

Original Research Article

Characterization of Virulence Gene in *Escherichia coli* Strains Isolated from Slaughtered Beef Using 16S rRNA Molecular Sequencing Method

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Received December 16, 2022; Revised December 24, 2022; Accepted December 27, 2022

ABSTRACT

The aims and objective of this research work is to determine the characterization of virulence gene in *Escherichia coli* strains isolated from slaughter beef in Akungba Akoko, Ondo State, Nigeria, using 16s rRNA sequencing method of analysis. Slaughtered Beef combines meat from many different animals, increasing the risk of contamination with *E. coli*. While most people experience a few days of upset stomach and then recover fully, *E. coli* infections can sometimes be life-threatening. The virulent gene under study in this research is *eaeA* gene, *easT* gene and *bfp* gene and the acquisition of virulence and resistance genes is believed to increase the pathogenicity of *E. coli* and the severity of infection with the great possibility of therapy failure. The bacterial isolates were isolated from obtained from 48 slaughter beef samples in several Local markets in Akungba Akoko, Ondo State, Nigeria. Fifteen isolates were taken from isolate stock kept in a freezer at -20°C . These were then grown in Brain Heart Infusion Broth (BHIB) media and incubated for 24 h at 37°C . DNA was extracted using the protocol stated by Pitout (2012). Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28°C . Primer sequences were as earlier documented. Using 16S rRNA Molecular Sequencing Method, PCR per primer set included (Reagent Volume μl) - 5X PCR SYBR green buffer (2.5), MgCl_2 (0.75), 10pM DNTP (0.25) were used for extraction of DNA, molecular sequencing and molecular integrity test of isolates. The integrity of the amplified gene fragment was tested on a 1.5% Agarose gel observed, to confirm amplification. Agarose gel showing the amplification of virulent gene, *eaeA* gene, *bfp* gene and *bfp* gene in selected *E. coli*. Band size of 248bp, 97bp and 324bp indicates positive amplification for all three virulent gene in *eaeA* gene, *easT* gene and *bfp* gene respectively. it was observed that the virulence determinant used to characterize this type of *E. coli* are not well known. But with the advent of 16S rRNA molecular sequencing, it was deduced that *easT* gene, *eaeA* gene, and *bfp* gene novel virulent genes are responsible for the virulent capability of *E. coli* strain present in Slaughtered Beef.

Keywords: 16S rRNA, Slaughtered beef, DNA sequencing, Pathogenic *E. coli*

INTRODUCTION

The danger in beef meat should always be a subject of discussion because slaughterhouse hygiene is a function and factor of its wholesomeness. The danger in the slaughterhouse is called abattoir, the presence of different microorganism wandering around the open space of the slaughterhouse. Some of the organisms are pathogenic, nonpathogenic, commensal, and parasitic organisms. One very good example is the *Escherichia coli* strains, which comprise of different strains with phylogenetic and genetic relatedness. The pathology and expression of clinical symptoms classify intestinal pathogenic *E. coli* into six pathogroups: (1) Enterotoxigenic (ETEC); (2) Enteropathogenic (EPEC); (3) Enterohaemorrhagic (EHEC); (4) Entero Aggregative (EAEC); (5) Enteroinvasive (EIEC); and (6) Diffusely adherent (DAEC). Strains of the bacteria that cause extraintestinal infection are called extraintestinal pathogenic *E. coli* (ExPEC) [1]. This pathotype is one of the

most commonly recognized associated with foodborne disease [2].

For clarity purposes, the slaughterhouse must have 90% microbial free and any trace of *E. coli* must be eliminated at all costs because *Escherichia coli* strains are mostly present as intestinal commensal bacteria in the gastrointestinal tract of humans, however, some can cause intestinal and extraintestinal infections [3]. We must find a way to reduce its scourge; they are the gateway to various clinical and

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Citation: Osuntokun OT. (2023) Characterization of Virulence Gene in *Escherichia coli* Strains Isolated from Slaughtered Beef Using 16S rRNA Molecular Sequencing Method. Int J Biopro Biotechnol Advance, 9(1): 484-491.

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subclinical infections.

Human infections occur through the consumption of contaminated food such as undercooked meat, drinking contaminated water, or direct person-to-person contact. In developing countries, like Nigeria, the major causes of infantile diarrhea are ETEC, EPEC and EAEC, while EHEC and EAEC are mainly associated with food poisoning, i.e. cooked and uncooked food. The presence of intestinal and extraintestinal pathogenic *E. coli* in food suggests a public health impact and inconsistency health hazard, and agencies such as the NAFDAC in Nigeria report food borne outbreaks associated with verotoxigenic (VTEC) and another pathogenic *E. coli*. There should be a concerted effort in reducing the microbial menace of *E. coli* strains with the enhanced general awareness of VTEC following reports of large outbreaks in the African country and worldwide [4].

