated Vero cells, only 7 samples were positive. The genogroup differentiation assay revealed that 10 samples out of 12 were of genogroup I. The main conclusion of this study is that SRSVs may be isolated by propagation in cell cultures previously treated with the water extract of *Aerva Javanica* plant, which facilitate this propagation. Also, detection of SRSVs in wastewater may be reflecting the importance of studying this group of viruses in our populations.

KeyWords: Small round structured viruses (SRSVs), Wastewater treatment plants, RT-PCR and Cell culture

Small rounded structured viruses (SRSVs) are positive sense single stranded RNA viruses (Jiang *et al.*, 1990) and have been classified as members of *Caliciviridae* family .The family *Caliciviridae* has been subdivided into three genogroup and SRSVs have been assigned to genogroup I and II, while classical caliciviruses belong to genogroup III (Green *et al.*, 1994; Wang *et al.*, 1994). Recently, the two major groups of human caliciviruses have been classified into

*Corresponding author: E-mail < esraa4@hotmail.com>

distinct genera within the family *Caliciviridae*, and they have been provisionally named the "Norwalk-like viruses" and the "Sapporo-like viruses" (Green *et al.*, 2000). Although all caliciviruses share a common ancestor in phylogenetic analyses,the "Norwalk-like viruses" and the "Sapporo-like viruses" form distinct genetic clads within the *Caliciviridae* (Berke *et al.*,1997; Noel *et al.*,1997). In addition, certain features of their RNA genome organization distinguish them from each other and from other genera in the *Caliciviridae*. The family has two additional genera, Lagovirus and Vesivirus, each of which includes caliciviruses of veterinary importance. SRSVs have high attack rate among both children and adults, especially in semi-closed communities such as families (Pickering *et al.*, 1982), hospitals (Chadwick and McCann, 1994), residential homes (Jiang *et al.*, 1996), schools (Kobayashi *et al.*, 1991), universities (Kingar et al., 1996), and cruise ships (Kobayashi *et al.*, 1992). Many of the outbreaks of SRSV enteritis that are reported (Kaplan *et al.*, 1982;Kappus *et al.*, 1982;Wilson *et al.*, 1982; Lawson *et al.*, 1991) are related to exposure to faecally contaminated water, either directly through recreational water and drinking water or indirectly through consumption of uncooked shellfish, that have accumulated the viruses from their growing waters or consumption of wastewater irrigated fresh vegetables. Other outbreaks are related to other food-products; these are mainly due to contamination by an infected food-handler (Lodder *et al.*, 2000; Appleton, 2000).The outbreaks of waterborne and shellfish-borne SRSV infections indicate that polluted water is an important vehicle for the transmission of SRSV. The most prevalent symptoms in adults are diarrhea, which may leads to death presumably from electrolyte imbalance (Lodder *et al.*, 2000; Wyn-Jones *et al.*, 2000). Attempts to culture SRSVs in vitro have been unsuccessful to date and no animal model of infection and disease exists. Laboratory diagnosis relies largely upon electron microscopy (EM) and reverse transcriptase (RT) PCR (Alison *et al.*, 1999, Wolfaardt *et al.*, 1995). However, organic and inorganic RT-PCR inhibitors in the environmental water samples limit the application of this method (Tsai and Olson, 1992). Removal of inhibitory compounds can be achieved by different methods such as antigen capture with specific antibodies bound to a solid phase (Grinde *et al.*, 1995; Schwab *et al.*, 1996). Some laboratories have developed enzyme-linked immunosorbent assay, but they are strain specific (Gary *et al.*, 1985; Hale *et al.*, 1996; Madore *et al.*, 1986). The use of plant extracts for virus replication inhibition is a worldwide attitude for drug discovery programs. On the other side, some plant extracts were found to increase virus titer in cell cultures. The aim of this study is to use a certain plant water extract that was found to enhance poliovirus (type 2) replication, for propagation of SRSVs, which isolated from wastewater. Also to determine the most frequent genogroup in our wastewater.

