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DETECTION OF ROTA VIRUS IN SOME MOLLUSCS IN ALEXANDRIA GOVERNORATE

(With 6 Tables and One Figure)

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الكشف عن وجود فيروس الروتا في بعض الرخويات فى محافظة الاسكندرية

بهجت زكريا يوسف ، يوسف ثابت عبد الشهيد

تم البحث عن مدى تواجد فيروس الروتا (مجموعةA) فى ١٧٥ عينة من الرخويات ذات الصدفتين وتشمل ١٠٢ عينة من أم الخلول و ٧٥ عينة من الجندفلى تم جمعها من ثلاث أسواق للسمك فى محافظة الإسكندرية بمصر فى عام ٢٠٠٨ م. تتكون كل عينة من ١٥ الى ٢٥ محارة وتم الفحص باستخدام أختبارى الإليزا واللاتكس وكذلك تم فحص عدد ٥٠ عينة بأستخدام أختبار تفاعل البوليميريز وتم كذلك معاينة الظروف الصحية لهذة الأسواق. وقد أثمرت هذة الدراسة عن تواجد أنتجن فيروس الروتا فى ٢٠ (١٩،٦١%) ، ١٥ (١٤،٧١%) وفى ١٣(١٧،٨١%) ، ١٠ (١٣،٧%) من عينات أم الخلول والجندفلى بأسخدام أختبارى الإليزا واللاتكس على التوالى وبدون أى إختلافات إحصائية بين الأسواق المختلفة. تم كذلك مقارنة نتائج أختبار تفاعل البوليميريز ونتائج كل من أختبارى الإليزا واللاتكس فى ٥٠ عينة مختارة من الرخويات والتي سبق فحصها (٣٣ عينة إيجابية بالإليزا و١٧ عينة سلبية بطريقتي الإليزا واللاتكس) وأسفرت هذة المقارنة عن أن النسب المئويه لكلا من الحساسية والتخصص والقيم الإيجابية والسلبية لأختبار الإليزا كانت ٨٦،١١ و ٨٥،٧١ و ٩٣،٩٢ و٧٠،٥٩ بينما كانت ٦٦،٤٤ و ١٠٠ و١٠٠ و ٥٦ لأختبار اللاتكس على التوالى. ونستنتج من هذه الدراسة عن وجود نسبة عالية من فيروس الروتا فى أم الخلول والجندفلى مما قد يتسبب فى مخاطر صحية لمستهلكى هذه الرخويات بحالتها الطبيعية ونوصي باستخدام أختبارى الإليزا أو اللاتكس فى الكشف عن تواجد فيروس الروتا فى المحاريات نظرا لنتائجهم القريبة الاحصائيات لطريقة تفاعل البوليميريز ذات التكلفة المادية العالية. وقد تمت مناقشة وإقتراح الإحتياطات الصحية اللازمة لحماية المستهلكين من مخاطر هذا الفيروس عند تناول هذه الرخويات.

SUMMARY

Rota viral antigen (Group A) was investigated in 175 molluscan shellfish pools including 102 Om-Elkohloul "*Donax tranculus*" and 73

Gandofelly "*Tapes decussates*" from three fish markets along Alexandria coast, Egypt, during 2008. Each pool was examined using ELISA and latex agglutination techniques. Fifty selected samples were examined using RT-PCR; also the hygienic conditions of fish markets environment were personally monitored. Rota viral antigen could be detected in 20 (19.61%) & 15 (14.71%) and in 13 (17.81%) & 10 (13.7%) of examined Om-Elkohloul and Gandofelly samples using ELISA & LA techniques respectively. No significance difference between different fish markets in detecting Rota viral antigen. The comparison between the results of RT-PCR and each of ELISA and Latex techniques for detection of Rota viral antigen was studied in 50 selected molluscs' samples (33 positives by ELISA and 17 negative by both ELISA and LA tests). Accordingly, the sensitivity, specificity, positive and negative predicative values for ELISA test were 86.11%, 85.71%, 93,94% and 70.59%, while it was 69.44%, 100.0%, 100.0% and 56.0% for LA test, respectively. In conclusion, this study revealed high detection rate of Rotavirus in molluscan shellfish, which may be one of the most important causative agent of gastroenteritis associated with the consumption of raw molluscs. The good sensitivity, specificity between the results of RT-PCR and each of ELISA and LA indicate that the latter two techniques can be used as reliable tools for detection of Rotavirus in shellfish. The corrective actions and hygienic precautions needed to protect consumers against possible hazard of Rotavirus through consumption of these products were discussed and clarified to be employed.