The virulent gene under study in this research work are 1, (*eaeA* gene), 2 (*easT* gene) and 3 (*bfpgene*) and the ability of virulence and resistance genes were believed to increase the degree pathogenicity of a microorganism and the prevalence of infection with the great possibility of therapy failure. *Escherichia coli* is an opportunistic pathogen, commensal bacteria that can be found as normal flora in humans and animals and contains the inherent ability of this virulent gene, *eaeA* gene, *easT* gene, and *bfpgene*. A subset of these genes will be key players in the ability of the bacterium to cause disease. The products of such genes that facilitate the successful colonization and survival of the bacterium in or cause damage to the host are considered as virulence or pathogenicity determinants. This is one of the pre-determinant factors to *E. coli* ability to cause infection [5].

The *easT* gene is expressed in the neurogenic region at the time of neuroblast segregation and in cells in the peripheral and central nervous system, *bfpgene* is the Blue fluorescent protein derived from the mutated purple chromoprotein isolated from the sea anemone *Stichodactyla haddoni* and *easT* gene while *eaeA*, a gene necessary for the characteristic intimate attachment of EPEC to epithelial cells. is the focal point of this research work, all together constitute the pathogenic factor and function for *E. coli* pathogenic tendencies.

E. coli bacteria in their intestines can get on the meat. Slaughtered Beef combines meat from many different animals, increasing the risk of contamination with *E. coli*. While most people experience a few days of upset stomach and then recover fully, *E. coli* infections can sometimes be life-threatening [6].

This research work will seek to educate the Nigerian populace and worldwide as a whole and deplete that the most way to get an *E. coli* infection is by eating contaminated food, such as: Slaughtered Beef from contaminated Slaughtered houses and other beef handler. When cattle are slaughtered and processed.

MATERIALS AND METHODS

Bacteria isolates

This research employed 15 *E. coli* isolates coded as samples AA1, AA3-2, AA-3, A2, A1-2, A2-2, A4-2, A1-3, A4-3, A1-2, A2-2, A2-3, AA-2, and A1-3. The bacterial isolates were obtained from 48 slaughter beef samples from slaughter house in several Local markets in Akungba Akoko, Ondo State, Nigeria. There were 12 meat samples from the Akoko North market (ASAN) and 6 isolates, 26 meat samples from Akoko south market (AAS) and 2 isolates, 6 meat samples from the Akoko East market (AAE) and 5 isolates, and 4 meat samples from the Akoko West market (AAW) and 2 isolates were found. These isolates were screened for the presence of target virulent gene and identified as pathogenic *E. coli* [7,8].

Cultivation of bacteria isolates

These fifteen isolates were taken from isolate stock kept in a freezer at -20°C. These were then grown in Brain Heart Infusion Broth (BHIB) media and incubated for 24 h at 37°C. Bacterial growth was evident with increasing turbidity of BHIB media [8,9].

DNA extraction of bacteria isolates

DNA was extracted using the protocol stated by Osuntokun [10]. DNA was extracted using the protocol stated by Pitout [1]. Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28°C. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37°C, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and vortexed. The suspension was incubated for 10 min at 65°C and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at -20°C for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer [9,11].

Molecular identification of bacteria isolates

Primer sequences were as earlier documented. Reaction cocktail used for all PCR per primer set included (Reagent Volume µl) - 5X PCR SYBR green buffer (2.5), MgCl₂ (0.75), 10pM DNTP (0.25), 10pM of each forward and backwards primer (0.25), 8000U of taq DNA polymerase (0.06) and made up to 10.5 with sterile distilled water to which 2µl template was added. Buffer control was also

added to eliminate any probability of false amplification Table below shows the primer sequence and PCR profile used in amplifying each fragment. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair [11,12].

Molecular Integrity of bacteria isolates

The integrity of the amplified gene fragments was checked on a 1.5% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the

slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 min to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliters (2 l) of 10X blue gel loading dye (which gives color and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 min visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel [9,11].

RESULTS (Table 1)

Table 1. Proposed Target gene, primer used, rRNA sequence and profile of test isolates (*E. coli*).

