Research Paper

Taqman hydrolysis probe application for *Escherichia coli*, *Salmonella enterica*, and *Vibrio cholerae* detection in surface and drinking water

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ABSTRACT

This study explored the applicability of using TaqMan qPCR (quantitative polymerase chain reaction) for *Escherichia coli*, *Salmonella enterica* and non-virulent *Vibrio cholerae* detection in surface and drinking water. One hundred and twenty water samples were collected monthly (January 2017–December 2017) from the surface water (input) and drinking water (output and distribution networks) of two drinking water treatment plants (DWTPs) in Damietta County, Egypt. The distribution of the studied bacteria based on their detection by TaqMan qPCR compared with membrane filtration (MF) technique showed that the higher positive samples were detected by TaqMan qPCR. The bacterial count was totally absent in all output samples. TaqMan qPCR assay (based on sequence detection of *uidA*, *invA*, and *ompW*) revealed 97.96%, 99.14%, and 98.3% specificity for *E. coli*, *S. enterica*, and non-virulent *V. cholerae*, respectively, compared with 100% specificity for all strains when MF cultures were applied. TaqMan qPCR exhibited 100% sensitivity for all strains, while it was 91.67%, 80%, and 50% using MF cultures for *E. coli*, *S. enterica*, and non-virulent *V. cholerae*, respectively. In conclusion, TaqMan qPCR sensitivity makes it a useful tool for urgent fast monitoring of water contamination, especially in network samples that contain low bacterial count.

Key words | bioindicators, *invA*, *ompW*, *uidA*, water treatment

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INTRODUCTION

Water is a convenient environment for microorganisms' growth including algae, fungi, bacteria, protozoa, and viruses. For many years, much effort has been devoted to study the bacterial diseases transmitted by drinking water pathogens such as cholera, gastroenteritis caused by vibrios, typhoid fever and other serious salmonellosis and acute diarrhea caused by *Escherichia coli*. These waterborne pathogens and their related diseases are a major worldwide public health concern (Ramírez-Castillo *et al.* 2015). They cause not only morbidity and mortality of the population, but also high costs that represent their prevention and treatment. WHO (2015) reported that improving water quality can reduce global disease by 4%.

Bioindicators are living microorganisms that play a vital role in water quality evaluation, and which are used to screen the health of the natural ecosystem in the environment (Parmar *et al.* 2016). Many bioindicators have been detected in water such as fecal *Streptococcus* and *E. coli*. Numerous methods have been developed for rapid detection of waterborne bacteria in water. These rapid methods can be classified into nucleic acid-based, immunology-based, and biosensor-based detection methods (Deshmukh *et al.* 2016).

doi: 10.2166/washdev.2019.137

Nucleic acid-based methods such as polymerase chain reaction (PCR), droplet digital PCR, quantitative polymerase chain reaction (qPCR), micro arrays, pyrosequencing, and fluorescence *in situ* hybridization (FISH) are rapid and can be performed without culturing the pathogens (Deshmukh *et al.* 2016). PCR methods can detect pathogens in a one-step reaction within 2–4 h (Botes *et al.* 2013). Different primers for specific genes have been used for detection of waterborne microbes. Maheux *et al.* (2014) used *lacZ*, *wecG*, and 16S rRNA for total coliform detection in water using PCR and compared them with culture methods. The *ompW* gene has been used for *V. cholerae* detection using SYBR green real-time PCR compared with conventional PCR and culture methods (Tirapattanun *et al.* 2015). Also, Ranjbar *et al.* (2016) used *ompW* for *V. cholerae* detection in addition to *invA* for *Salmonella* spp. using multiplex PCR. Hydrolysis TaqMan probe is a qPCR method that has been used for the detection of pathogens. For instance, *uidA* sequence was probed for detection of *E. coli* in water samples (Frahm & Obst 2003). Also, Ishii *et al.* (2014) determined *E. coli* using TaqMan probes labeled with different fluorophores. TaqMan probe for *invA* has been used for *S. enterica* monitoring in environmental samples (Kasturia & Drgon 2017).

Membrane filtration (MF) method is the main technique used for bacterial detection and quantification in Damietta drinking water treatment plants (DWTPs). This is a time-consuming technique, which suggested being enhanced by using another more rapid and sensitive technique. In this work, we explored the possibility of TaqMan qPCR application as a fast method for detection of *E. coli, S. enterica*, and non-virulent *V. cholerae* in DWTPs.

