Anti-biofilm and Antibacterial Activity of *Allium sativum* Against Drug Resistant Shiga-Toxin Producing *Escherichia coli* (STEC) Isolates from Patient Samples and Food Sources

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Abstract *Escherichia coli* (*E. coli*) colonizes human intestinal tract and is usually harmless to the host. However, several strains of *E. coli* have acquired virulent genes and could cause enteric diseases, urinary tract and even brain infections. Shiga toxin producing *Escherichia coli* (STEC) is an enterohaemorrhagic *E. coli* (EHEC) which can result in bloody diarrhoea and could potentially lead to deadly haemolytic uremic syndrome (HUS). STEC is one of the important food borne pathogens that causes food poisoning leading to diarrhoea and number of STEC outbreaks have occurred across the world. The use of standard antibiotics to treat STEC infection is not recommended as it increases the production of shiga toxin which could lead to HUS. Therefore, use of alternative approaches which include use of plant products to treat STEC infections have been gaining attention. The objective of this study was to evaluate the antibacterial and anti-biofilm activity of garlic

(*Allium sativum*) against STEC strains isolated from various patient and food samples using in vitro assays. The microbiological isolation of STEC from various patient and food samples resulted in eight STEC isolates of which seven strains were multidrug resistant. Antibacterial assay results indicated that all the strains exhibited dose dependent sensitivity towards garlic with zone of inhibition diameters ranging from 7 to 24 mm with 15 µl of fresh garlic extract (FGE). Minimum inhibitory concentration (MIC) of FGE for isolates ranged from 30 to 140 µl/ml. Interestingly, the biofilm formation of all isolates in presence of 4% of FGE decreased by 35 to 59%. FTIR analysis indicated that treatment with 1% FGE results in compositional and content changes in the biofilm. In addition, the total carbohydrate content of biofilm was reduced by 40% upon 1% FGE treatment. The results of the present study report for the first time the antibacterial and anti-biofilm activity of garlic against STEC. The findings will enable development of novel garlic organosulfide based drugs for the prevention and treatment of STEC infections.

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Abbreviation

STEC	Shiga toxin producing <i>E. coli</i>
MIC	Minimum inhibitory concentration
FGE	Fresh garlic extract
EMB	Eosin methylene blue agar
SMAC	Sorbitol MacConkey agar
CT-SMAC	Cefixime tellurite sorbital MacConkey agar
QS	Quorum sensing

Introduction

Escherichia coli are commensal bacteria that reside in human colon and rarely cause any disease. However, certain clones of *E. coli* are pathogenic by the virtue of acquiring virulence factors and are referred as *E. coli* pathotypes. Shiga toxin producing *E. coli* (STEC) constitute to enterohaemorrhagic *E. coli* (EHEC) class of intestinal pathotype. STEC infections cause bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhoea and could lead to potentially deadly haemolytic uremic syndrome (HUS) [1]. The intestine of ruminants is the main reservoir of STEC and consumption of contaminated meat and produce is the main cause of infection. Shiga toxin (Stx) is the primary toxin produced by STEC and based on lipopolysaccharide (O) and flagellar (H) antigens they are categorized into various serotypes. O157:H7 strain produces Stx and contains pathogenicity island called locus of enterocyte effacement (LEE) that codes for other virulence factors resulting in human disease [2]. Most of the non-O157:H7 STEC strains are also associated with human illness but go unreported due to lack of laboratory tests and clinical awareness. Stx1 and Stx2 are two subgroups of Stx family of toxin that cause apoptosis of intestinal cells and necrosis leading to enterocolitis, bloody diarrhoea and intestinal perforation. In addition, Stx can enter the blood stream and cause fatal renal failure due to kidney endothelial damage, microvascular occlusion and inflammation [3, 4].

A report estimated that around 2.8 million acute illnesses were caused by STEC infections annually between 1990 and 2012 in 21 countries effecting children of age 0–4 years more severely [5]. In USA alone O157:H7 strains causes 100,000 infections yearly whereas in Europe 6109 confirmed cases of STEC infections were reported in 2014 [6, 7]. The estimation of STEC infections in India is obscure due to paucity of data. However, according to some studies STEC was isolated from 1.4 to 1.6% of

bloody diarrhoea samples [8]. A sizeable number of STEC infections also occur in Africa [9]. In addition, 1999 outbreak in China caused due to a new O157:H7 strain resulted in 177 deaths due to HUS out of 195 infected individuals, mainly elderly females [10]. It has been noticed that children < 5 years are particularly more susceptible to STEC infections [11, 12]. Altogether, STEC is one of the main enteropathogens with prevalence all over the globe that can result in severe disease conditions with potential to cause death [13].