Material and Methods

Wastewater samples

Wastewater samples were collected as 5 L from the intake and the treated effluent of three wastewater treatment plants (Zeinen, El-Berka and Mostorod) in Great Cairo. The survey was for one year from November 1999 to October 2000.

The collected raw wastewater samples were left for two hours to separate the sediments, and the effluents of both raw and outlet were adjusted to pH 3.5 and filtered through nitrocellulose membrane of 0.45 µm pore size, 142 mm diameter for virus concentration (APHA, 1995). The adsorbed viruses were eluted from the membrane in 1.5% beef extract, pH 9.5 and reconcentrated by precipitation of the viruses by 7.5% polyethylene glycol 6000-2.5% NaCl as described by Vilagines *et al.* (1997).

The concentrated samples were extracted twice with freon and 50 µl of antibiotic-antimycotic mixture (10,000 U penicillin G sodium, 10,000 µg streptomycin sulfate and 25 µg amphotericin B, GIBCO BRL) were added per each 5 ml concentrated sample.

Cell Cultures

Three types of cell cultures were used for cultivation of SRSVs, these were: I) African green monkey kidney cells (Vero), ii) African green monkey kidney-derived cells (BS-C-1), and iii) Human hepatoma cells (Huh-7). All cell cultures were kindly provided by Prof. Verena Gauss-Muller at Medical Institute for Molecular Biology, The medical University of Luebeck, Germany. The cells were grown and maintained in Dulbecco's minimum essential medium (DMEM) with Glutamax II (GIBCO-BRL), supplemented with 10% foetal bovine serum (FBS) for growth medium and 2% FBS for maintenance medium, and 1% antibiotic-antimycotic mixture (GIBCO-BRL). The concentrated wastewater samples were inoculated into the previous cells for three successive passages of cell harvests at 4 days each, and then cells were frozen and thawed three times to release viruses followed by low speed centrifugation to remove cell debris. Viruses were concentrated and purified by freon extraction, followed by PEG 6000 precipitation as described by Vilagines et al. (1997).

Preparation of Plant extract

Aerva Javanica plant (Burm.F.) Juss.Ex Schult, was collected in May 20,1999 from south of Sinai, Egypt . The plant was air dried in shade using a solar oven at temperature not exceeding over 40^{o}C,to avoid degradation of the natural ingredients. The dried plant was then powdered to sieve No.36 to be ready for the extraction step. The powdered plant was exhaustively extracted with successive portions of water for 5 hrs then filtered. Aqueous extract was combined and water was removed by freeze-drying. The lyophilized extract (100 mg) was dissolved in 10 ml sterile deionized water.

Cytotoxicity assay

Cytotoxicity experiments for the plant extract at two fold dilutions from 5µg to 320µg, were carried out on Vero, BS-C-1 and Hugh 7 cells. Non-radioactive cell proliferation system (Promega) was used and processed as described by the manufacturer. The color reaction was read at 570 nm wavelength with reference

650 nm wave length. The non-cytotoxic concentration (40µg) was selected for the virus replication enhancement assay.

Virus replication enhancement

To improve virus replication in the different cell cultures used, the cells were treated with 40 µg of water extract of the *Aerva Javanica* (Burm.F.) Juss.Ex Schult, which was found as the high concentration non-cytotoxic for all cell cultures used. After the treatment, cell cultures were incubated at 37°C for 2hrs followed by inoculation of the concentrated wastewater samples and then 3 successive passages of cell harvest each for 4 days and viruses were harvested. Untreated cells were also inoculated by the same samples as a control.

Oligonucleotide primers

The primers used for PCR amplification were Ni-sense (5'- GAA TTC CAT CGC CCA CTG GCT-3') and E3-antisense (5'-ATC TCA TCA TCA CCA TA-3') (Green *et al.*, 1995), which amplify a 113 bp product for the detection of both genogroup I and II SRSV. For differentiation between genogroup II, and I two primers were used; GI (5'-TCN GAA ATG GAT GTT GG-3') and GII (5'-AGG CNT NGA AAT NAT GGT GGT –3') (Green *et al.*, 1998); where E3, amplify 190 and 270-bp products of the RNA polymerase gene of genogroup I and genogroup II viruses, respectively as shown in Table (1).

