Review Article

Entero-Aggregative- Haemorrhagic Escherichia coli (EAHEC) Serotype O104:H4 -The Evolving "Superbug".

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Abstract

The rare, E.coli strain O104:H4 has been identified as the causative agent of one of the largest ever reported food-borneoutbreaks of gastroenteritis and Hemolytic-uremic syndrome (HUS) in Germany this year. This hypervirulent pathotype possess a unique combination of two pathogens: enterohemorrhagicE.coli (EHEC) and enteroaggregative E.coli (EAEC) strains. The serotype has rarely been described previously in humans and never associated with any earlier large scale EHEC outbreaks. It is now being referred to as the Entero-Aggregative-Haemorrhagic Escherichia coli (EAHEC).Advances in high-throughput sequencing technologies helped in rapid complete genome sequencing of the outbreak strains by different laboratories.Comparison of the genome sequence of the outbreak strain with other diarrhea-associated EAEC serotype O104:H4 indicate that the chromosome of the outbreak strain is most similar to that of an early isolated EAEC strain 55989 and has evolved to become more virulent by the acquisition of a Shiga toxin 2 encoding prophage, a plasmid encoding CTX-M beta-lactamases, and substituting the aggregative adherence fimbria III (AAF/III)with the rarer aggregative adherence fimbria I (AAF/I). The present article reviews the virulent traits ofthe outbreak strain, and also presents an update of the different intervention strategies that are being tested for the treatment of infections by such highly pathogenic strains.

Key words: EHEC outbreak, EAHEC, Shiga toxin, gastroenteritis

Introduction

Escherichia coli (E.coli) are gram negative bacilli that are the predominant facultative anaerobe of the human gastrointestinal tract. Most E.coli strains are harmless, but some strains have acquired genetic determinants (virulence genes) rendering them pathogenic and can cause intestinal and extraintestinal infections in humans[1]. Evolution of new virulent strains of E.coli is mainly by horizontal gene transfer of genetic elements encoding virulent traits through an existing population[2]. The pathogenic enteric E.Coliare classified on the basis of serological characteristics, virulence factors, clinical symptoms and epidemiologic and pathogenic profiles. Gastroenteritis causing virotypes include: enterotoxigenic E.coli (ETEC), which are characterized by producing heat-labile(LT) and heat stable (ST) enterotoxins, enteropathogenic E.coli (EPEC), which use an adhesin known as intimin to bind intestinal host cell, and elicit characteristic attaching and effacing lesions, enteroinvasive E.coli (EIEC), which has the ability to invade epithelial cells and causes a syndrome that is identical to shigellosis ,enterohemorrhagic E.coli (EHEC) which is moderately invasive, induces characteristic lesions known as attaching and effacing (A/E) lesions and produce Shiga toxins (Stxs)/ verotoxins (VT), enteroaggrega-

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tive E.Coli(EAEC), whose aggregative adherence is characterized by a 'stacked brick' formation of bacterial cells attached to HEp-2 cells, and diffusely adherent E.coli (DAEC), which demonstrate pattern of diffuse adherence [3]. Extraintestinal infections are caused by three separate E.coli pathotypes: uropathogenic (UPEC) strains that cause urinary tract infections, strains involved in neonatal meningitis (MENEC), and strains that cause septicemia [4].

Clinical Presentation

EAEC are a heterogeneous subgroup of emerging food borne pathogens that has been implicated for watery, mucoid ,secretory diarrhea and malnutrition in children in developing countries [5]. It is also responsible for diarrhea in adults, including travelers and patients infected with human immunodeficiency virus (HIV) [3]. EAEC initially adheres to the intestinal mucosa mostly that of terminal ileum and colon aided by the aggregative adherence fimbriae (AAF) and forms a thick mucoid biofilm, followed by secretion of enterotoxins and cytotoxins that result in mucosal toxicity, mucosal inflammation and diarrhea [(4)]. EHEC colonize the gastrointestinal tract and after an incubation period of 2-3 days cause diarrhea and abdominal pain. In about 80% of cases after a 2-4 day interval there is progression into hemorrhagic colitis (HC) or bloody diarrhea [6]. Within 3-13 days after the beginning of diarrhea about 10-15% of patients with HC go on to develop a life-threatening condition known as hemolytic uremic syndrome (HUS) which includes the triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure [6,7]. It is the most frequent cause of acute renal failure and mortality in children[8]. The most significant serogroups among EHEC strains are O26:H11, O91:H21 O103:H2, O111: H8, and 157:H7 [8]. Among them, E.Coli O157:H7 is the predominant serotype that has caused major outbreaks of gastroenteritis worldwide and is of global concern to public health [9]. Extrarenal complications such as rhabdomylosis, myocardial and CNS damage are associated with increased mortality in E.coli O157:H7 infections [10]. E.coli O157:H7 belongs to the larger category of Shiga toxin producing E.coli (STEC).Shiga toxins (Stxs) produced by EHEC are the major virulence factors that are thought to be responsible for the pathogenesis of HUS [1]. Domestic animals especially healthy cattle and sheep act as reservoirs of this virulent strain and ingestion of meat products, vegetables, milk, juice or water contaminated with bovine waste result in human infections [11].

