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OCCURRENCE AND MOLECULAR DIVERSITY OF *ESCHERICHIA COLI* O157:H7 VIRULENT MARKERS ISOLATED FROM IRRIGATED CABBAGE

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ABSTRACT
Shiga-like toxin-producing Escherichia coli O157:H7 isolated from vegetables can be of public health importance especially when they harbor shiga-like toxin 2.It has in recent times attracted worldwide attention because of the rapidly increasing morbidity and mortality in humans. It is along this background that the occurrence and diversity of 22 strains of E. coli O157:H7 virulent factors were investigated. Multiplex polymerase chain reaction was used for the determination of shiga-like toxins 1 and 2, aea A and hlyCa genes. The results of the PCR assays showed only 4.5% of the strains harboring both Stx1 Stx2, eaeA and hlyCa. Stx2 was 100% while eaeA and hlyCa were 90.91% and 59.09% respectively. Isolates were clustered into 4 different clusters using Dice similarity coefficence by complete linkage method. The identification of 100% Stx 2 was subject of high public health concern, especially when fresh (ready to eat) agricultural produce are involved. The results of this study suggest that cabbage has a high value as a reservoir of E. coli O157:H7, which poses potential risk of infecting human and transmission of pathogenic E. coli O157:H7 to the environment as well as pathogenic genes to other bacterial strains.

Key:Shiga-like toxin (Stx), enterohemolysin (hlyCa), intimin gene (eaeA), multiplex PCR, dendrogramm.

INTRODUCTION

Shiga-like toxin (*Stx*1) producing *E. coli* (EHEC) strains carry *Stx*genes and produce *Stx* but not associated with disease, although some strains are capable of causing hemorrhagic enteritis and hemolytic uremic syndrome(HUS). EHEC may produce two immunologically-distinct toxins, *Stx*1 or *Stx*2, alone or in combination, *Stx* can inhibit protein synthesis (Ogasawara *et al*., 1988). O157:H7 strains that produce *Stx*2 may be associated with an increased risk of systemic complications (Donohue-Rolfe *et al*., 2000). *Stx*, produced by EHEC during colonization of the intestinal tract, gains entry to the host through epithelial cells and acts on submucosal immune cells that release cytokines; these induce inflammation and increase the expression of the *Stx* receptor globotriaosylceramide (Gb3) (O'Loughlin and Robins-Browne, 2001). *Stx*-mediated endothelial injury activates coagulation and inhibition of fibrinolysis leads to accumulation of fibrin and thrombosis (Tarr*et al*., 2005). The combination of *Stx* and O157 lipopolysaccharide (LPS) induces platelet-leukocyte aggregates and tissue factor release and thus contributes to a prothrombotic state (Stahl *et al*., 2009). *Stx*genes are encoded in bacteriophages and may have different variants based on their genetic sequence (Friedrich *et al*., 2002). Upon induction, *Stx*-encoding bacteriophages increase toxin production and play a role in horizontal transfer of *Stx*genes by infecting other bacteria, as showed in vivo and in vitro experiments (Herold*et al*., 2004).

In Nigeria, there has been no report on the outbreak of food-borne disease cause by *E. coli* O157:H7, largely, due to non inclusion of the bacteria in disease notification forms used by health ministries and agencies and lack of procedures for routine diagnosis in both public and private laboratories. This serotype was isolated from fruits and vegetables and beef samples (Dahiru*et al*., 2008a; Uzeh and Adepoju, 2013;Enabulele*et al*., 2014) in different parts of the country. The incidence of *E. coli* O157:H7 in vegetables and meat samples in local market (Dahiru*et al*., 2008a; Enabulele*et al*., 2014) indicated the exposure of these bacteria to the environment as threat to public health and can serve as a vehicle for the transmission of disease to man.