TABLE 1. Primer locations of small round structured-viruses.

Primer	Orientation	Genogroup	Genomic location*
Ni	Sense	I	4756-4776
		II	4492-4512
E3	antisense	I	4869-4853
		II	4605-4588
GI	Sense	I	4679-4696
GII	Sense	II	4338-4355

• Genomic locations for genogroup I and II strains refere to Norwalk virus and Lordsdale virus nucleotide sequences, respectively (Green, *et al.*, 1998).

RNA extraction, cDNA synthesis and PCR

Concentrated wastewater samples and the third cell culture passages of both treated and untreated cells were tested for the presence of SRSVs by RT-PCR with both Ni-E3 general primers and genogroup primers GI and GII as follow:
To 5µl sample, 4µl 5X RT buffer (250 mM Tris-HCl,pH 8.3, 375 mM KCl, 40mM MgCl2, 50 mM DTT), was added and covered with 50 µl mineral oil. The mixture was heated at 99°C for 5 min to release viral RNA by heat shock then chilled onice and 1µl (40U) Rnase inhibitor (Promega) was added. Reaction mixture was prepared by mixing 2µl of 10 mM each dNTP,1µl (0.25µmol/l)

antisense primer E3 and 0.5 µl (10U) of reverse transcriptase from Rous-associated virus 2 (RAV-2, TaKaRa Biomedicals, Japan), 4 µl of 5X RT buffer and 7µl DEPC-treated water. The mixture was incubated at 37°C for 1 hr followed by 99°C for 5min, then chilled on ice. The produced cDNA was used for PCR reaction by mixing the following reagents to a final volume of 80 µl: 2 µl of 10 mM dNTPs, 2 µl of each sense (Ni) and antisense (E3) primer, 8µl of 10x PCR buffer containing 2mM MgCl2 and 1µl (5U) TaKaRa Ex Taq DNA polymerase, 47µl DEPC-treated water. The PCR mixture was subjected to the following temperature condition, 94°C, 50°C and 72°C for 1 min for 40 cycles followed by an extention cycle at 72°C for 10 min. The PCR product was detected in 2% agarose gel containing 0.5µg ethidium bromide and visualized under UV light comparing to the 100 bp DNA ladder (Promega). The same procedure was applied with both genogroup primers (GI and GII) against antisense primer E3 for positive RT-PCR samples in all cell culture harvests.

Results

RT-PCR of wastewater samples

SRSV-specific amplification segments were detected in 11 out of 72 samples (15.27%) as shown in fig (1-A). Although, Zeinin and El-Berka WWTPs used a secondary treatment and sometimes a disinfection step by chlorine, SRSVs were detected in both raw wastewater and treated effluent as shown in Table (2).

TABLE 2. Effect of the treatment of Vero, BS-C-1 and Huh-7 cell lines with water extract of *Aerva Javanica* (Burm.F.) Juss.Ex Schult, on SRSV propagation enhancement.

Wastewater treatment plant	No. of positive RT-PCR						
	Concentrated wastewater	Untreated cells			Treated cells		
		Vero	BS-C-1	Huh-7	Vero	BS-C-1	Huh-7
Zeinen Raw	3/12	0	0	1	1	3	3
Treated effluent	2/12	0	0	0	2	2	2
El-Berka Raw	2/12	0	0	0	1	3	3
Treated effluent	2/12	0	0	0	1	2	2
Balaks Raw	1/12	0	0	0	1	1	1
Treated effluent	1/12	0	0	0	1	1	1