Key words: *Virology, Rota virus, molluscs*

INTRODUCTION

Despite major advances and improvements in food and water quality, diagnostic methods, and surveillance systems; food-borne diseases remain a global public health problem (Costantini *et al.,* 2006). Enteric viruses contaminating the environment represent a danger hazard for public health notably that are excreted in stools and can contaminate water and shellfish (Sdiri *et al.,* 2006). The main enteroviruses that cause gastroenteritis are Norovirus, Rotavirus, Sapovirus, Astrovirus, and enteric Adenoviruses (Hansman *et al.,* 2008). Rotavirus is the leading etiologic agent of severe diarrheal disease in infants and young children world wide (Kapikian and Chanock 1990, Patton 1995, Man *et al.,* 2005). Globally, it is the cause of about 600,000 diarrheal deaths every year, in addition to 125 millions cases of Rotavirus diarrhea have been

estimated to occur annually in children under the age of 5 years most of them in developing countries (Midthun and Kapikian 1996). In developed countries, Rotavirus has been detected in 35 to 52% of infants and young children hospitalized with acute diarrhea (Konno *et al.*, 1983).

Rotavirus infection is spread primarily by feco-oral route. Although it is a relatively acid labile, it can survive the pH of a stomach after a meal while it inactivate rapidly at fasting stomach of pH 2.0 (Weiss and Clark, 1985). However at pH 3.0, inactivation of the Rotavirus is much slower where the infant gastric pH tends to be approximately 3.2, this probably explain the efficient transmission of Rotavirus in infants (Christensen, 1989). After ingestion the Rotavirus particles are carried to the small intestine where they infect enterocytes leading to change in their structure from columnar to cuboidal. The severity of these changes is correlated with the severity of the resulting illness (Lundgren and Svensson, 2001). The incubation period for Rotavirus disease is approximately 2 days. The disease is characterized by vomiting, watery diarrhea for 3 - 8 days, fever and frequently abdominal pain. Immunity after infection is incomplete, but repeat infections tend to be less severe than the first attack (CDCP, 2007).

Rotavirus is a member of the family Reoviridae (Mathews, 1979), non-enveloped, double stranded RNA, icosahedra structure and 70 nm in diameter (Desselberger, 1996). There are 7 known subgroups of Rotavirus lettered A through G. Groups A, B and C have been known to infect human and 95% are caused by group A (Kostouros *et al.*, 2003).

Diseases caused by the consumption of bivalve molluscs shellfish containing pathogenic viruses of human origin is a well known phenomenon, particularly in connection with raw oysters (Lees, 2000). Molluscs are filter feeders that can concentrate more than fourfold in their tissue particles present in the surrounding water, i.e., the filtering of large water volumes, when water is contaminated with human feces, viral pathogens may get trapped in the shellfish (Potasman *et al.*, 2002, Butt *et al.*, 2004). Therefore, the objective of this study is; Detection of Rota viral antigen in Om-Elkhlol and Gandofelly sold in regional fish markets along Alexandria coast, which were destined for human consumption, using ELISA, Latex (LA) and RT-PCR techniques. RT-PCR technique is used as gold standard method for confirmation of the results. The corrective actions and hygienic precautions needed to protect consumers against possible hazard of Rotavirus through

consumption of these molluscs were discussed and clarified to be employed.

MATERIALS and METHODS

Molluscs sampling: A total of one hundred and seventy five molluscs' pools samples comprising 102 Om-Elkholoul "*Donax tranculus*" and 73 Gandofelly "*Tapes decussates*" were collected from three fish markets along Alexandria coast, Egypt, during 2008. Forty two, 22 and 38 of Om Elkhlol and 33, 17 and 23 Gandofelly samples were collected from Shedia, Bakous and Rateb fish markets, respectively. Each pool sample was composed of 15-25 oysters. The samples were kept on ice during shipment and processed immediately after arriving to the laboratory.

Molluscs Processing: The processing was done according to Costantini *et al.* (2006) as following: shellfish samples were rinsed in water prior to opening. Oysters were shucked with a sterile knife, and the oyster tissues were removed and dissected with sterile scissors and forceps then homogenized with 3.0 ml. phosphate buffer saline (pH 7.4) in sterile homogenizer. The homogenate were then subdivided into aliquots and frozen at -20° C. until used.