Gene	Primer	Sequence 5'-3'	Profile
eaeA	eaeF	ATGCTTAGTGCTGGTTTAGG	An initial denaturing 5min at 94°C, then 35 cycles of 94°C for 30s, 60°C for 30s 72°C for 60s and terminate at 72°C for 10min
	eaeR	GCCTTCATCATTTTCGCTTTC	
easT	easF	CACAGTATATCCGAAGGC	An initial denaturing 5min at 94°C, then 35 cycles of 94°C for 30s, 60°C for 30s 72°C for 60s and terminate at 72°C for 10min
	easR	CGAGTGACGGCTTTGTAG	
bfp	bfpF	AATGGTGCTTGCGCTTGCTGC	An initial denaturing 5min at 94°C, then 35 cycles of 94°C for 30s, 52°C for 30s 72°C for 30s and terminate at 72°C for 10min
	bfpR	GCCGCTTTATCCAACCTGGTA	
hly	hlyF	AACAAGGATAAGCACTGTTCTGGCT	An initial denaturing 5min at 94°C, then 35 cycles of 94°C for 30s, 53°C for 30s 72°C for 60s and terminate at 72°C for 10min
	hlyR	ACCATATAAGCGGTCATTCCCGTCA	

Characterization of Virulence Gene analysis from fifteen isolates under investigation were depleted in **Figures 1-3**, showing the Agarose gel showing the amplification of eaeAgene, bfp gene and bfp gene in selected *E. coli*. Band

size of 248bp, 97bp and 324bp indicates positive amplification for all three virulent gene in eaeAgene (Sample 2, 6), easT gene (8, 1, 4, 5) and bfp gene (7. 1, 4, 5 and 7) respectively.

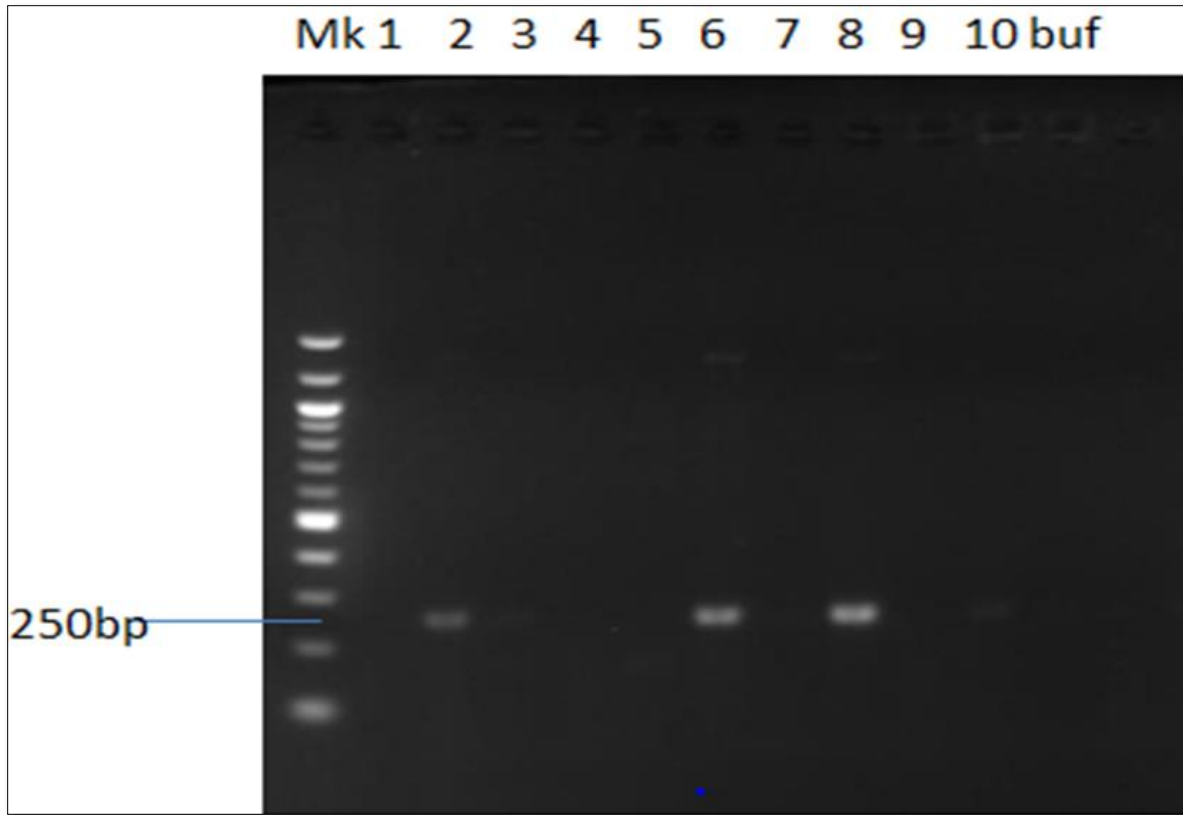


Figure 1. Agarose gel showing the amplification of eaeA gene in selected *E. coli*. Band size of 248bp indicates positive amplification. Presence of eaeA gene is positive only in Sample 2, 6 and 8.