RT-PCR for detection of virus propagation enhancement

The results revealed that the plant water extract was highly effective at a concentration of 40 µg/2X10^{5}cells. The three cell culture types were differed in their efficiency for SRSV propagation. Where BS-C-1 and Huh-7 cells became more susceptible after plant extract pretreatment than Vero cells. SRSVs were detected in 11 of concentrated wastewater samples but, the untreated cell culture harvests of Vero, BS-C-1 and Huh-7 cells were negative by RT-PCR except one sample in Huh-7 cells. In contrast, the treated cell cultures were much susceptible for SRSV especially BS-C-1 and Huh-7 cells where 12 samples were positive by RT-PCR, one of them was negative in concentrated wastewater sample. The treated Vero cells were less susceptible than the other two cell lines where only 7 samples out of 12 were positive by RT-PCR (Table-2 and Fig.1). This meant that some sort of propagation has been achieved which amplified the low titer of SRSV in the water samples that was beyond RT-PCR detection limit.

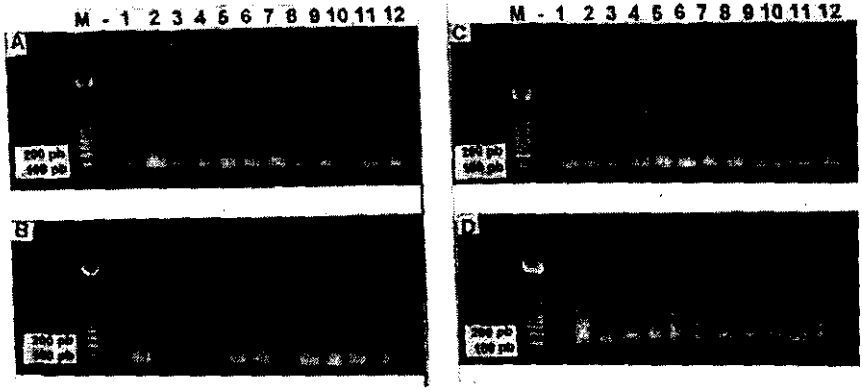

Fig. 1. RT-PCR amplification products of SRSVs with Ni-E3 primers for A) concentrated wastewater samples, B) for experimentally infected cell culture (Vero) harvest, C) for BS-C-1 cells and D) for Huh-7 cells. Samples showed a 113 bp bands against 100 bp DNA ladder (Promega). Lane (-) is the negative control.

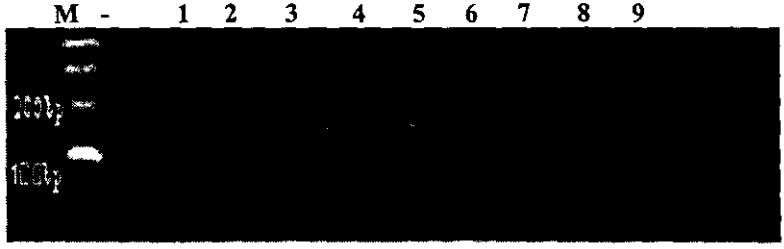

Fig. 2. RT-PCR amplification products with GI-E3 primers of experimentally infected BS-C-1 cell harvests, which showed positive response with Ni-E3 primers by RT-PCR. Lane (M) is the 100pb DNA ladder (Promega) and all samples showed 190 pb bands except sample no 10 and 12 which were negative. Lane (-) is the negative control.

SRSV Genogroup Differentiation

The experimentally infected BS-C-1 cell culture harvests of the wastewater samples that were found to be positive for SRSV by RT-PCR with the broadly reactive NI-E3 primers were genotyped by RT-PCR using GI and GII primers. The results revealed that all the samples were of genogroup I and two samples were negative as shown in Fig.2.