The current treatment strategy for STEC infections is mainly supportive therapy such as rehydration due to lack of effective treatment options [4]. The use of antibiotic is not recommended due to the risk of HUS progression [9, 14, 15]. It has been reported that antibiotic treatment increases Stx production either by releasing intracellular Stx due to lysis or by induction of Stx expression from prophage due to genomic insult caused by antibiotics [16]. Thus, there is a lot of interest in developing novel alternative therapies to treat STEC infection such as antibodies, Stx receptor analogs, novel antibacterial agents, probiotics, phages and plant derived natural products [17–19]. The antibacterial activity of Chinese cinnamon, Spanish oregano and savory essential oils against STEC was found to be mediated by compromising the cell wall and membrane [20]. Green tea and cranberry has also been reported to exhibit antibacterial activity against STEC [21]. The antibacterial activity of garlic (*Allium sativum*) is well documented against many pathogenic bacteria including antibiotic resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) [22–26]. A very important aspect of STEC infection is survival of the bacteria outside the host or reservoir under harsh condition until it infects a new host or reservoir. Biofilm formation facilitates such survival on contaminated food surfaces and plays a major role in foodborne bacterial infections [27]. Biofilm is accumulation of microorganisms on animate and inanimate surfaces with the help of extracellular polymeric substance which is composed of proteins, polysaccharides and nucleic acids which plays an important role in infection and bacterial resistance [28]. Quorum sensing (QS) is chemical communication system through which bacteria interact with each other when present in high densities. Among other phenomenon, formation of biofilm is mediated by QS and can lead to expression of attachment and virulent genes that are critical for colonization and pathogenesis [17]. Inhibition of biofilm formation by inhibiting QS is a clever approach to fight bacterial infections and diseases. Extensive research and recent advancements in technology has enabled development of various assays to monitor QS which can be used to screen and discover QS inhibitors (QSI) as antipathogens [29]. Natural products are of great importance as many of them can be used as therapeutics to

treat bacterial infections by their virtue to inhibit biofilm formation by interfering with QS. It is recently reported that phytochemicals such as limonoids, coumarins, harmaline and nonharmaline along with antibacterial activity also exhibit anti-biofilm activity against *E. coli* O157:O7 [30, 31]. The need to keep looking for new antibacterial and anti-biofilm agents is always there due to emergence of drug and QSI resistant bacterial strains [32]. It is suggested that biofilm formation could have an influence on the pathogenesis of STEC, however more studies are required to better understand the relevance of biofilm in the STEC pathogenesis [33].

In the present study, we isolated STEC from patient samples and common food samples such as water, milk and food from local stalls. The sensitivity of these isolates against antibiotics and garlic was investigated. As attachment and formation of biofilm plays an important role in STEC infection and pathogenesis, we investigated the effect of garlic on the biofilm formation of STEC isolates.

Materials and Method

Isolation of STEC

Food and water samples were collected from different sites in and around Sagar district, Madhya Pradesh, India. Stool samples from patients suffering from diarrhoea were collected from Bundelkand Medical College, Sagar, Madhya Pradesh, India and King George's Medical University, Lucknow, Uttar Pradesh, India. In brief, the samples were transported in sterile container and initially plated on eosin methylene blue (EMB) agar media (selective and differential media) and were incubated at 37 °C overnight to selectively grow Gram-negative bacteria. Lactose fermenting *E. coli* were differentiated from non-fermenters due to their metallic green colonies following overnight incubation. In order to differentiate between non-pathogenic and enterohaemorrhagic *E. coli*, these colonies were picked and streaked on MacConkey agar with Sorbitol (SMAC) differential media and were incubated overnight at 37 °C [34]. The following day colorless colonies were picked and were further confirmed by plating on cefixime and tellurite (CT)-SMAC selective media [35]. The colonies which grew on (CT)-SMAC were collected and considered as STEC.