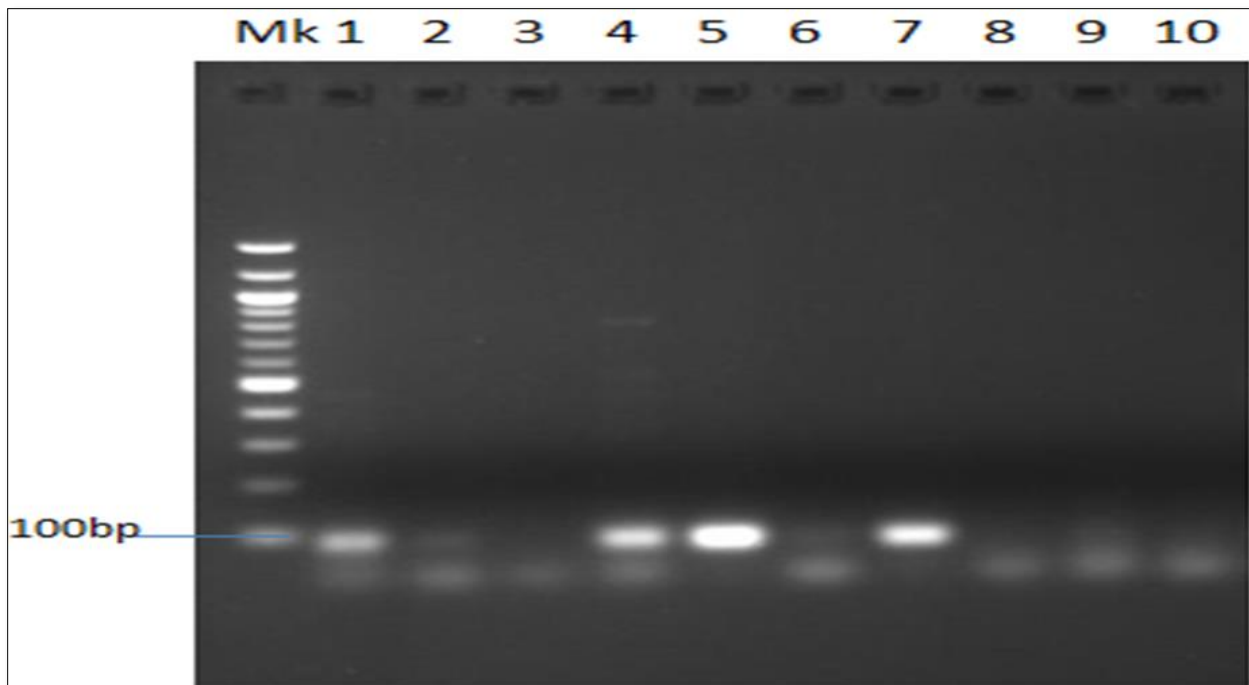


Figure 2. Agarose gel showing the amplification of easT gene in selected *E. coli*. Band size of 97bp indicates positive amplification. Presence of easT gene is positive only in Sample 1, 4, 5 and 7.