MATERIALS AND METHODS

Sample collection protocol

To investigate *E. coli*, *S. enterica*, and non-virulent *V. cholerae* in DWTPs using TaqMan qPCR, 120 water samples (24 influent samples, 24 output samples, and 72 samples from six different distribution networks related to the examined DWTPs) were collected under sterile conditions from two DWTPs: DWTP1 (El-Bostan) that depends on Lamella settler clarification and DWTP2 (Kafr-Soliman) that works by conventional clarifiers in Damietta governorate, Egypt through four successive seasons between January and December 2017 according to APHA (2012). The output and distribution network samples were de-chlorinated with 0.1 mL of 3% $Na_2S_2O_3.5H_2O$ at the time of sampling. The collected samples were stored at 4 °C until analysis within 2 hours of collection.

Membrane filtration culture method

One hundred mL of each sample were filtered within 2 hours of collection on 0.45 μm Millipore membrane filter then transferred for culturing on specific selective chromogenic medium. Modified membrane thermotolerant *E. coli* (m-TEC) agar medium were used for detection and enumeration of *E. coli*. After 2 hour incubation at 35 ± 0.5 °C, the m-TEC plates were transferred to a sealed plastic bag and placed onto a water bath rack at 44.5 ± 0.2 °C for 22–24 hours. *Salmonella–Shigella* (SS) medium was used for *S. enterica* detection and isolation at 35 ± 2 °C for 18–24 hours. Thiosulfate citrate bile salt sucrose (TCBS) agar medium was used for detection and isolation of non-virulent *V. cholerae* at 35 ± 2 °C for 24–48 hours.

Taqman quantitative PCR method

Genomic DNA extraction

One hundred mL to 1,000 mL of water samples depending on their type and level of contamination were filtered using 0.45 μm sterile Millipore membrane. The filter membrane was washed with 5 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

The resulting suspension of the wash was centrifuged for 10 min at 10,000 rpm. The bacterial pellet was re-suspended in 20 μL DNA/RNA free distilled water and was kept at −20 °C until DNA extraction. Bacterial genomic DNA extraction was carried out using QIAamp DNA mini purification kit according to the manufacturer's instructions by QIAcube Extraction system (QIAGEN, Germany).

The quality of the extracted chromosomal DNA was examined on 1% agarose gel electrophoresis. The high quality of cDNA appeared as a sharp and intense band without smear of degradation. Total extracted DNA concentration

and purity were measured by UV at A260/280 using double beam spectrophotometry.

Taqman quantitative PCR (amplification)

The qPCR assay reactions were carried out in a MxPro Mx3000P Real-Time thermal cycler (Stratagene, USA) according to the manufacturer's instructions using 20 μL of extracted DNA sample. Each run included 20 μL DNA specific for each strain as positive control. Also, 20 μL of RNase-/DNase-free water was run as negative control.

The amplification conditions for all target sequences *(uidA, invA*, and *ompW)* were one initial cycle at 95 °C for 2 min (TaqMan polymerase enzyme activation) followed by 50 cycles each one of 95 °C for 10 sec (template denaturation) followed by 60 °C for 1 min (annealing). TaqMan probes used for the target sequences were 5′ labeled with 6-FAM reporter dye and 3′ labeled with a TAMRA quencher.

Taqman qPCR standardization

Bacterial strain detection was carried out by TaqMan qPCR kit (genesig® Advanced Kit PrimerDesign™ Ltd) following its instructions. The target sequences for *E. coli* (all strains) detection and quantification was glucuronidase gene (*uidA*), for *S. enterica* invasine A gene (*invA*), and for non-virulent *V. cholerae* (all subtypes) outer membrane protein W gene (*ompW*).

Data analysis

Amplification efficiency and quantity (copies) were calculated as described by MxPro Mx3000P Real-Time thermal cycler manual using MxPro software v3.20 (Stratagene, USA).

The equations calculating sensitivity, specificity, accuracy, positive predictive value (PPV), negative predictive value (NPV), and prevalence are described by Baratloo *et al.* (2015). Microsoft Excel 2010 and two-way analysis of variance (ANOVA) of SPSS ver. 22 were used for data management and calculations.