EHEC outbreak in Germany, May to June 2011

Outbreaks of Shiga- toxin producing EHEC infections are reported every year mainly in summer and autumn from different parts of the world. The world's biggest outbreak was recorded in Japan from May to October, 1996 affecting children mainly in elementary and junior high schools and included over 8000 cases (108 with HUS) [12]. White radish-sprouts contaminated with E.coli O157:H7 was the source of the pathogen [12]. This year Germany was the starting point of one of the largest ever reported ood-borne EHEC outbreaks of gastroenteritis and HUS. The latest update indicate that as of July 22, 2011, a total of 4075 outbreak cases, 908 of which (23 %) involved HUS, including 34 fatal cases (3.7%) have been confirmed [13]. Germany accounted for over 95% of the STEC cases. Other than Germany and other EU states, few cases have also been reported from United States of America and Canada and can be linked to travel-related exposures in north Germany shortly before becoming ill [14]. Isolation of the strain and PCR-based detection of specific marker genes O104 lipopolysaccharide (LPS) gene (rfb0104), H4 flagellin- encoding gene (fliCH4), Shiga- toxin[2] encoding gene (stx[2]),and tellurite resistance encoding gene (terD) identified the rare, virulent, enterohemorrhagic E.coli strain O104:H4 as the causative agent of the EHEC infection outbreak [2]. It belongs to the B1 phylogenetic group and combines virulence potentials of two pathogens: Shiga-toxin-producing E.coli and enteroaggregative E.coli. E coli O104:H4 serotype has rarely been described previously in humans and never associated with any earlier large scale EHEC outbreaks. In the past decade the other reported cases of HUS associated with this strain include two isolates from patients with HUS in Germany in 2001, one in France in 2004, another single case in 2006 from Korea,two cases of HUS in the Republic of Georgia in 2009, and one case from Finland in 2010[15].Compared to the previous large EHEC outbreaks the present outbreak has certain unique clinical and microbiological distinctiveness which are listed in Table [1].

- The causative agent is the much rare, hybrid serotype O104:H4, rather than the more common O157:H7 associated with previous EHEC outbreaks.
- EHEC associated haemolytic uremic syndrome (HUS) occurs more frequently in children, rare in adults, but in the present outbreak most of those afflicted (89%) are adults.
- Among adults, for reasons unexplained, two thirds are women(60% of the EHEC cases and 69% of the HUS cases)
- The proportion of patients who developed HUS was about 25 %, unusually higher as compared to previous outbreaks (1-15%), indicating increased virulence of the strain.
- Neurological complications ranging from, delirium to status epilepticus was observed in about 30% of the cases.
- Median incubation period of O104:H4 is 8 days, which is longer than the 3-4 days incubation period that is observed for O157:H7.
- The strain has an extended spectrum beta-lactamases (ESBLs) genotype and harbours TEM-1 and CTX-M-15 beta-lactamase genes that makes it resistant to numerous antibiotics including third generation cephalosporins .

Table I: Unique characteristics of the O104:H4 outbreak, 2011.(Adapted from references[2, 15, 16]. Epidemiological evidence pointed to contaminated bean sprouts as the outbreak source. On June 12th this was confirmed by isolation of O104:H4 from sprouts [17].Since, 10 June, there has been a decline in the number of newly reported cases which indicate the gradual tapering of the outbreak .