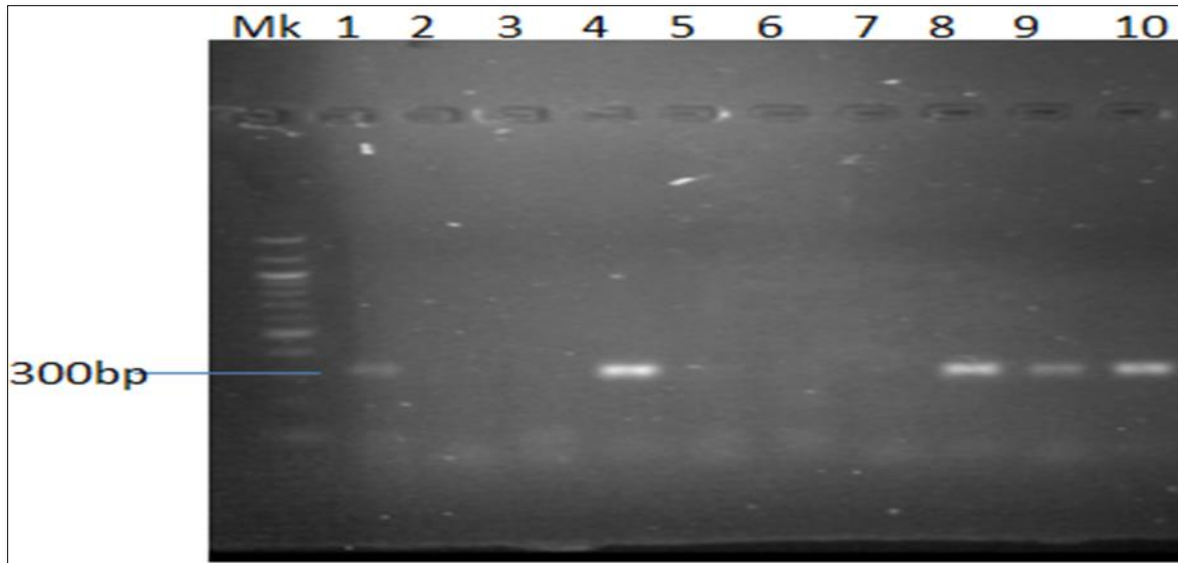


Figure 3. Agarose gel showing the amplification of bfp gene in selected *E. coli*. Band size of 324bp indicates positive amplification. Presence of bfp gene is positive only in Sample 1, 4, 5, 8, 9 and 10.

DISCUSSION

The aim and objective of this research work are to determine characterization of virulence gene in *E. coli* organisms isolated from slaughtered beef using 16s rRNA molecular sequencing method of analysis. *Escherichia coli* are an emerging and reemerging cause of food-borne infection. An estimated 10,000 to 20,000 cases of infection occur in the in Nigeria each year. Infection often leads to diarrhea, and occasionally to kidney and liver failure. Most enteric infection has been associated with eating undercooked, contaminated Slaughtered Beef under the investigation of this research work, *E. coli* is naturally found in the intestinal tracts of many farm animals, including healthy cattle, sheep, goats and etc., as a commensal organism ready to harm if opportunity arises [13].

The discovery of diarrheagenic *E. coli* in cow meat indicates a critical role as a reservoir for strains that transmit disease from animals to humans. Food produced from such animals as cows may be contaminated with DEC at the slaughterhouse, processing facility, or the consumer's kitchen. DEC infection may also come from the consumption of raw and unpasteurized milk and vegetables contaminated with feces [14].

Diarrheagenic *E. coli* (DEC) (is one of the first pathogens to be observed using today's advanced molecular diagnostic of 16S rRNA molecular gene sequencing methods. These methods are the most popular and reliable in differentiating *E. coli* (DEC) strains from those of non-enteric pathogenic bacteria. Moreover, the phylogeny and taxonomy of bacteria can also be studied using 16S rRNA gene sequence as differential genetic markers [6,15-18].

The gene molecular sequence of 16S rRNA is used because the 16S rRNA gene is found in almost all bacteria, the 16S rRNA gene does not change its function over time, and the 16S rRNA gene (1500 bp) is large enough for information purposes, one of the most interesting potentials of 16S rRNA gene is its ability to provide genus and species identification for isolates and also predetermined the virulent gene adherent in the gene clusters of the cell. This virulent gene plays an important role in the ability of *E. coli* to infect and establish infection and diseases [19-21]. The effectiveness of 16S rRNA should be stressed as a matter of urgency, Characteristics of molecular targets from these 16S rRNA methods allow the study of bacterial phylogenetics, both for bacteria detection or identification in clinical laboratories [22,23]. It is difficult to overlook the activity of the virulent gene under the scope of this research. The *east* gene is expressed in the neurogenic region at the time of neuroblast segregation and in cells in the peripheral and central nervous system of *E. coli* [24], this is one focal point of this research work.

However, the pathogenesis of *E. coli* should be mentioned, for understanding the activity of these virulent genes. *Escherichia coli* (EPEC) strains were the first *E. coli* strains associated with gastroenteritis and continue to be a leading cause of diarrhea. pathogenesis of EPEC infections and other *E. coli* strains three-stage model [25]. We can mention one or two stages under the scope of this research, for clarity purposes.