Discussion

Raw wastewater is still the very accurate monitor for enteric viruses shedding and virological examination of it may reflect to some extend the public health of a certain population. In the present study SRSVs were detected in 16.6% of the collected raw wastewater samples, which justifies the treatment of wastewater as an urgent need to prevent the distribution of these viruses, which infect all age groups and are frequently associated with gastroenteritis. Many studies dealt with the characterization of SRSVs by nucleotide sequencing of the viral RNA and different strains have been evolved (Ando *et al.*, 1994; Dingle *et al.*, 1995; Green *et al.*, 1998). In the study by Alison et al.(1999), 98% of the SRSV strains detected in fecal speciemens by RT-PCR were of genogroup II and had greater than 96% identity with Grimsby virus. In our study almost all SRSV detected were of genogroup I. The choice of diagnostic procedures for SRSV detection have been limited to EM which was widely used. Also, enzyme linked immunosorbent assays were used but most of them are strain specific (Gary *et al.*,1985; Hale *et al.*,1996; Madore *et al.*,1986) and RT-PCR (Alison *et al.*,1999; Kojima, 2001; Otsu *et al.*, 2000). The sensitivity of SRSV detection has been increased much since Green *et al.*(1998) have introduced the nested RT-PCR. The introduction of a material which may enhance virus propagation will facilitate the characterization studies and increase the possibility of virus isolation in cell cultures. Our work is continued to fractionate the plant extract to separate the active material which will be evaluated and the mechanism of virus propagation enhancement will be studied.

The main conclusion of our study is that SRSVs are representing a health hazard factor and the treatment of water and wastewater is necessary for elimination of waterborne viral enteritis.

Acknowledgment: The authors thanks Dr. El-Menshawi B., Professor of Pharmacognosy, NRC, the principal investigator for the project of Drug Discovery for his help. We also thank all those who helped us to make this study possible.

References

Alison, J.M., Jon, G., David, W.G.B. Ulrich, D. and James, J.G. (1999) Molecular Epidemiology of outbreaks of gastroenteritis associated with small round-structured viruses in east Anglia,United Kingdom, during the 1996-1997 season. *J.Clin. Microbiol.*, **37**(1), 81.

Ando, T., Mulders, M.N., Lewis, D.C., Estes, M.K., Monroe, S.S. and Glass, R.I. (1994) Comparison of the polymerase region of small round structured virus strains previously classified in three antigenic types by solid-phase immune electron microscopy. *Arch Virol*; **135**, 217.

APHA (1995) *Standard Methods for Examination of Water and Astewater.* 19th ed. American Public Health Association, Washington, DC.

Appleton, H. (2000) Control of food-borne viruses.*Br.Med.Bull.* **56**(1), 172.

Berke, T., Golding, B. and Jiang, X. et al. (1997) Phylogenetic analysis of the caliciviruses. *J. Med Virol;* **52**, 419.

Chadwick, P.R. and McCann, R. (1994) Transmission of a small round structured virus by vomiting during a hospital outbreak of gastroenteritis. *J. Hosp. Infect.* **26**, 251.

Dingle, K.E., Lambden, P.R., Caul, E.O. and Clarke, I.N. (1995) Human enteric calcivirus: the complete genome sequence and expression of virus-like particles from a genetic group II small round-structured virus. *J.Gen.Virol.* **76**, 2349.

Gary, G.W., Jr., Kaplan, J.E., Stine, S.E. and Anderson, L.J. (1985) Detection of Norwalk virus antibodies and antigen with a biotin-avidin immunoassay. *J. Clin. Microbiol.* **22**, 274.

Green, K.Y., Ando, T., Balayan, MS., et al. (2000) Taxonomy of the caliciviruses. *J. Infect Dis*; 181, S322.

Green, J., Gallimore, C.I., Norcott, J.P., Lewis, D. and Brown, D.W.G. (1995) Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. *J. Med. Virol.* **47**, 392.

Green, J., Henshilwood, K. Gallimore, C.I. Brown, D.W.G. and Lees, D.N. (1998). A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish. *Appl. Environ. Microbiol.* **64**, 858.

Green, S.M., Dingle, K.E., Lambden, P.R., Caµl, E.O., Ashley, C.R. and Clarke, I.N. (1994) Human enteric *Caliciviridae*: a new prevalent SRSV group defined by RNA-dependent RNA polymerase and capsid diversity. *J. Gen. Virol.* **75**, 1883.