Preparation of Fresh Garlic Extract

The garlic bulbs were washed with double distilled water and wiped with ethanol before homogenizing by crushing in a mortar and pestle. The crushed garlic was placed in a muslin cloth and pressed to obtain fresh garlic extract. The

extract was centrifuged at 6000 rpm for 10 min and supernatant was collected as fresh garlic extract (FGE), which was stored at – 80 °C till further use.

Antibacterial Activity Assays

Antibiotic sensitivity and antimicrobial activity of FGE was tested with disc and well diffusion method respectively. In brief, 100 µl of 0.5 McFarland bacterial culture was evenly spread on Luria–Bertani (LB) agar plates. The growth of the isolates was not uniform on Mueller–Hinton Agar (MHA) therefore LB agar plates were used for antibacterial studies. Antibacterial sensitivity was performed using discs of Amoxicillin (Amx-10 µg), Vancomycin (Va-10 µg), Ciprofloxacin (Cip-10 µg), Linezolid (Lz-10 µg), Clindamycin (Cd-10 µg), Lincomycin (L-10 µg), Teicoplanin (Te-30 µg), Nfacillin (Nf-1 µg) and Rifampicin (Rif-15 µg). The antibacterial activity of FGE was tested by puncturing wells in the plates and different volumes (5, 10 and 15 µl) of FGE was added [36, 37]. The plates were incubated at 37 °C for 18 h. The zone of inhibition was measured and recorded around the disc and well after the incubation. Broth serial dilution technique was used to determine MIC in 24 well plates. 750 µl FGE serially diluted with LB media to a final volume of 1500 µl to which 500 µl of 0.5 McFarland inoculum of STEC isolates were added and incubated at 37 °C for 18 h. The concentration where no turbidity was noticed was recorded as the MIC [38].

Biofilm Formation

Biofilm assay was performed as per the protocol described by Merritt et al. [39]. In brief, different STEC isolated were grown in LB media in the presence of 0, 2, 3 and 4% of FGE in 96 well plates for 18 h at 37 °C. After incubation, the wells were washed twice with phosphate buffered saline and once with double distilled water. The biofilm was stained with 100 µl of 0.1% crystal violet for 10 min and rinsed once with double distilled water. Finally, crystal violet was solubilized using 33% acetic acid and OD at 570 nm was recorded (Multiskan GO, Thermo Fisher Scientific) to determine the biofilm formation.

Protein and Carbohydrate Estimation

Protein and carbohydrate content of the biofilm were estimated using protocol mentioned by Kim et al. [40] with some modifications. LN12 isolate (0.5 McFarland) was incubated in 6 wellplate with 0, 0.5 and 1% FGE for 72 h at 37 °C and resultant biofilm was pelleted by centrifugation at 6000 rpm for 10 min and washed once with PBS. Collected samples were dried for 24 h and sonicated in PBS

followed by centrifugation at 6000 rpm for 5 min and supernatant was used for protein and carbohydrate estimation. Protein content was estimated by adding Bradford reagent to the supernatant and after 30 min incubation absorbance was recorded at 595 nm (Multiskan GO Thermo Fisher Scientific). To estimate total carbohydrate content, the supernatant was incubated with H_2SO_4 and 5% phenol for 10 min and absorbance was recorded at 490 nm (Multiskan GO ThermoFisher Scientific).

FTIR Analysis

The powder sample of LN12 biofilm was prepared in the presence of 0 and 1% FGE as mentioned earlier. The powder samples were characterized using Bruker Vertex 70 V FT-IR spectrometer (Bruker Optics), equipped with KBr beam splitters and IR spectra were recorded in transmission mode from 4500 to 1000 cm^{-1} at a spectral resolution of 4 cm^{-1} . OPUS software version 7.8 was used to perform data analysis.

Statistical Analysis

Experiments were repeated in triplicates three times. The data were subjected to analysis of variance, and mean values in each treatment were compared using Duncan's multiple range test (DMRT) using SPSS (version 18) at 5% probability ($p \leq 0.05$) [41].