RESULTS

Membrane filtration culture method

The *E. coli* count on m-TEC was detected in all raw water samples except El-Bostan in February and Kafr-Soliman in December 2016 (Table 1), but was completely absent in all treated output and network samples. The maximum *E. coli*

Table 1 | Average counts of *E. coli* using m-TEC medium and TaqMan qPCR of (surface water): input water samples of El-Bostan and Kafr-Soliman DWTPs

	El-Bostan input			Kafr-Soliman input		
		TaqMan qPCR values			TaqMan qPCR values	
Month	Culture count ($\times 10^2$CFU/100 mL)	Cq (dRn)	Quantity (copy/μL)	Culture count ($\times 10^2$CFU/100 mL)	Cq (dRn)	Quantity (copy/μL)
January	2.0	38.05	2.46	2.4	38.37	2.01
February	ND	39.40	1.05	2.1	39.00	1.35
March	2.4	33.26	5.42	2.8	28.33	1.31×10^2
April	3.0	26.15	5.39×10^2	2.6	33.30	5.28
May	2.4	29.33	6.89×10^1	2.9	26.32	4.83×10^2
June	2.9	28.86	9.34×10^1	2.7	29.35	6.80×10^1
July	4.2	25.75	6.98×10^2	3.9	26.23	5.12×102
August	3.2	28.23	1.40×10^2	3.8	28.84	9.46×10^1
September	4.0	27.72	1.95×10^2	3.8	28.46	1.21×10^2
October	3.2	32.73	7.64	2.4	38.68	1.65
November	2.6	37.66	3.14	2.8	30.38	3.49×10^1
December	2.6	38.87	1.47	ND	38.79	1.54

CFU, colony forming unit; Cq, quantification cycle; dRn, baseline subtracted fluorescence reading normalized to the reference dye; ND, not detected.

count occurred in El-Bostan input in July (420 CFU/100 mL), whereas the minimum *E. coli* value (<1 CFU/100 mL) occurred at El-Bostan input in February and Kafr-Soliman in December. The maximum *E. coli* count occurred in Kafr-Soliman input in July (390 CFU/100 mL), whereas the minimum *E. coli* value (210 CFU/100 mL) occurred at Kafr-Soliman input in February.

Detection of *S. enterica* on SS medium (Table 2) showed that it was absent in all output and distribution network samples. It was found only in El-Bostan input in June, July, and August and in Kafr-Soliman input in July (2 CFU/100 mL). The maximum *S. enterica* count was in El-Bostan input in August (3 CFU/100 mL), whereas its minimal count was in June and July (1 CFU/100 mL) at El-Bostan input.

Non-virulent *V. cholerae* was totally absent in all samples except El-Bostan input in July (2 CFU/100 mL) and August (3 CFU/100 mL), as reported in Table 3.

Taqman qPCR

The standard curves of TaqMan qPCR for *E. coli*, *S. enterica*, and non-virulent *V. cholerae* are represented in Figure 1. Standard curves were generated by plotting the copy number of bacteria against Cq values.

Generally, the three tested bacteria, *E. coli, S. enterica*, and non-virulent *V. cholerae* could not be detected using TaqMan qPCR in all treated output water samples for both DWTPs. Table 1 shows that the maximum *E. coli* count in El-Bostan input was in July (6.98×10^2 copy/μL), while the minimum was in February (1.05 copy/μL). On the other hand, the maximum *E. coli* value of Kafr-Soliman input occurred in July (5.12×10^2 copy/μL), with the minimum value in February (1.35 copy/μL).

S. enterica occurred in summer months (June, July, and August) in El-Bostan input and in July at Kafr-Soliman input, as reported in Table 2. It was also detected in Kafr-Soliman network 6 (1.65 copy/μL) in July (Table 4).

Non-virulent *V. cholerae* was maximally detected by TaqMan qPCR in August in El-Bostan input (3.43×10^2 copy/μL) (Table 3), in July in the samples taken from the input of El-Bostan and Kafr-Soliman DWTPs (64.6 copy/μL and 26.3 copy/μL, respectively). Also, it was found in July within El-Bostan network 3 (4.08 copy/μL) (Table 4).