Hale, A.D., Lewis, D. Green, J. Jiang, X. and Brown, D.W.G. (1996) Evaluation of an antigen capture ELISA based on recombinant Mexico virus capsid protein. *Clin. Diagn. Virol.* **5**, 27.

Jiang, X., Graham, D.Y., Wang, K. and Estes, M.K. (1990) Norwalk virus genome cloning and characterization. *Science* **250,** 1580.

Jiang, X., Turf, E., Hu, J., Barrett, E., Dai, X.M., Monroe, S., Humphrey, C., Pickering, L.K. and Matson, D.O. (1996) Outbreaks of gastroenteritis in elderly nursing homes and retirement facilities associated with human caliciviruses. *J. Med. Virol.* **50,** 335.

Jiang, X.I., Wang, M. Wang, K. and Estes, M.K. (1993) Sequence and genomic organization of Norwalk virus. *Virology.* **195,** 51.

Kaplan, J.E., Goodman, R.A., Schonberger, L.B., Lippy, E.C. and Gary, G.W. (1982) Gastroenteritis due to Norwalk virus: an outbreak associated with municipal water system. *J. Infect. Dis.* **146,** 190.

Kappus, K.D., Marks, J.S., Holman, R.C., Bryant, J.K., Baker, C., Gary, G.W. and Greenberg, H.B. (1982) An outbreak of Norwalk gastroenteritis associated with swimming in a pool and secondary person-to-person transmission. *Am. J. Epidemiol.* **116,** 834.

Kilgore, P.E., Belay, E.D., Hamlin, D.M., Noel, J.S., Humphrey, C.D., Gary Jr, H.E., Ando, T., Monroe, S.S., Kludt, P.E., Rosenthal, D.S., Freeman, J. and Glass, R.I. (1996) A university outbreak of gastroenteritis due to a small round-structured virus. *J. Infect. Dis.* **173,** 787.

Kobayashi, S., Morishita, T., Yamashita, T., Sakae, K., Nishio, O., Miyake, T., Ishihara, Y. and Isomura, S. (1991) A large outbreak of gastroenteritis associated with a small round structured virus among schoolchildren and teachers in Japan. *Epidemiol. Infect.* **107,** 81.

Kojima, T. (2001) Studies of acute gastroenteritis among infant and young children in a pediatric clinic. *Kansenshogaku Zasshi,* **75**(4), 333.

Lawson, H.W., Brayn, M.M., Glass, R.I.M., Stine, S.W., Monroe, S.S., Atrah, H.K., Lee, L.E. and Englander, S.J. (1991) Waterborne outbreak of Norwalk virus gastroenteritis at a s outhwest US resort: role of geological formations in contamination of well water. *Lancet* **337,** 1200.

Lodder, W.J., Vinje, J., Mulders, M.N., Leenen, E.J.T.M., Medema, G.J. and Koopmans, M.P.G. (2000) Molecular methods for the detection of small round structured viruses in water. *OECD worhshop Molecular Methods for Safe Drinking Water.*

Madore, H.P., Treanor, J.J., Pray, K.A. and Dolin, R. (1986) Enzyme-linked immunosorbent assays for Snow Mountain and Norwalk agents of viral gastroenteritis. *J. Clin. Microbiol.* **24,** 456.

Noel, J.S., Liu B.L., Humphrey C.D., et al. (1997) Parkville virus: A novel genetic variant of human calicivirus in the Sapporo virus clade, associated with an outbreak of gastroenteritis in adults. *J. Med. Virol*; **52,** 173.

Otsu, R., Ishikawa, A., Mukae, K. (2000) Dtection of small round structured viruses in stool specimens from outbreaks of gastroenteritis by electron microscopy and reverse transcription- polymerase chain reaction. *Acta Virol.* **44**(1), 53.