Thus, determining the public health risk of *E. coli* 0157:H7, may still be unconfirmed without detection of the presence of virulent factors usually involved in the disease pathogenesis cause by this organism, virulent factors such as *Stx*1and 2 *gene, eaeA* gene and enteroheamolysin (*hlyC*) gene in the bacteria. Shiga-like toxin 2 (*Stx*2) is considered the most important virulence factor associated with the human infection. It is about 400 fold more toxic to mice than *Stx*1 and were shown to induce feto-placental re-absorption, intra-uterine heamatoma, fibril deposition and neutrophil infiltration (Gaddad*et al*., 2011). *E. coli* O157:H7 are defined by the presence of specific virulence factors; all strains produce hemolysin and many produce intimin, a 97kDa attachment-and-effacement protein encoded by *eaeA gene* (Fagan *et al.,*1999).The 60-MDa plasmid commonly found in O157:H7 strains contain genes encoding a hemolysin (termed enterohemolysin) *hly*. Enterohemolysin is found in nearly all O157:H7 strains.

Enterohemolysin belongs to the RTX toxin family members, expressed by uropathogenic*E. coli*, *Pasteurellahaemolytica*, and other human and animal pathogens. The gene encoding the hemolysin (*ehxA*) has ca. 60% identity to the *hlyA* gene encoding hemolysin expressed by uropathogenic*E. coli*. Similarly, *eae* gene encodes for the only potential *E. coli* O157:H7 adherence factor that was found to play a role in intestinal colonization *in vivo* in an animal model (the 94- to 97 kDa OMP intimin) (Nataro and Kaper, 1998).

García-Aljaro*et al*, (2009) observed that horizontal transmission of pathogenic factors through mobile elements, such as bacteriophages, plasmids or transposable elements is important in the emergence of new pathogenic bacteria. In view of the epidemiological importance of identifying possible sources of infection and monitoring the spread of bacteria, especially emerging infectious pathogens, the research examined the presence and diversity of Shiga- toxin 1 and 2 (*Stx*1 and *Stx*2) genes, *eaeA* gene, enteroheamolysin (*hlyC*) genes in 22 *E. coli* O157:H7 strains isolated from pre-harvest cabbage irrigated with wastewater in Kano. Cabbage is consumed as ready to eat food in Kano State, prepared in form of salad, served alone or with other foods.Because of the high demand, cabbage production has turned to be a preferred commodity for cultivation by irrigation farmers. These directly increase the potential role it could play as a vehicle for the transmission of *E. coli* O157:H7.

MATERIAL AND METHODS

Bacterial strains

Twenty two (22) *E. coli* O157:H7 strains isolated on cabbage were collected from the stock culture collection of Department of Microbiology Laboratory Bayero University Kano (Dahiru*et al*., 2015) and sub cultured onto sorbitol macconkey agar (SMAC) and subjected to standard biochemical tests (indole, vogesprokauer, methyl red, citrate, and utilization of potassium cyanide, fermentation of rhamnose and production of β-glucuronidase), and latex agglutination test (Oxoid LTD Hamspshire, England) for confirmation(Nataro and Kaper, 1998).

Preparation of whole-cell DNA for PCR

Genomic Deoxynucleotide (DNA) extraction was carried as per Bio Basic Inc (Canada) extraction Kit instructions (Ref). Harvested cells were transferred to RNase-free centrifuge tube and 1 ml of lyses buffer (DNlysis-F) was added and inverted to mix immediately and incubated at 21^0 (room temperature) for 5 minutes. Two hundred micro liters [200 µl] of chloroform was added to the tube inverted several times and mixed again. The suspension was centrifuged at 12,000 g for 5 minutes at 4^0C and the suspension was transferred into a new tube. Half milliliter (0.5 ml) of ethanol was then added to the tube and vortexed for 30 seconds and centrifuged at 12,000 g for 5 min at 4^0C. The supernatant was discarded. The pellets were washed with 1 ml of 75% ethanol and then centrifuged at 12,000 g for 1 min.The supernatant was discarded. Lastly, the pellets were air dried at 21^0(room temperature) for 5 minutes.