Virus elution and concentration for ELISA & LA TESTS: Virus elution and concentration for detection of Rota viral antigen from shellfish was done according to Atmar *et al.* (1995); the homogenate was transferred to a centrifuge tube and the choloroform-butanol was added to remove tissues.

Virus elution and concentration for RT-PCR technique: The shellfish homogenate was kept frozen in deep freeze at -20°C after addition of RNA late™ (Ambion cat. no. # 7620) as a preservative and were subjected to RT-PCR technique for detection of Rotavirus according to Gouvea *et al.* (1990).

Laboratory Techniques

1- ELISA technique: RIDASCREEN® Rotavirus (C 0901) ELISA kits, was supplied by R-Biopharm AG, Germany. In this test monoclonal antibodies against a capsid protein of gene 6 (VP6) of the Rotaviruses are applied to the surface of the well in the micro-well plate. One hundred µl of each positive, negative controls and Shellfish extract and were pipetted into each corresponding well. Then 100 µl of the enzyme conjugated antibody (Peroxidase conjugated monoclonal antibody prepared in mouse against Rotavirus in stabilized protein solution; contains 0.1% Kathon) was added and incubated 60 min. at room

temperature. After washing, 100 µl of substrate solution (Urea peroxide/TMB) was added to each well and the plates were incubated at room temperature for 15 min. in the dark. The stopping solution (1N sulphuric acid) was added and the O.D of each well was measured at 450 nm. Cut-Off value was calculated as follow:

Cut-Off value = O.D. of negative control + 0.15.

The sample was considered positive if their O.D. was greater than 10% above the calculated Cut-Off value, and considered negative if O.D. was more than 10% below the calculated Cut-Off value. The equivocal values between positive and negative should be repeated.

2- Rapid latex agglutination test (LA): Latex test was carried out according to Haikala *et al.* (1983) and Hughes *et al.* (1984) using VIROTECT® ROTA diagnostics Kit (Omega House, United Kingdom). The latex particles are coated with rabbit antibodies prepared against Rotavirus group A. Shellfish extract when added to test latex reagent in the presence of Rotavirus group A antigen agglutinates the latex particles.

3- RT-PCR technique: RT-PCR assay and primers used in this study was done according to Gouvea *et al.* (1990) using Omni-script Reverse Transcriptase kit (Qiagen).

Primers used

Prime	Sequence (5' -3')	Position	Strain serotype
Beg 9	GGTTTTAAAAGAGAATGGTTTCCTGG	1-28	Wa (1)
End 9	GGTCACATCATACAATTCTAATCTAAG	1062-1036	SA11 (3)

RNA Extraction: RNA extraction was done according to Ibrahim *et al.* (1997) and as follow: One hundred µl of shellfish-RNA late™ were processed for RNA extraction by Trizol method (Gibco-BRL) which is a monophasic solution of phenol and guandine isothiocynate. The outline method for RNA isolation by Trizol reagent as follow:

Homogenate shellfish -RNA- late™ in Trizol reagent (50 min.→ Separate phases (add chloroform-5 min.) → Precipitate RNA → wash and solubilize.

(The elapsed time less than one hour)

The purified RNA was subjected immediately to reverse transcriptase process.

Reverse transcription: The (ds RNA) of Rotavirus was first denatured by heating the mixture first at 97°C for 5 minutes in the thermocycler

(Beco). Then the tube was transferred quickly to an ice bath to prevent reannealing of the ds RNA, then the reverse transcriptase was added and the tube was transferred back to the thermocycler for incubation at 37°C/60 min. followed by 94°C/5 min. The complementary DNA copies (cDNA) from Rotavirus RNA strands were synthesized in 20 µl solution (8 µl RNase-free water, 2 µl of 10x buffer RT, 2 µl of d NTPs deoxynucleotide triphosphate, 2 µl of both primers, 1 µl of 4 IU Omni-reverse transcriptase enzyme and 5 µl of the RNA samples

RT-PCR amplification: The component of the mixture used was; 1 µM of each primer, 10 µM of each deoxynucleotide triphosphate (d ATP, d GTP, d TTP, d CTP), 10 µM tris Hcl, 50 µM KCl, 1.5 µM MgCl2, 2.5 units of Tag DNA polymerase (Qiagen), and 10 µl of cDNA.