Pickering, L.K., DuPont, H.L., Blacklow, N.R. and Cukor, G. (1982) Diarrhea due to Norwalk virus in families. *J. Infect. Dis.* **146,** 116.

Vilagines, P.h., Suarez, A., Sarrette, B. and Vilagines, R. (1997) Optimization of the PEG reconcentration procedure for virus detection by cell culture or genomic amplification. *Wat.Sci.Tech.* **34** (11-12), 455.

Wang, X., Jiang, X., Madore, H.P., Gray, J., Desselberger, U., Ando, T., Seto, Y., Oishi, I., Lew, J.F., Green, K.Y. and Estes, M.K. (1994) Sequence diversity of small, round-structured viruses in the Norwalk virus group. *J. Virol.* **68,** 5982.

Wilson, R., Anderson, L.J., Holman, R.C., Gary, G.W. and Greenberg, H.B. (1982) Waterborne gastroenteritis due to the Norwalk agent: clinical epidemiological investigation. *Am. J. Public Health* 72, 72.

Wolfaardt, W., Moe, C.L. and Grabow, O.K. (1995) Detection of small round structured viruses in clinical and environmental samples by polymerase chain reaction. *Wat.Sci.Tech.* **31**(5-6), 375.

Wyn-Jones, Pallin, A.P., Dedoussis, R., Shore, C., Sell, J., wood, J. (2000) The detection of small round-structured viruses in water and environmental materials. *J.Virol.Methods.* **87**(1-2), 99.

(*Received* 3 / 11 / 2002;
accepted 25 / 5 / 2003)

إكثار الفيروسات الصغيرة التركيب على مزارع الأنسجة والتعرف عليها بإستخدام التفاعل النووى المتسلسل العكسى فى مياه الصرف الصحى.

محمد احمد احمد على و محمد الجندى

قسم بحوث تلوث المياه و قسم الصناعات الدوائيه – المركز القومى للبحوث – القاهرة – مصر.

يعتبر تلوث مياه الشرب والأغذيه بالفيروسات صغيرة التركيب من الأسباب الرئيسيه لإنتشار الاوبئه الخاصة بهذا الفيروسات. لذا فإن مياه الصرف الصحى هى المؤشر الرئيسى لتواجد هذه الفيروسات بين الناس فى المجتمعات .

فى هذه الدراسه تم جمع ٧٢ عينة صرف صحى من مداخل ومخارج ثلاثه محطات صرف بالقاهره الكبرى وتم تركيز و إستخلاص الفيروسات الموجوده بالترشيح خلال أغشية النيتروسيليلوز. كما تم التخلص من السميه بهذه العينات المركزة وكذا الحمل الميكروبى بها واعيد تركيز العينات بإستخدام طريقة البولى إيثيلين جليكول ٦٠٠٠وحققت العينات المركزه فى ثلاثة أنواع من مزارع الأنسجه . ولكى نحسن من طريقة عزل الفيروسات وأكثارها تم معالجة الخلايا لمدة ساعتين بمستخلص نبات *Aerua jauanica*. وتكرر حقن العينات المركزه فى الخلايا المعالجه لثلاثة مرات متعاقبه وجمعت الفيروسات المعزوله وأجرى عليها إختبار التفاعل النووى المتسلسل العكسى لتحديد تواجد الفيروسات صغيرة التركيب بها.

وقد وجد أن ١٢ عينه أظهرت نتائج إيجابيه أى بها الفيروسات صغيرة الحجم فى نوعين من الخلايا المعالجه ولكن فى النوع الثالث كانت العينات الإيجابيه ٧ عينه.

وتم التعرف على المجموعه الجينيه التى ينتمى اليها الفيروسات المعزوله بإستخدام التفاعل النووى المتسلسل وقد وجد أن ١٠ عينات من المجموعه الأولى، ٢ عينه من المجموعه الثانيه.

ويعتبر إكثار الفيروسات صغيرة التركيب من أهم النجاحات فى هذا العمل حيث أن إكثارها على مزارع الأنسجه من الأمور الصعبة جدا.