Distribution of the bacterial strains

The overall results for distributions of *E. coli*, *S. enterica*, and non-virulent *V. cholerae* within the input (surface), output,

Table 2 | Average counts of samples that gave positive results with *S. enterica* using SS medium and TaqMan qPCR in inputs of El-Bostan and Kafr-Soliman DWTPs

	El-Bostan input			Kafr-Soliman input		
		TaqMan qPCR values			TaqMan qPCR values	
Month	Culture count (CFU/100 mL)	Cq (dRn)	Quantity (copy/μL)	Culture count (CFU/100 mL)	Cq (dRn)	Quantity (copy/μL)
June	1.0	37.20	2.74	ND	ND	ND
July	1.0	36.78	3.57	2.0	36.20	4.83
August	3.0	35.1	10.3	ND	ND	ND

CFU, colony forming unit; Cq, quantification cycle; dRn, baseline subtracted fluorescence reading normalized to the reference dye; ND, not detected.

Table 3 | Average counts of samples that gave positive results with non-virulent *V. cholerae* using TCBS medium and TaqMan qPCR in El-Bostan and Kafr-Soliman inputs

	El-Bostan input			Kafr-Soliman input		
		TaqMan qPCR values			TaqMan qPCR values	
Month	Culture count (CFU/100 mL)	Cq (dRn)	Quantity (copy/μL)	Culture count (CFU/100 mL)	Cq (dRn)	Quantity (copy/μL)
July	2.0	29.8	64.6	ND	31.2	26.3
August	3.0	27.2	3.43×10^2	ND	ND	ND

CFU, colony forming unit; Cq, quantification cycle; dRn, baseline subtracted fluorescence reading normalized to the reference dye; ND, not detected.

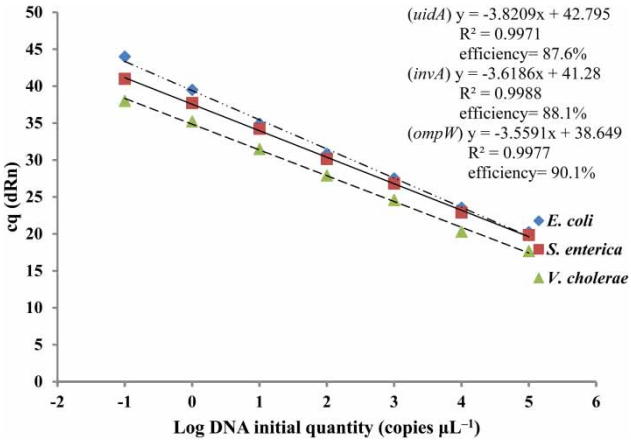

Figure 1 | Standard curves for the detection and quantification of the *E. coli* (*uidA*) gene, *S. enterica* (*invA*) gene, and non-virulent *V. cholerae* (*ompW* gene) by TaqMan qPCR. The equations, correlation values (R^2) and efficiency for each curve are reported.

Table 4 | Network samples that gave positive results with *S. enterica* and non-virulent *V. cholerae* in July using TaqMan qPCR only and not detected with the MF method

	S. enterica		Non-virulent *V. cholerae*	
Site	**Cq (dRn)**	**Quantity (copy/µL)**	**Cq (dRn)**	**Quantity (copy/µL)**
El-Bostan network 3	ND	ND	34.1	4.08
Kafr-Soliman network 6	38	1.65	ND	ND

Cq, quantification cycle; dRn, baseline subtracted fluorescence reading normalized to the reference dye; ND, not detected.

and networks (drinking) samples using culturing media and TaqMan qPCR tests are summarized in Table 5. Generally, among 120 water samples (24 input, 24 output, and 72 networks), the input samples exhibited a higher rate of positive results reaching 100% for detection of *E. coli* using TaqMan qPCR based on *uidA* gene compared with culturing on m-TEC medium (91.67%). The positive rates of detection for *S. enterica* present in input samples was the same (16.67%) for both TaqMan qPCR based on *invA* gene and culturing on SS medium. A low positive rate of detection was observed for non-virulent *V. cholerae* when either TaqMan qPCR based on *ompW* gene was used (12.5%) or culturing on TCBS medium (8.33%).