The full length of the gene segment 9 (1,062), encoding the VP7 glycoprotein in human group A Rotavirus, was amplified using primers Beg 9 in the forward direction and primer End 9 in the reverse direction. Amplification was performed in a final volume of 50 µl of PCR mixture and the reaction was applied as follow: denaturation at 94°C/1 min., annealing at 42°C/2 min. and extension at 72°C/1 min. (25 cycles), followed by a final extension at 72°C. Then 10 µl of the PCR products were analyzed by electrophoresis on 1.5% agarose gel containing 0.5ug/ml of ethidiun bromide and the DNA was visualized by U.V. light. Positive results showed lane at 1062 base pair as shown in Figure (1).

Personal monitoring of Fish markets: hygienic environment of fish markets, molluscs sellers practices, sewage drainage, Water Cycles (WCs) and seller locations were noticed and evaluated during collection of molluscs samples.

RESULTS

Tables (1&2) showed the detection rate of Rota viral antigen in Om-Elkohloul "*Donax tranculus*" and Gandofelly "*Tapes decussates*" molluscs at some different fish markets of Alexandria governorate using ELISA and Latex techniques. A total of 20 (19.61%) and 15 (14.71%) out of 102 examined Om-Elkholoul pools were positive using ELISA and LA tests, respectively. The detection rates in Om-Elkhlol samples were 19.05%, 22.73% and 18.42% in Shedia, Bakous and Rateb fish markets, respectively, using ELISA technique. Also, a total of 13 (17.81%) and 10 (13.7%) out of 73 tested Gandofelly pools were positive using ELISA and LA techniques, respectively with detection

rates of 15.15%, 17.65% and 21.74% in Shedia, Bakous and Rateb fish markets, respectively, using ELISA technique.

Tables (3&4) represent the percent of agreement, sensitivity, specificity, positive and negative predictive values of LA technique, regarding ELISA. There were 95.43, 100, 94.67, 75.76 and 100, respectively, for the detection of Rotavirus in molluscs' pools.

Tables (5&6) show the comparison between the results of RT-PCR and each of ELISA and Latex techniques for detection of Rotavirus in 50 selected molluscs' pools (33 positive by ELISA and 17 pools negative by both ELISA and LA techniques). Out of 36 RT-PCR positive molluscs pools, 31 (86.11%) were positive by ELISA and 2 (14.29%) out of 14 RT-PCR negatives were positive by ELISA, while 5 pools out of 17 negative by ELISA were positive by RT-PCR. On the other hand, only 25 (69.44%) out of 36 RT-PCR positives were positives by latex test, whereas all negatives RT-PCR were negatives by latex test. Regarding RT-PCR, the sensitivity, specificity, positive and negative predicative values were 86.11%, 85.71%, 93.94% and 70.59% for ELISA test, while it was 69.44%, 100.0%, 100.0% and 56.0% for latex test respectively.

Figure (1): shows Gel electrophoresis for PCR products.

Personal monitoring of different fish markets showed that: poor hygienic environment and very poor condition of WCs without enough water supplies. Unclean and low quality of packaging materials in addition to imperfect cleaning of markets streets and improper periodical removal of waste. Molluscs' sellers had no enough personal hygienic knowledge.

Table 1: Detection rate of Rotavirus in OM-Elkholoul molluscs at different fish markets of Alexandria, Egypt, using ELISA and Latex techniques

Fish markets	No. of tested samples	ELISA		Latex test	
		No. of +ve samples	percent	No. of +ve samples	percent
Shedia*	42	8	19.05	7	16.67
Bakous	22	5	22.73	3	13.64
Rateb	38	7	18.42	5	13.16
Total	102	20	19.61	15	14.71

X^2 =0.17 (No significance between Shedia and Bakous markets; P =0.98). X^2 = 0.07 (No significance between Shedia and Rateb markets; P = 0.79)

Table 2: Detection rate of Rotavirus in Gandofelly molluscs at different fish markets of Alexandria, Egypt, using ELISA and Latex techniques.

Fish markets	No. of tested samples	ELISA		Latex test	
		No. of +ve samples	percent	No. of +ve samples	Percent
Shedia*	33	5	15.15	4	12.12
Bakous	17	3	17.65	3	17.65
Rateb	23	5	21.74	3	13.04
Total	73	13	17.81	10	13.70

X^2 =0.03 (No significance between Shedia and Bakous markets; P =0.86). X^2 = 0.04 (No significance between Shedia and Rateb markets; P = 0.83)

Table 3: Percentages of agreement between ELISA and Latex techniques for detection of Rotavirus in mollusc's pools in Alexandria, Egypt.