Multiplex PCR for detection of Shiga-toxin 1 and 2 (*stx*1 and *stx*2) genes,

Amplification of shiga-like toxin 1 and 2 were carried out in accordance with the method described by Yaron*et al* (2000) and Galland*et al* (2001). Genes for *Stx*1 and *Stx*2 virulence markers were amplified in 20 µl reaction volume, which consisted of 1 µl of DNA, 2 µl of primer (1 µl each of *Stx*1 and *Stx*2 genes), 10 µl pre-mix (Maxima Hot Start Green PCR Master Mix Fermentas) and 7.0 µl deionize (dH_2O water) for the first round. The green PCR master mix consists of $MgCl_2$, deoxynucleoside triphosphates (dNTPs), and *Taq* polymerase. Amplification was done with a thermal cycler (GeneAmp 9600 Thermal Cycler, Cert. GeneTool, Inc, Pleasanton), which was set for 35 cycles at 94°C for 5 min, 94°C for 1 min, 54°C for 1 min, 53°C for 1 min 72°C for 1 min, and 72°C for 1 min. The primers sequences used for the reaction were *Stx*1 forward primer (AAA TCG CCA TTC GTT GAC TAC TTC T), *stx*1 reverse primer (TGT CCA TTC TGG CAA CTC GCG ATG CA), *Stx*2 forward primer (CAG TCG TCA CTC ACT GGT TTC ATC A), *Stx*2 reverse primer (GGA TAT TCT CCC CAC TCT GAC ACC) synthesized by Integrated DNA Technology, Company USA. Second round consist of 2.0 µl DNA template from the first round reaction, 2.0 µl primer (1 µl each from *Stx*1 and *Stx* 2) and 36 µl dH_2O, making a reaction volume of 40 µl. Same processes and reaction conditions were used for *eaeA* with following primer sequences as adopted from Galland*et al* (2001) forward sequenceCAG GTC GTC GTG TCT GCT AAA, reverse sequenceTCA GCG TGG TTG GAT CAA CCT and *hlyCA* forward sequenceCTT TTG ACG TCA TGG GGA AGG, reverse sequenceCGA ATA TTG CAA CAC CAC GTT CAG adopted from Yaron*et al* (2000). The PCR products were compared with 100 bp marker (Farmentas) on 1% agarose gel by Gel electrophoresis. Fragments sizes (molecular weight) were calculated by comparison of the distance travelled by the unknown samples fragment with the standard (rulers) during electrophoreses(Kek*et al*., 2009).

Data analyses

Descriptive statistics and cluster analysis using Dice similarity co-efficience by complete linkage method (Kosman and Leonard,2005; Kek*et al*., 2009)were employed to determine the relationship between the strains.

RESULTS

The results of PCR assays for the 22 isolates of *Stx*1 *Stx*2, *eaeA* and *hlyCa* demonstrated only 4.5% of the strains harbored the 4 virulent markers tested (Table 1). However, other strains had only *Stx*2 while presence of *eaeA* and *hlyCa* were observed in 20(90.91%) and 13(59.09%) respectively.

As for the presence of these markers in a strain, 45.45% isolates had 10 (45.45%) the *Stx*2, *eaeA* and *hlyCa* genes, similarly 20 (90.91%) harbor *Stx*2 and *eaeA* and 11(50.00%) harbor *eaeA* and *hlyCa*. The results of the comparison of the genes detected among the isolates showed close relationship using Dice similarity analysis. Isolates were clustered into 4 different clusters, the first contained 10 isolates, which harbored*Stx*2, *eaeA* and *hlyCa* genes, the 2nd cluster had 1 membership (whose member harbored the 4 genes), 3rd cluster had 2 isolates (whose members harbored*Stx*2 and *hlyCa*)while the 4th cluster had 9 members (whose members harbored*Stx*2 and *eaeA*) (figure 1). Interestingly, in each of the two largest clusters (1 and 4), members from both sources were observed.