According to a model, postulated by a researcher [26]. The first model initial adherence of the (*Escherichia coli*) organism to the epithelial cell, recognized in tissue cultures as localized adherence, is characterized by the formation of

micro-colonies on the cell surface and is dependent on a large plasmid common to *Escherichia coli* strains [26]. The second model postulated is the initial contact, the bacterium transduces, a signal to the epithelial cell that results in the activation of host cell tyrosine kinase activity [27], and the third model postulated is the elevation of intracellular calcium concentrations [28]. *E. coli* becomes intimately attached to the membrane of the epithelial cell, with damage to host cell microvilli and accumulation of cytoskeletal proteins beneath the adherent organism [29]. The latter two steps are collectively referred to as attaching and effacing. Subsequent to these events, a subset of the bacteria enters the epithelial cell of host tissues.

However, there is a need to mention the virulent activity of the characterized gene responsible for the virulent factor in the species of *E. coli* under the scope of this research work. eaeA gene, initially termed eae, isolated as a locus this gene is necessary for the attaching and effacing activity of *E. coli*. Sighting an example in EPEC [30]. This eaeA gene is found on the chromosome of *E. coli* strains. The novel gene is responsible display attachment activity of all *E. coli* strain, which gives *E. coli* the ability to cause rapid infection [31,32]. eaeA gene for required intimate attachment to host epithelial cells and identification of this novel gene is a function of virulence determinant of *E. coli* strains. We can say that eaeA gene is the second chromosomal gene necessary for the virulent capability of *E. coli*.

Biologists use GFP as a marker protein. Gfp refers to the gene that produces green fluorescent protein. Fluorescent proteins help whole-body imaging of tumors on internal organs. These multicolored proteins have allowed the color-coding of cancer cells to grow in vivo with the distinction of different cell types, including host from the tumor, with single-cell resolution. Photoactivatable fluorescent proteins enable the tracking of photo labeled molecules and cells in space and time and can also be used for super-resolution imaging. Gfp can be termed genetically encoded sensors which makes it possible to monitor the activity of enzymes and the concentrations of various analytes [33].

The major disadvantage of studying GFP fusion proteins is that they are generally over-expressed relative to endogenous proteins. The GFP tag can, in principle, affect protein function which makes them more predominantly virulent genes, two advantages of GFP as a reporter of gene expression are that i. protein accumulation can be directly observed in living cells prior to quantitative analysis, and ii. GFP gene expression can be measured on a cell-by-cell basis [34].

BFP gene is the third virulent gene under the scope of this research work. They completely cleaved into the 201 and 123 bp fragments, and no 324 bp fragment remained in 16S rRNA sequencing profiling. We should mention the base pair which the gene function properly with positive amplification it was observe that the function between 97bp

to 324bp. (easT gene band size of 97bp, eaeA gene 248bp and bfp gene 324bp respectively in **Figures 1-3**, 324 bp band was observed due to the editing where both the enzyme and gRNA were applied together, 201 bp and 123 bp in the same sample of the same lane where restored GFP was expressed (**Figure 3**) we could see the cleaved bands which restored for genetic code is not possible which we have also observed from our confocal images. when an expression vector is introduced into the cell, an endogenous transcription factor binds to the vector promoter, Therefore, the transcription of endogenous genes is totally suppressed. Necessary transcription factors are depriving, the easT gene was found in *E. coli* strains they are detected more frequently in strains of *E. coli*, and other *E. coli*, family mention above. This suggests that their toxin is insufficient to cause diarrhea unless another virulence factor is present as an adjuvant which is demonstrated in this research work. These results agree with those reported by Savarino [35] who found the *astA* gene in a significant proportion of *E. coli* intestinal isolates from children without diarrhea in developing countries [35]. For this gene to function properly, the novel gene must work in conjunction with other novel gene like BFP, eaeA gene and etc. if we find one, other can also present in the race to make *E. coli* more virulent.

CONCLUSION

In conclusion, *E. coli* strains have received increased attention as a cause of human diarrhea in many populations. However, the virulence factors that characterize this type of diarrheagenic *E. coli* are not well known but with the advent of 16S rRNA molecular sequencing, it was deduced that easT gene, eaeA gene, and bfp gene novel genes are responsible for the virulent capability of *E. coli* strain present in Slaughtered Beef. This has been proved beyond all reasonable doubt and extra caution should be taken in the Slaughtered house to prevent Slaughtered Beef contamination, and to reduce the scourge of *E. coli* and its other species infestation.

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