No. of tested samples	ELISA technique	Latex test	Results of:	
			Agreement	Disagreement
25	Positive	Positive	25	0
8	Positive	Negative	0	8
142	Negative	Negative	142	0
175 (Total)			167	8

Percent of agreement = 167 / 175 X 100 = 95.43 %

Table 4: Percentages of sensitivity and specificity of ELISA in comparison to Latex technique for detection of Rotavirus in mollusc's pools in Alexandria, Egypt.

Test results	ELISA technique	Latex test		Total
		Positive	Negative	
Positive	33	25 (A)	8 (B)	33
Negative	142	0 (C)	142 (D)	142
Total	175	25 (A+C)	150 (B+D)	175

Percent of sensitivity = A / (A+C) X 100 = 25 / 25 X 100 = 100 %
Percent of specificity = D/ (B+D) X 100 = 142/150 X 100 = 94.67%
Positive predictive value = 75.76%
Negative predictive value = 100%

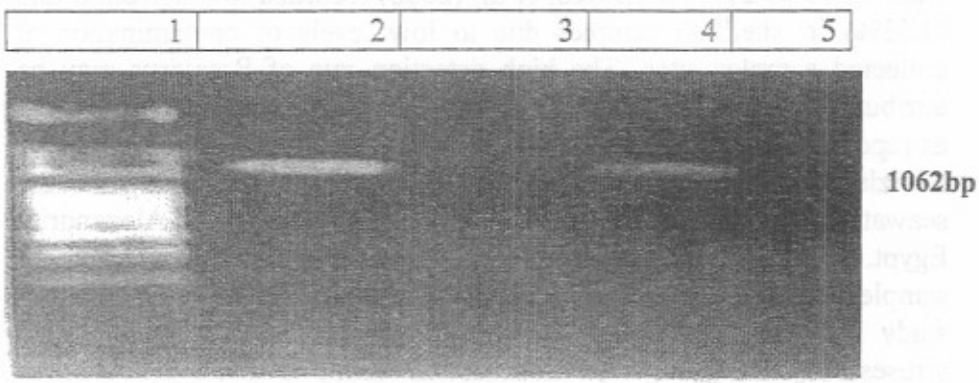
Table 5: Comparison between the results of RT-PCR, ELISA and Latex techniques for detection of Rotavirus in 50 selected mollusc's pools in Alexandria, Egypt.

Techniques	RT-PCR				Total
	positive	Percent	negative	percent	
ELISA					
Positive	31	86.11	2	14.29	33
Negative	5	13.89	12	85.71	17
Total	36	100.0	14	100.0	50
Latex					
Positive	25	69.44	0	0.00	25
Negative	11	30.56	14	100.0	25
Total	36	100.0	14	100.0	50

Table 6: Sensitivity and specificity of ELISA and Latex tests versus RT-PCR

Parameters	Percent	
	ELISA	Latex test
Sensitivity	86.11	69.44
Specificity	85.71	100.0
Positive predictive value	93.94	100.0
Negative predictive value	70.59	56.0

Fig. 1: Gel electrophoresis for PCR products



- Lane 1: 100 base pair marker.
- Lane 2 and 4: Rota irus positive samples.
- Lane 3 and 5: Rotavirus negative samples.

DISCUSSION

Human health problem associated with bivalve shellfish are well documented (Koopmans and Duizer 2004). Viruses are strict intracellular pathogens that can not replicate in food or water. Therefore, food borne viral infectious diseases depends on the initial concentration of virus in the food, host susceptibility, virus stability and the dose required for infection (Costantini *et al.*, 2006). A major public health concern posed by virus contaminated bivalves is that shellfish are often eaten raw or improperly cooked. Heat can render many viruses non infectious, however Rotavirus may be found in cooked shellfish (Abad *et al.*, 1997, Hansman *et al.*, 2008). Rotavirus is non enveloped virus and is generally more environmentally resistant than enveloped virus (Gerba *et al.*, 1996).