Results

Antibiotic Sensitivity of STEC Isolates

The treatment of STEC infections with antibiotics is generally not recommended due to risk of progression to HUS [9, 42]. Although the use of antibiotics for the treatment of STEC infections is discouraged and controversial, to understand the general antibiotic resistance of the isolates antibiotic sensitivity of all eight STEC isolates was performed with eight different antibiotics. According to the results shown in Supplementary Table 1, isolate CKN1 is sensitive to two antibiotics i.e. ciprofloxacin and rifampicin with multiple antibiotic resistances (MAR) index of 0.77 and isolates CKN2, LN12, LN2, LN9.1, LN10.1 and ECD were sensitive to only one antibiotic i.e. rifampicin with MAR index of 0.88 out of nine antibiotics that were tested. Isolate LN6.2 was sensitive to eight antibiotics out of nine that were tested with MAR index of 0.11. The antibiotic sensitivity data indicates that except LN6.2 all isolates had MAR index more than 0.6 making them highly drug resistant STEC isolates.

Antibacterial Activity of Garlic Against STEC Isolates

The antibacterial activity of FGE was tested against all of the eight STEC isolates. The MIC value of FGE ranging from 30 to $140\text{ }\mu\text{l/ml}$ against each isolates (Supplementary Table 2) indicates that garlic exhibits antibacterial activity against all STEC isolates. In addition, we also tested the antibacterial activity of FGE using well diffusion assay and Fig. 1 shows the zones of inhibition with 5, 10 and $15\text{ }\mu\text{l}$ of FGE against all the isolates. All the STEC isolates exhibit susceptibility to FGE extract in a dose dependent manner. Both MIC and well diffusion antibacterial assays indicate that garlic exhibits antibacterial activity against STEC isolates including highly drug resistant isolates.

Anti-biofilm Activity of Garlic Against STEC Isolates

The ability of garlic to inhibit biofilm formation of STEC isolates was also investigated by treating STEC with sub-MIC concentration of FGE. The effect of 2, 3 and 4% of FGE on the biofilm formation ability of STEC isolates is shown in Fig. 2. The data indicates that the FGE reduced biofilm formation of all STEC isolates in a dose dependent manner and the data shown is the average of three independent experiments. The ability of biofilm formation by all STEC isolates was reduced significantly in the presence of 4% of FGE. The data clearly indicates that FGE extract significantly reduces the biofilm formation of all the STEC isolates.

Garlic Effects the Composition and Content of STEC Biofilm

In order to get more insights into the mode of action through which garlic inhibits biofilm formation ability of STEC isolates FTIR analysis was performed. The FTIR analysis of biofilm formed by LN 12 in absence and presence of 1% FGE was analyzed using FTIR. The biofilm sample was obtained with 1% FGE since large amount of sample is required for FTIR analysis which is difficult to obtain considering that in the presence of higher concentration of FGE formation of biofilm is reduced by almost 50%. As expected the FTIR data (Fig. 3) shows peaks in four distinct regions: I-3000 to 2800 cm^{-1} , II-1700 to 1500 cm^{-1} , III-1500 to 1200 cm^{-1} and IV-1200 to 900 cm^{-1} that correspond to membrane fatty acids; amides of protein and peptides; proteins, lipids, carbohydrates and phosphate containing molecules (nucleic acids and phospholipids); and polysaccharides and carbohydrates respectively [43]. Although FGE treatment resulted in shifts in all peaks, prominent shifts occurred in peaks 1540, 1235 and

Fig. 1 Antibacterial activity of garlic against STEC isolates. The graph represents average zone of inhibition (mm) with increasing volume of FGE against STEC isolates as labelled in the figure. The graph shows that there is dose dependent increase in the antibacterial activity of FGE against STEC isolates. The graph represents the data of three sets of triplicate data

Fig. 2 Anti-biofilm activity of garlic against STEC isolates. The graph shows the average 590 nm reading of crystal violet staining of biofilm which is normalized to untreated control sample. The effect of increasing percentage of FGE against STEC isolates shows decrease in biofilm formation as labelled. Different letters indicate mean values ($n = 3$) that are significantly different at $p < 0.05$, according to DMRT for each different parameter

1054 cm^{-1} that correspond to amide-II of peptides and proteins, nucleic acids and polysaccharides respectively indicating changes in chemical composition of the biofilm. In addition, notable decrease in the intensity of peaks 3275, 1630, 1235 and 1054 cm^{-1} indicate that amounts of proteins and polysaccharides, proteins, nucleic acids and polysaccharides decreased respectively. Although the amide-I peak intensity decreased there was no change in overall protein content of the biofilm (data not shown). The total carbohydrate content of the biofilm was estimated to understand the effect of FGE on the polysaccharide content of LN12 biofilm. In agreement with the FTIR data the total carbohydrate of biofilm in the presence of 0.5 and 1% of

FGE decreased approximately by 20 and 40% respectively compared to untreated control (Supplementary Fig. 1). The data indicates that garlic inhibits biofilm formation ability of STEC by changing the composition of the biofilm and also by reducing the amount of polysaccharide content of biofilm.