Table 1: Distribution of *Shigella*-like toxin (*Stx*) 1and 2, attaching and effacing gene (*eae*) and enteroheamolysin (*hly*) genes isolated by PCR method from cabbage

S/No	Source	*Stx* 1	*Stx* 2	*eaeA*	*hlyCAa*
1	JK16	A	P	P	P
2	JK20	A	P	P	P
3	JK21	P	P	P	P
4	JK23	A	P	P	A
5	JK24	A	P	P	P
6	JK25	A	P	P	A
7	JK33	A	P	P	P
8	JK38	A	P	P	A
9	JK39	A	P	P	A
10	JK41	A	P	P	A
11	JK48	A	P	P	P
12	SH4	A	P	P	A
13	SH5	A	P	P	A
14	SH7	A	P	P	A
15	SH11	A	P	P	A
16	SH14	A	P	A	P
17	SH18	A	P	P	P
18	SH20	A	P	P	P
19	SH24	A	P	A	P
20	SH25	A	P	P	P
21	SH26	A	P	P	P
22	SH29	A	P	P	P
	Total Present	1(4.54%)	22(100)	20(90.91%)	13(59.09%)

Legend: JK = Jakara sample, SH = Sharada sample, *Stx* 1 = Shiga like toxin 1, *Stx* 2 = Shiga like toxin 2, *eaeA*= Effacing and attaching gene, *hlyCa* = Enteroheamolysin gene, A = Absent, P = Present.

Dendrogram using Complete Linkage

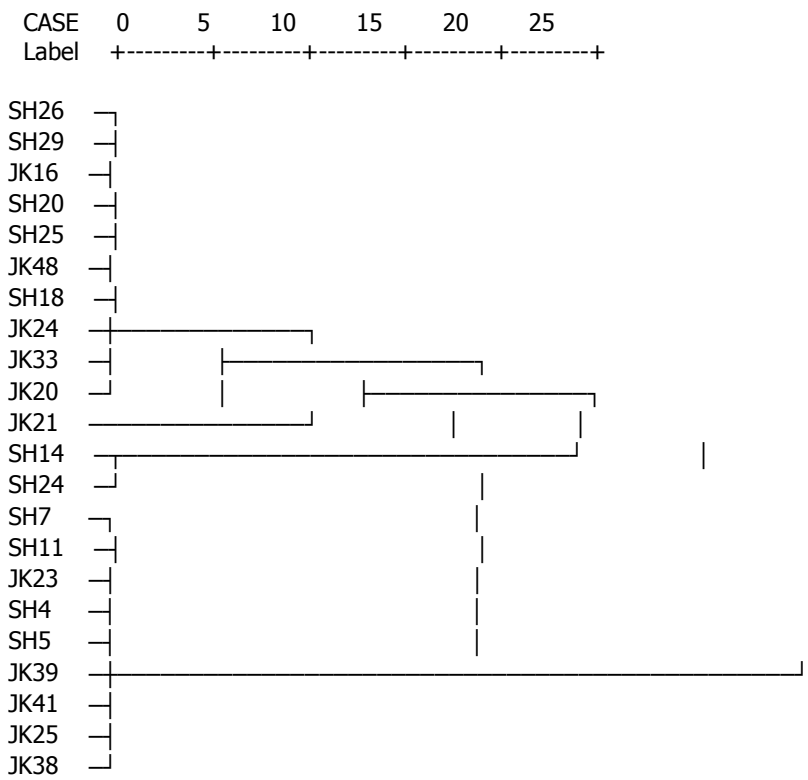

Figure 1: Dendrogram showing the similarities of *Esherichia coli* O157:H7 strains as a factor of presence or absence of *stx*1, *stx*2, *eaeA* and *hlyCa* using Dice similarity co-efficience by complete linkage method of cluster analyses