Escherichia coli O104:H4: The new "Chimeric Superbug"

Characterization of seven isolates, Danish isolate (EU-RL, Rome), LB222692 (University of Munster, Germany), GOS1 and GOS2 (Gottingen Genomics Laboratory, Germany, TY-2482 (Beijing Genomics Institute, China), C227-11 (Institute of Genomic Studies, University of Maryland School of Medicine), and H112180280 (Health Protection Agency, Cambridge, United Kingdom) have all provided insights into the genomic sequences of chromosomes and plasmids, virulence traits and phylogeny of O104:H4 Complete genomic sequence analysis of six isolates derived from the German outbreak has shown about 99.5% similarity in their genetic makeup suggesting that the outbreak is clonaland single-sourced [13,18,19,20]. The outbreak strain has one circular chromosome of about 5.31Mbp in length and harbors a total of three plasmids; onelarge plasmid (~95kb), an intermediate size plasmid (75 kb) and a smaller third plasmid of 1.5 kb [(13)]. The chromosome has a total of approximately 5,215 protein-coding sequences covering about 87% of the genome, and has a GC content of 50.6%. [19].The large plasmid is almost identical to the pEC_Bactec plasmid and carry the genes including TEM-1 and CTX-M-15 for extended spectrum beta-lactamases (ESBLs) phenotype (resistant to all penicillins and cephalosporins)[13]. The intermediate-size plasmid is similar to pAA-type plasmid of EAEC strains and harbors the gene cluster required for biogenesis of a rare type of aggregative adherence fimbria (AAF/1) [13,20].

Genes encoding manyEAEC virulent factors such as the protein coat secretion system (Aat), the dispersin (aap), AggR transcription activator, and serine protease autotransporters of Enterobacterriaceae (SPATEs)are also located on this plasmid which point to its decisive rolein the pathogenicity of the German outbreak strain[13].

Genes encoding known virulence factors were not found on the large and small plasmids. Genomic characterization of the O104:H4 outbreak strain showed that it carry genes frequently found in two types of pathogenic E.coli, the EAEC and EHEC[18].

Comparison of the genome sequence of the outbreak strain with seven diarrhea-associated EAEC serotype O104:H4 indicate that the chromosome of the outbreak strain is most similar(nucleotide identity of 99.8%)to that of EAEC strain 55989 which was first isolated a decade ago from the stool samples of adults infected with human immunodeficien-

cy virus (HIV), in Bangui, Central Africa [13]. Phylogenetic analysis also revealed its close relationship to a strain 01-09591, which was isolated in 2001 from a child with HUS in Germany [13,18].The present outbreak strain is unique from typical EAEC strains due to the presence of a prophage integrated in the chromosome encoding the Shiga toxin 2 (Stx2), which is a distinctive for EHEC strains[2]. Sequence analysis of Stx2 showed it to be 99% homologous to Stx2a from E.coli O157:H7 strain

EDL933[13]. Thus the outbreak strain is a hybrid pathotype combining the virulence potentials of both EHEC and EAEC strains: production of Stx2 and its ability to aggregate and colonization of epithelial cells. This ability to adhere to intestinal epithelium might assist systemic absorption of Shiga toxin and could explain the high number of associated cases of HUS. The German outbreak strain seems to be a typical EAEC strain that has become extremely pathogenic by the acquisition of a Stx-encoding prophage,a plasmid encoding CTX-M_15 ESBL, and substituting the AAF/III fimbriae with the rarer AAF/I fimbriae[13,18,19,20]. Presence of three SPATE encoding genes(pic, SigA,and SepA) whose products promote intestinal colonization, rounding and exfoliation of enterocytes, and increased cytotoxicity has enhanced the virulence of the strain [(13)] The hypervirulent phenotype is now referred to as Entero-Aggregative-Haemorrhagic Escherichia coli (EAHEC)[18].Comparison of the serotype/ virulence loci of the different outbreak isolates with the reference strains 55989 HUS causing strain 01-09591 is listed in Table [2].

Serotype/ Virulence loci	German Isolates			Reference strains			
	LB226292 (EAHEC)	GOS2 (EAHEC)	TY248 (EAHEC)	C227-11 (EAHEC)	112180280 (EAHEC)	01-09591 (EAHEC)	55989 (EAEC)
O104:H4							
rfb$_{O104}$	+	+	+	+	+	+	+
flic$_{H4}$	+	+	+	+	+	+	+
STEC							
Stx2	+	+	+	+	+	+	--
subAB	--	--	--	--	--	--	--
eae	--	--	--	--	--	--	--
hyla	--	--	--	--	--		
iha	+	+	+	+	+	+	+
lpf026	+	+	+	+	+	+	+
terD	+	+	+	+	+	+	--
saa	--	--	--	--	--	--	--
fyuA	+	+	+	+	+	+	--
EAEC							
aatA	+	+	+	+	+	+	+
aggA	+	+	+	+	+	--	--
agg3A	--	--	--	--	--	+	+
aggR	+	+	+	+	+	+	+
aap	+	+	+	+	+	+	+
sigA	+	+	+	+	+	--	--
pic	+	+	+	+	+	+	+
astA	----------	+	+				
sepA	+	+	+	+	+	--	--
ESBLs							
TEM-1	+	+	+	+	+	--	--
CTX-M-15	+	+	+	+	+	--	--