Discussion

The first STEC infection occurred in 1983 due to consumption of undercooked hamburgers in United States [44]. Since then the occurrence and severity of STEC

Fig. 3 FTIR analysis of LN12 biofilm. The graph shows that FTIR spectra of LN12 biofilm in the absence of FGE (control-black line) and in the presence of 1% FGE (blue/gray line) (color figure online)

infections has increased and incidence has spread all over the world. Haemorrhagic colitis and diarrhoea caused by STEC is one of the important emerging infectious diseases of recent past. Although, STEC is not considered as one of the main reasons of diarrhoea in India but several articles have been published describing isolation of STEC from patient samples and various food sources [45]. In this study we isolated eight STEC strains from patient and food samples indicating the prevalence of STEC in central and western India. It was noticed that seven of these eight isolates were highly drug resistant. Rifampicin was the only antibiotic to which all the isolates were sensitive and CKN1 was sensitive to both ciprofloxacin and rifampicin. Whereas, isolate LN6.2 was sensitive to all the tested antibiotics except for Nfacillin. The antibiotics resistance was much higher in our isolates compared to a previous study by Khan et al. [46].

The current treatment options of STEC infection is supportive care in the form of rehydration and administration of antibiotics is discouraged due to exacerbation of Stx production and/or release increasing the chances of HUS progression [7]. Thus, alternative treatment such as antibodies against Stx [47], use of probiotics [48] and divalent metals are being evaluated [49]. In addition, phytochemical such as essential oils and other phytochemicals are also being evaluated for anti-STEC activity [21, 44, 45]. Novel alternative treatment options for STEC infections using natural products have been investigated with encouraging results [19]. Recently, derivatization of

cationic polysaccharides has been shown to exhibit activity against *E. coli* [50]. Similarly, we also wanted to test the anti-STEC activity of garlic which has been reported to have excellent antibacterial activity against many pathogenic bacteria. It has also been reported that garlic exhibits excellent antibacterial activity even against multi drug resistant pathogenic bacteria. As seven of the eight STEC isolates were highly drug resistant. Therefore, we also wanted to test, if garlic exhibits similar antibacterial activity against highly drug resistant STEC isolates. As expect garlic was highly effective against all the STEC isolates including the highly drug resistance isolates. The amount of FGE required for anti-STEC activity was higher for CKN1 isolate compared to other isolates. The MIC for CKN1 was 140 µl/ml whereas zone of inhibition started from 10 µl of FGE. The antibacterial activity of garlic against STEC has been studied earlier [4, 12, 51]. However, these tests were done using standard strains and not with isolates. In addition, in these studies the anti-STEC activity was noticed when garlic was used in combination with other plant products. In the present study, we report anti-STEC activity of FGE against STEC isolates that were highly drug resistance.

Further, we have reported the anti-biofilm activity of garlic against STEC for the first time. On an average the biofilm formation of STEC isolates was decreased by approximately 48 percent at 4% FGE. The anti-biofilm activity of garlic against STEC is of great importance as formation of biofilm plays a very important role in

colonization of STEC in reservoir animal and also in persistence at other sites (green leafy vegetables). It was found that garlic alters the biofilm composition and in particular reduces the carbohydrate content of the biofilm while it had no effect on the protein content indicating that garlic reduces biofilm formation by reducing the amount of carbohydrate in the biofilm. This observation is very important because cellulose along with curli protein has synergistic effect on attachment and biofilm development of *E. coli* [52] and expression of polysaccharide by STEC play a major role in attachment [53]. In addition, expression of cellulose was noticed in *E. coli* isolated human gastrointestinal tract suggest the role of cellulose in attachment and colonization [54]. Development of novel garlic-organosulfide based agents with anti-biofilm activity against STEC could help in evolution of strategies to tackle the problem of STEC infection and spread. Overall, the present study comprehensively indicates that garlic exhibits antibacterial and anti-biofilm activity against STEC isolates. Thus more research should be directed towards understanding the mechanism of action through with garlic is exhibiting antibacterial and anti-biofilm activities.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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