Our surveillance showed high percent of detection of Rotavirus in Alexandria molluscan shellfish, Om-Elkohloul "*Donax tranculus*" and Gandofelly "*Tapes decussates*", the highest popular sea food molluscs. There was a higher detection rate of Rotavirus antigen in Om-Elkohloul samples (19.61% and 14.71%) than in Gandofelly samples (17.81% and 14.70%) by ELISA and LA techniques, respectively. There was no significance difference in detection rate between different fish markets. These results were nearly agreed with the results recorded by Le Guyader *et al.*, 1994, Croci *et al.*, 2000, Le Guyader *et al.*, 2000, Macaluso *et al.*, 2004, Gabrieli *et al.*, 2007 with detection rates ranged from 13.89 to 29.9 %. Kittigul *et al.* (2008) recorded low detection rate (3.33%) in shellfish samples due to low levels of contamination at collected samples sites. The high detection rate of Rotavirus may be attributed to fecal pollution in sea water along Alexandria coastal area, as reported by many authors (Lackany 1963, El-Sharkaway *et al.*, 1977, Divizia *et al.*, 1997.). The later authors evaluate the quality of the seawater samples taken at different points on the coast of Alexandria, Egypt. Samples were collected in 6 different points from which three samples were positive for the presence of enteric viruses. In another study in India, the authors studied the prevalence of human enteric viruses in bivalve molluscan shellfish and shrimp to assess the extent of fecal pollution of coastal environment (Umesha *et al.*, 2008). High detection rate reaching 44% was detected by Show (1999) and Hansman *et al.* (2008) due to contamination of water by sewage

The higher detection rate of Rotavirus by ELISA (19.61% & 17.81%) than Latex test (14.71% & 13.7%) in Om-Elkohloul and Gandofelly samples, respectively may be attributed to the following: the

viral titer could be lower than technique sensitivity, so ELISA requires $10^5 - 10^6$ viral particles/gram that may not enough for LA test and the shellfish extract may contain non specific inhibitors resulting in weak agglutination reaction, not detected by ELISA (Arguelles *et al.*, 2000).

In the present study, the percent of agreement, sensitivity, specificity, positive and negative predictive values of LA test when compared to ELISA were 95.43%, 100.0%, 94.67%, 75.76% and 100.0% respectively. LA test was of low complexity, easy to interpret and provided a rapid diagnosis in a short time. It showed a reasonable amount of sensitivity and a high degree of specificity, proving to be a suitable test for diagnosis of Rotavirus in the examined samples. Nearly similar results were reported by many other workers (Ibrahim *et al.*, 1990, Raboni *et al.*, 2002, Altindis *et al.*, 2004).

RT-PCR results, inspite of its cost, showed a highly sensitive and specific method for detection of Rotavirus, which provides a rapid and efficient means of obtaining large quantities of cDNA. It is important to note that empty particles of Rotavirus can also be detected by VP6-ELISA, thereby enhancing the sensitivity of the assay by detecting virus fragments inaccessible by RT-PCR (Adler *et al.*, 2005). That may explain our findings as 2(14.29%) of negative by RT-PCR were positive by ELISA. These proteins (VP6) are more stable compared to RNA and therefore allow for detection of Rotavirus in samples where the RNA is lost due to harsh conditions.

The observed poor hygienic environment of fish markets and unhygienic molluscs' handlers practices (Personal monitoring); may add an access load of the Rotavirus contaminations to Om-Elkholoul and Gandofelly molluscs.

In conclusion, this study showed a high detection rate of Rotavirus in shellfish that may increase the incidence of diarrheal gastroenteritis due to consumption of Om-Elkholoul and Gandofelly molluscs. There was a good sensitivity and specificity between the results of RT-PCR and each of ELISA and LA techniques, therefore; it could be recommended the following:

- The use of either ELISA or LA technique as reliable tool for detection of Rotavirus in shellfish.
- The applying of corrective actions and hygienic precautions to avoid Rotavirus infection and ensure safety and fitness of these molluscs to consumers that clarified to be employed as following:

1- Prevention of sewage drain into sea water, even treated, to prohibit the main source of Rotavirus contamination. The stability of Rotavirus

in environmental waters, and their resistance to physical, chemical treatment processes in sewage treatment plants may facilitate their transmission (Carol Shieh *et al.,* 2000).

2- Application of obligatory hygienic training programs, personal hygiene and good hygienic practices, for fishermen and molluscs sellers.

3- Periodical hygienic inspection of fish markets to ensure perfect cleaning and removal of waste, continual upgrading of hygienic certificates for sellers and renewal of all WCs with enough water supplies.

4- Awareness for customers to avoid eating raw Om-Elkholoul or Gandofelly and should apply hygienic practices in handling and efficient cooking of these molluscs to ensure getting red off possible risk of Rotavirus and other enteric viruses.

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