Table II: Comparison of the serotype/virulence loci of with the reference strains (EAEC:Enteroaggregative E.coli,EAHEC: Enteroaggregative haemorrhagic E.coli).

Gene products:rfbO104 : lipopolysaccharide (LPS) gene; flicH4 : H4 flagellin- encoding gene ;Stx2 : Shiga toxin2; subAB : subtilase cytotoxin; eae : intimin ; hyla :EHEC hemolysin ;iha : IrgA homologue adhesion; lpf026 : Structural subunit of long polar fimbrie of STEC 026;terD : tellurite resistance ;saa : STEC agglutinating adhesin ;fyuA : component of iron uptake system;aatA: EAEC virulence plasmid (pAA) ; aggA : Pilin subunit of AAF/I; agg3A:Pilin subunit of AAFIII;aggR : transcription regulator AggR;aap : dispersin;sigA: serine protease autotransporter; pic : protein involved in intestinal colonization ;astA : EAEC heat stable enterotoxin1;sepA : serine protease A TEM1:TEM beta-lactamases ; CTX-M-15: CTX-M beta-lactamases. Positive(+), Negative(--). (Adapted from Ref:2, [13, 18, 19, and 20]

Shiga toxin 2-the critical virulence factor of EAHEC O104:H4

Shiga toxins are produced by the bacteria in the gut lumen, released into the circulation and then carried to different target sites such as the kidneys, brain and other organs where they damage the microcirculation, causing vasculitis and induces other toxic effects [6,21]. It has been observed that enteric STEC infections are usually not accompanied by bacteraemia[7]. The Stx family comprises a group of structurally and functionally related exotoxins that have an AB5 subunit structure. Both A and B subunits combine to form the 70 kDa 'Shiga holotoxin' (Fig 1A)[22]. The enzymatically active A subunit, StxA is non-covalently attached to the pentamer of identical B fragments that form the B subunit, StxB (Fig1B). B-subunits form a doughnut-shaped structure which enfolds the carboxy terminus of the StxA subunit (Fig1B and 1C)[23]. The B-moiety is responsible for binding to the neutral glycosphingolipid globotriaosylceramide (Gb3) cell surface receptors of target cells [(24)]. There is a relatively limited expression of Gb3 in normal human tissues and its functions are not fully elucidated. Gb3 occurs mainly on renal glomerular endothelial, mesangial, and tubular epithelial cells, microvascular endothelial cells in intestinal lamina propria, in platelets, and in subsets of germinal centre B lymphocytes[6,7,21]. Lower level of Gb3 expression has been observed in neurons,endothelial cells in the central nervous system, monocytes, and monocyte-derved macrophages and dentritic cells[21]. The molecular structure of Gb3 with its ceramide backbone and sugar molecules is shown in Fig 1D.