Output samples gave negative results for detection of *E. coli*, *S. enterica*, and non-virulent *V. cholerae* using both methods. Network samples gave the lowest positive rate of detection (1.39%) only when TaqMan qPCR was used for *S. enterica* and non-virulent *V. cholerae* based on *invA* and *ompW* genes, respectively.

Taqman qPCR and MF culture evaluation

Comparative statistical evaluation of TaqMan qPCR and MF culture methods are represented in Table 6, where their sensitivity, specificity, accuracy, PPV, NPV, and prevalence are calculated.

TaqMan qPCR detection and quantification for *E. coli* using *uidA* primer set showed relatively high specificity (97.96%) with maximum sensitivity and NPV reaching 100%. The PPV and accuracy of TaqMan qPCR were calculated to be 91.67% and 98.33%, respectively. The sensitivity of m-TEC medium for *E. coli* culturing was 91.67% with maximum specificity and PPV reached 100%. The NPV was 97.96% and the accuracy was 98.33%.

The detection and quantification of *S. enterica* by TaqMan qPCR using *invA* primer set showed relatively high specificity reaching 99.14% with maximum sensitivity and NPV (100%). The PPV and accuracy of TaqMan

Table 5 | Distribution of *E. coli*, *S. enterica*, and non-virulent *V. cholerae* within 120 samples from input, output, and networks using MF culturing media and TaqMan qPCR

		E. coli						*S. enterica*						Non-virulent *V. cholerae*					
		Culturing on m-TEC medium			TaqMan qPCR (*uidA* gene)			Culturing on SS medium			TaqMan qPCR (*invA* gene)			Culturing on TCBS medium			TaqMan qPCR (*ompW* gene)		
Sample type	**Sample no.**	+	−	+ %	+	−	+ %	+	−	+ %	+	−	+ %	+	−	+ %	+	−	+ %
Input	24	22	2	91.67	24	0	100	4	2	16.67	4	20	16.67	2	22	8.33	3	21	12.5
Output	24	0	24	0	0	24	0	0	24	0	0	24	0	0	24	0	0	24	0
Networks	72	0	72	0	0	72	0	0	72	0	1	71	1.39	0	72	0	1	71	1.39

Table 6 | Comparative statistical evaluation of MF culture and TaqMan qPCR methods

Bacteria	*E. coli*		*S. enterica*		Non-virulent *V. cholerae*	
Parameter	**MF culture**	**TaqMan qPCR**	**MF culture**	**TaqMan qPCR**	**MF culture**	**TaqMan qPCR**
True positive	22	22	4	4	2	2
True negative	96	96	115	115	116	116
False positive	0	2	0	1	0	2
False negative	2	0	1	0	2	0
Specificity (%)	100	97.96	100	99.14	100	98.3
Sensitivity (%)	91.67	100	80	100	50	100
Accuracy (%)	98.33	98.33	99.17	99.17	98.3	98.3
PPV (%)	100	91.67	100	80	100	50
NPV (%)	97.96	100	99.14	100	98.3	100
Prevalence (%)	18.33	20	3.33	4.17	1.65	3.67

qPCR were 80% and 99.17%, respectively. The sensitivity of SS medium for *S. enterica* culturing was 80% with maximum specificity and PPV reaching 100%. The NPV was 99.14% and the accuracy was 99.17%.

TaqMan qPCR detection and quantification using *ompW* primer set for non-virulent *V. cholerae* showed relatively high specificity (98.3%) with maximum sensitivity and NPV reaching 100%. The PPV and accuracy of TaqMan qPCR were 50% and 98.3%, respectively. The sensitivity of TCBS medium for non-virulent *V. cholerae* culturing was 50% with maximum specificity and PPV (100%). The NPV was 98.3% and the accuracy was 98.3%.

DISCUSSION

Public health is directly linked to the occurrence of pathogens in water (Li *et al.* 2017). Thus, pathogen detection techniques need to be updated using modern rapid techniques. Distribution of the bacteria occurrence in water samples based on either TaqMan qPCR or culture method revealed that all output treated water samples of both examined DWTPs were free from *E. coli*, *S. enterica*, and non-virulent *V. cholerae*. The most likely explanation for the absence of any detectable bacterial strains in the output of DWTPs might be attributed to the stable performance of the treatment processes: coagulation, sedimentation, filtration that can physically remove most of the microorganisms, and disinfection with chlorine. Similarly, Holinger *et al.* (2014) agreed that filtration and disinfection had more significant effects on the microbial community and inactivated more microorganisms than coagulation and sedimentation.