DISCUSSION

The PCR analyses of the 22 *E. coli* 0157:H7 isolates revealed the presence of pathogenic genes *Stx*1 and *Stx*2, *hlyCa* and *eaeA,* which play a significant role in the pathogenesis of diseases caused by this bacterium. Pathogenicity of *E. coli* O157:H7 is based on the ability to produce one or both *Stx* (*Stx*1 and *Stx*2).The presence of the *eaeA*, known to be located on the EHEC chromosome, encodes the 94-97 KDa outer-membrane protein intimin, an intestinal adherence factor, which plays a role in intestinal colonization (Sohn*et al*., 2000). And produce extensive A/E lesions in the large intestine, featuring intimate adherence of the bacteria to the epithelial cells (Nataro and Kaper, 1998), is very important in determining the potential risk of these isolates in causing disease when consumed. Similarly, The*hlyCa* gene reported to be located on the EHEC pO157 plasmid encodeshemolysin (termed as enterohemolysine) is widely distributed among *E. coli* O157:H7, that lyses erythrocytes and significant determiner in the identification of *E. coli* O157:H7 (Sohn*et al*., 2000).

The major and defining characteristic of EHEC virulence factor is (*Stx*),which leads to death and many other symptoms in infected individuals. The *Stx* family contains two major, immunologically non-cross-reactive groups called *Stx1* and *Stx*2. A single EHEC strain may express *Stx*1 only, *Stx*2 only, or both toxins or even multiple forms of *Stx*2(Nataro and Kaper, 1998). Among the *Stx*1 and *Stx*2, *Stx*2 is considered the most important virulence factor associated with the human infection. It is about 400 fold more toxic to mice than *Stx*1 and has been shown to induce feto-placental re-absorption, intra-uterine heamatoma, fibril deposition and neutrophil infiltration (Gaddad*et al*., 2011). Therefore the 100% amplification of *Stx*2 is a subject of high public health concern, especially when fresh agricultural produce are involved. In contrast to the results of this survey,Gaddad*et al* (2011) observed predominance of *Stx*1 over *Stx*2 in *E. coli* O157:H7 isolated from diverse sources though the implication of *Stx*2 is more important than *Stx*1 in the progression of *E. coli* O157:H7 infection to HUS (Nataro and Kaper, 1998). As shown earlier, some strains had virulent maker genes that are characteristic of a typical *E. coli* 0157:H7, Sohn*et al* (2000), similarly reported *E. coli* O157:H7 KM with only *Stx*2 gene no *Stx*1. Jacek (2002) also reported similar finding on the analysis of virulent makers from *E. coli* O157:H7 isolated from Pigs using BOX PCR fingerprint with presence of variable number of these virulent markers.

In a similar work by Feder*et al* (2003) none of the *E. coli* O157:H7 recovered from swine contained *Stx*1, *Stx*2, *eaeA* and *hlyCa*, at the same time, it either had one or two without the other.Nevertheless, this finding does not prevent the identification of these isolates as O157:H7. Thus not all O157:H7 strains were found to poses the four amplified virulent factors.

Although large percentages of strains were *eaeA* positive and may be thought to be potentially atypical EPEC but the presence of *Stx*2 has seriously posed a great public health risk and indicated the potential risk of transmission of these pathogenic genes to other bacterial strains. For this reason the present research recommends for the continuous monitoring of the epidemiological dynamics of *E. coli* O157:H7 strains especially in vegetables. The isolates although were not from the same source but showed similarities, demonstrating the ability of each isolate tendency of causing a disease, by the presence of two or more virulent makers. The strains, revealed a low degree of heterogeneity among the different strains of *E. coli* O157:H7 as shown in the cluster analyses.This factor could influence the degree of their pathogenicity. The degree of heterogeneity was limited to four clonal leneages, with each clonal lineage having specific combination of virulent markers, suggestingthat the strains are likely from the same source because of the occurrence of *Stx*2 among the entire strains tested.This could make epidemiological contact tracing of human infection quite simple. It is noteworthy that some of the strains having same origins had different virulent markers, suggesting that the horizontal transfer of these markers could have been a recent event or that the markers are subjected to high selective pressure that could not have amounted to increase in high genotypic diversity.

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