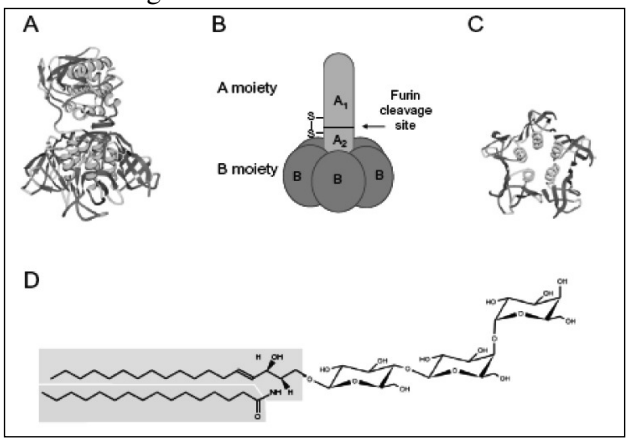

Fig I: Shiga toxin structures. A: Side view of 70 kDa holotoxin, B. Graphic representation of Shiga toxin, C: Ribbon diagram of the doughnut-shaped B moiety composed of five identical 7.7 kDa subunits, D: Chemical structure of Gb3. The ceramide backbone (indicated in blue) has a relatively invariable chain length of 18 carbons, whereas the fatty acyl chain (indicated in pink) occurs with both variable lengths and degrees of saturation, here shown as C16:0.
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After binding of the pentameric B subunit to its cellular toxin receptor Gb3, Stx toxin is internalized into target cells mainly by clathrin-dependent endocytosis[25]. Internalization of the Stx by clathrin- independent mechanism as a result of the toxin driven clustering of Gb3 receptors have also been reported [26]. Following internalization, Stx is delivered to the trans-Golgi network and then routed by retrograde transport to the endoplasmic reticulum and finally to the cytosol [27] During this transport, a protease sensitive loop located at the C-terminal region of StxA moiety is nicked by the membrane-associated endoprotease furin into the catalytically active N- terminal A1 fragment and a C-terminal A2 fragment (Fig 1B). The A1 fragment remains linked to the StxA2-StxB complex by a disulfide bond which is subsequently reduced in the ER, releasing the active A1 component [28]. The toxin A1 fragments has N-glycosidase activity and specifically depurinate the 28S eukaryotic rRNA of the 60S ribosomal subunit, inhibiting protein synthesis[27]. Modification of the rRNA also induce a ribotoxic stress response that can lead to activation of several signaling cascades,

thereby triggering cytokine release and apoptotic cell death [29]. Stx injury to the endothelial cells generates thrombin and increased microvascular deposition of fibrin [6]. It has recently been reported that Stx and lipopolysaccharide (LPS) can induce platelet-leukocyte aggregation formation and stimulate the release of tissue factor (TF) thereby inducing aprothrombotic state[3]. The kidney and the CNS are the organs which are most frequently involved in the life-threatening extraintestinal complications [6,7].

The prototype Shiga toxin (Stx) is produced by Shigella dysenteria serotype1, whereas the Shiga-like toxins Stx1 and Stx2 are produced by EHEC strains [27]. Stx1 is almost identical to the prototypical Shiga toxin, differing in only one amino acid residue in the catalytic A subunit of the toxin [24]. The A and B subunits of Stx2 shows more variance and has 53% and 64% sequence similarity respectively to Shiga toxin [27]. Comparison of Stx1 and Stx2 show that they are only 56% identical at the amino acid level [31].

Within the toxin types, there are three Stx1 subtypes: Stx1a, Stx1c, Stx1d, and seven Stx2 subtypes: Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g [27]. Stx2 variants are 84-99% homologous to Stx2 [25].The high degree of variability in Shiga toxins probably represents the mosaicism of the lambdoid bacteriophage encoding the toxin operons [23].

Epidemiological and experimental studies indicate thatSTEC that carry the Stx2 genotype are more virulent and cause systemic complications such as HUS in humans than the Stx1-producing strains [6, 32, 33]. Stx1 has a 10-fold affinity for Gb3 in cells than Stx2 [32]. However, studies in mice have shown that the LD50 of Stx2 is approximately 400-fold lower than Stx1 signifying its increased toxicity [34].

Treatment of human renal endothelial cells with purified Stx1 or Stx2 revealed that Stx2 is 1000-fold more toxic than Stx1 [35] Strains which produce only Stx1 induce only diarrhea and no systemic complications [27]. Ironically strains that produce both Stx1 and Stx2 are less virulent than those that produce only Stx2 [35,36]. The combination of the Stx2 encoding genes in the outbreak isolates along with the other virulence factors discussed earlier make the German outbreak strain extremely pathogenic and could explain the reason for the increased proportion of patients who developed HUS [2].