It is unexpected to detect non-virulent *V. cholerae* in Egyptian water. However, the WHO (2011) reported that usually non-toxigenic *V. cholerae* is widely distributed in water environments. Non-virulent *V. cholerae* was detected in July within Kafr-Soliman input sample using TaqMan qPCR rather than MF culture method. Furthermore, *S. enterica* and non-virulent *V. cholerae* were also detected separately by TaqMan qPCR in July at Kafr-Soliman and El-Bostan networks, respectively. The pathogens detected in the networks might be attributed to the cross-connections or intrusion of pathogens through leaks and cracks. Detection of bacteria by Taqman qPCR and absence by MF might be due to the non-viability of their cells which could not be detected by culturing, as shown in a recent study by Vital *et al.* (2017), who stated that while quantitative real-time PCR can be used to determine levels of bacteria in water, it might detect non-viable organisms. Also, this might be due to the high sensitivity of Taqman qPCR.

The distribution of the tested bacteria based on the sites using both techniques demonstrated that the examined bacteria were most commonly associated with the input rather than the output of both studied DWTPs. This could be due to the pollution of the intake water source with waste

from houses besides intakes and/or the disposal of huge quantities of fish farms in the Nile River Damietta branch during the study period. This complies with the work of Mohamed *et al.* (2015), who revealed that the water source of Damietta DWTPs contains high nutrient concentrations, particularly NO_3, NH_4, and PO_4, which enrich the microbial growth.

The distribution of the bacteria in water with respect to seasonal variation is negotiable as several studies showed different seasonal patterns (Wilkes *et al.* 2011). In contrast, Sadik *et al.* (2017) reported that surface water and drinking water sources contained little seasonal variation in the quantity of microbes. In our study, it was obvious that *E. coli* gave a positive result in all seasons, while *S. enterica* and non-virulent *V. cholerae* occurred only in the summer months. This might be because the water temperature became close to the optimal which promotes more bacterial nutrients in hot seasons.

Analysis of data showed that the sensitivity of the TaqMan qPCR technique was higher than the MF culture method. The lower sensitivity of the culture method could be due to the treatment conditions, which probably introduced a type of bacterial growth inhibition as a result of metabolic stress and, as such, bacterial cells may enter into a viable but non-culturable state.

Both techniques, MF and real-time PCR, provide qualitative and quantitative results. MF is low cost, but its result is not available for at least 1–3 days and pathogens have to grow in artificial media.

The advantage of the TaqMan qPCR is not only to quantify the investigated bacteria directly from water samples in one step from low amounts of samples with higher technology rather than the time-consuming culture method, but also it possesses a high sensitivity and specificity as it can estimate the level of contamination due to detection of damaged or destroyed bacterial cells and their contents. Chern *et al.* (2015) supported the usage of TaqMan qPCR as an alternative technique instead of culture method for detection and enumeration of *Mycobacterium* in drinking water as it demonstrated 100% specificity and sensitivity. Mendes Silva & Domingues (2015) stated that although the advantages of TaqMan qPCR are being used as a fast monitoring tool, MF method should not be excluded as TaqMan is costly and needs highly priced kits, extraction, and real-time PCR thermocycler instruments.

CONCLUSIONS

TaqMan qPCR is a more sensitive technique than traditional routine methods. It allows rapid detection and quantification of the microbes present in surface water, putting an end to drinking water microbial contamination. Furthermore, unknown species cannot be detected by real-time PCR.

As both methods have advantages and limitations, we recommend using a combination of TaqMan PCR that generates rapid, sensitive results and MF for confirmation, in daily raw and treated drinking water analysis to help reduce microbial risk for public health.

Also, our study confirmed the presence of *E. coli*, *S. enterica*, and non-virulent *V. cholerae* as waterborne pathogens in River Nile water and *S. enterica* and non-virulent *V. cholerae* in distribution network waters of Egypt. The present study demonstrates the important public health problem in Egypt.

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First received 25 July 2018; accepted in revised form 21 December 2018. Available online 8 April 2019