Intervention strategies

As discussed in the previous section all outbreak strains have an ESBL phenotype. They are resistant to beta-lactam antibiotics including penicillins and cephalosporins but susceptible to carbapenems (ertapenam, imipenam, meropenam) [2]. The strain is also resistant to co-trimazole(trimethoprim-sulfamethazole, partially resistant to the fluroquinone, nalidixic acid and sensitive to ciprofloxacin and aminoglycosides (gentamycin and tobramycin) [2,19]. Emergence of novel,aggressive and resistant strains of bacteria reflects the vertical transmission of mutations and horizontal gene transfer between different bacterial strains that occurs during the evolutionary processes [37,38]. It has been recommended that antibiotics, antimotility agents, and narcotics should not be given to patients with definite or possible STEC infections [7]. These conventional therapies may be counterproductive and increase the risk of development of HUS or neurological complications of the disorder [6,7]. Since anti-inflammatory drugs diminish the renal blood flow their use in the treatment of STEC infections are also not advised [8]. Use of anticoagulant heparins, asprin, prostacyclin, fibrinolytics streptokinase and intravenous immunoglobulins are of no significant benefit in the treatment of HUS [8,39]. The mechanisms by which antibiotics increase the risk of HUS include phage induction leading to multiplication of Stx genes and phage-mediated bacterial lysis which can accelerate the toxin release [8,13,27]. This has been proved to be correct with the outbreak strain where an increased expression of the Stx2 genes by a factor of about 80 was observed when grown in a medium containing ciprofloxacin (25 ng per milliliter) [13].

As the management of HUS remains supportive and no specific therapies to prevent its complications are currently available, a need exists for the development of new therapeutic modalities [6,40]. The prime target for the development of new drugs is Shiga toxin since it is the major virulence factor of the infectious disease and is linked to the complications such as HC and HUS [27, 41]. Synthetic Shiga toxin B subunit binders, toxin A subunit inhibitors, and transport inhibitors with various structural characteristics have been designed and tested as possible therapeutic agents [27]. Stx neutralizers that bind the toxin directly thereby inhibiting its binding to the Gb3 receptor in target cells have been developed. These

include those functioning in the circulation such as the oligovalent water soluble globotriose ligands, termed STARFISH, and carbosilane dendrimers with variously oriented trisaccharides referred to as SUPER TWIG [42,43]. An orally applicable Stx-neutralizer named Synsorb-Pk consisting of clustered trisaccharides moiety covalently coupled to inert silica particles has also been tested for its protective activity against Shiga toxins [44]. In-vitro studies with these synthetic Shiga toxin binders have shown promising results, but clinical trials in children with diarrhea associated HUS did not show the expected protective effects [45]. Oral administration of specifically designed recombinant probiotic bacteria that display Stx receptors on its surfaceefficiently adsorbed and neutralized Shiga toxins and protected mice from lethal toxin dose [46]. The neutralization efficiency of monoclonal antibodies produced against both Stx1 and Stx2 have been recently confirmed in studies in piglet models and mice [41]. The neutralization efficiency of A subunit specific human monoclonal antibodies (HuMAbs) against Stx2have also recently been demonstrated [47]. Small molecule compounds such as Exo1 and Golgicide A that could inhibit toxin transport along the retrograde route have been designed and developed [48]. However, their efficacy to counteract the toxic effects of Shiga toxin and protect the host organism remains to be established [27].

Preventive measures

Prevention is better than cure. Preventing primary infection with Shiga toxin producing bacteria is the best way to protect oneself from the aftermath of this infection. The World Health Organization (WHO) recommends following strict hygienic measures with regard to personal hygiene and food safety: washing hands thoroughly before handling food and eating and aftervisiting the toilet, and washing fresh produce that are to be eaten without further heat treatment [49]. The five keys to safer food as advised by them is critical to prevent infection with EHEC. These include: 1.Keep clean,[2]. Seperate raw and cooked,[3]. Cook thoroughly until all parts reach a temperature of 70oC or higher,4. Keep food at safe temperatures,[5]. Use safe water and raw materials [49]. The importance of basic hand hygiene is highlighted by the fact that those who did not wash their hands before meals were nearly nine times more likely to get infected than were those who did[50]. Personal hygiene is also very important in preventing secondary transmissions. Isolation of hospitalized patients and laboratory safety in the handling on EHEC are also highly effective preventive measures [40,51].

Concluding Remarks

The outbreak clone O104:H4 is important pathogen that has evolved itself dramatically over the years to acquire a deadly combination of virulence traits. This has led to an increased proportion of patients who developed HUS in the present outbreak. The emergence of such highly virulent, multiresistant microorganisms show their genomic plasticity and pose challenges to both the scientific and medical community[52,53]. There exists an urgent need for development of new simpler, affordable, techniques that can help in rapid pathogen detectionfrom clinical, food or environmental samples [54,5)]. The swift genome sequencing of the outbreak strains demonstrate the power of high-throughput sequencing technologies. This should facilitate the development of newer interventional strategies that could help us combat these emerging superbugs.

Conflict of interest statement

We declare that we have no conflict of interest

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