



Shift in Phenotypic Characteristics of Enterotoxigenic *Escherichia coli* (ETEC) Isolated from Diarrheal Patients in Bangladesh

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Abstract

Background: Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of bacterial diarrhea. Over the last decade, from 1996 to 2012, changes in the virulence antigen properties of ETEC such as heat labile (LT) and heat stable (ST) toxins, colonization factors (CFs), and 'O'-serogroups have been observed. The aim of this prospective study was to compare changes in antigenic profiles of ETEC strains isolated from a 2% surveillance system at the icddr,b hospital in Dhaka, Bangladesh between 2007–2012 and an earlier time period of 1996–1998 conducted at the same surveillance site.

Methodology: In the surveillance system every 50th patient attending the hospital was screened for major enteric pathogens including ETEC, *Vibrio cholerae*, *Shigella* spp. and *Salmonella* spp. from January 2007 to December 2012.

Principal Findings: Of the 15,152 diarrheal specimens tested between 2007–2012, the overall rate of ETEC isolation was 11%; of these, 43% were LT/ST, 27% LT and 30% ST positive. Isolation rate of ST-ETEC ($p < 0.009$) and LT/ST ETEC ($p < 0.011$) during 2007–2012 period differed significantly compared to those seen between 1996–1998. In comparison to the 1996–1998 period, difference in CF profile of ETEC isolates during 2007–2012 was observed particularly for strains expressing CS7 (12.4%), CS14 (9.5%) and CS17 (10.0%). The predominant CF types were CS5+CS6, CFA/I, CS7, CS17, CS1+CS3, CS6 and CS14. The most common serogroups among the CF positive ETEC isolates were O115, O114, O6, O25 and O8. A strong association was found between CFs and 'O' serogroups i.e. between CS5+CS6 and (O115 and O126); CS7 and (O114), CFA/I and (O78 and O126), CS17 and (O8 and O167) and CS1/CS2+CS3 and (O6).

Conclusion: The analyses show a shift in prevalence of antigenic types of ETEC over the study period; the information is important in designing effective ETEC vaccines with broad protective coverage.

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is one of the leading causes of bacterial diarrhea in children and adults in developing countries. ETEC is a multivalent pathogen and have been sub-classified into different virulence types based on production of different enterotoxins, colonization factors (CFs) and the 'O' antigen group of the lipopolysaccharide. At least 160 'O' serogroups have been found among ETEC strains isolated from humans, although a more restricted number of serogroups are detected among strains isolated from patients requiring medical intervention.

Moreover, the ETEC phenotypic heterogeneity is also illustrated by different enterotoxin profiles and expression of different CFs as well as additional putative adhesion factors [1–4]. ETEC strains express one or both of two enterotoxins, the heat labile toxin (LT)

and the heat stable toxin (STh, STp variants) [5] and more than 25 different CFs including CFA/I and CS1–CS22 [2,6,7]. In this analyses, we have compared changes in antigenic profiles of ETEC strains isolated from a 2% surveillance system of all stool specimens from patients seeking care for diarrhea at the icddr,b hospital in Dhaka, Bangladesh between 2007–2012 compared to that seen during an earlier time period between 1996–1998 under the same surveillance system.

Methods

Study Site

This 2% systematic routine surveillance system was carried out between 2007 to 2012 in patients ($n = 15,152$) attending the diarrheal hospital at the icddr,b, Dhaka, Bangladesh. All patients were assessed for clinical conditions, including degree of dehydra-

Author Summary

Diarrheal diseases constitute a major health problem in Bangladesh, where *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) are two most important causes of bacterial diarrhea. Prevention through vaccination is helpful to reduce the incidence and severity of diarrheal disease due to ETEC, particularly among children in low-resource settings. In this context, we collected stool and/or rectal swab (RS) specimens from patients with diarrhea between 2007 to 2012 under the 2% systematic routine surveillance system at the icddr,b hospital in Dhaka, Bangladesh and screened for ETEC infection. We tested the specimens for two major virulence factors of ETEC: toxins and colonization factors. In this research article, we have focused on changes in toxin as well as colonization factor profiles of ETEC strains isolated from diarrheal patients seeking care at the icddr,b hospital between 2007–2012 and an earlier time period of 1996–1998. We concluded that, such shift in antigenic profile of ETEC over the study period is important in designing effective ETEC vaccines with broad protective coverage.

tion according to WHO guidelines. The clinical criteria for admission were moderate to severe diarrhea requiring hospitalization.

Ethics Statement

The 2% surveillance system was a routine ongoing activity of the icddr,b Dhaka Hospital which has been approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of icddr,b. Since most of the patients were illiterate, informed oral consent was obtained from the caregivers or guardians on behalf of the patients for collecting stool specimens only, following the hospital policy. The information was stored in the hospital database and used for conducting research. The verbal consent was documented by keeping a check mark in the questionnaire which was again shown to the patient or the guardians. At the same time, patients or the guardians were assured about the non-disclosure of information collected from them, and were also informed about the use of data for analysis and using the results for improving patient care activities as well as publication without disclosing the name or identity of the participants. ERC was satisfied with the voluntary participation, maintenance of the rights of the participants and confidential handling of personal information by the hospital audit committee and approved this consent procedure.

Microbiological Evaluation

Under the 2% systematic routine surveillance system stool and/or rectal swab (RS) specimens were collected from every 50th patient attending the icddr,b hospital and screened for major enteric pathogens including ETEC, *Vibrio cholerae*, *Shigella* spp. and *Salmonella* spp. by standard microbiological and biochemical methods [8,9]. For the detection of ETEC, each stool/rectal swab specimens were plated on MacConkey agar and the plates incubated overnight at 37°C. Six lactose fermenting, individual colonies from each stool or rectal swab were tested by multiplex PCR and ganglioside GM₁-Enzyme-Linked Immuno Sorbent Assay (ELISA) for the presence of LT and ST of ETEC. Multiplex PCR was used to detect both variants of ST toxin, STh and STp.

Multiplex PCR for Detection of ETEC Toxin

Multiplex PCR for enterotoxins was performed as previously described [10,11]. Single band gene products of the correct sizes for LT, STh and STp toxin genes were obtained in 25.5 μL PCR reactions containing 1 Unit Taq polymerase (Takara), 10× PCR reaction buffer containing 25 mM MgCl₂ (Sigma Aldrich, St. Louis, MO), 2.5 mM dNTPs (Roche, Mannheim, Germany) and 4 pmol of each primer.

Multiplex PCR for Detection of ETEC CFs

In order to simplify and accelerate the detection of six CFs—CS13, CS15, CS18, CS17/CS19, CS20 and CS22 specific primers designed to target corresponding CF genes. These were assembled into two panels. In panel I, CS15 (*nfaA*), CS19 (*csdB*), CS20 (*csnA*) genes and in panel II, CS13 (*cshE*), CS18 (*folG*), and CS22 (*cseA*) genes were amplified, respectively. In each panel, single band gene products of the correct sizes for CS15, CS19, CS20 as well as CS13, CS18 and CS22 genes were obtained in 25.0 μL PCR reactions containing 5 U/μL Taq polymerase (Takara), 10× PCR reaction buffer containing 25 mM MgCl₂ (Sigma Aldrich, St. Louis, MO), 2.5 mM dNTPs (Roche, Mannheim, Germany) and 10 pmol of each primer. The PCRs were amplified by an initial denaturation at 94°C (1 min), followed by 35 cycles of amplification (94°C for 30 s, 52°C for 30 s, and 72°C for 1 min), and finally, 5 min at 72°C. Amplified products were analyzed by agarose gel electrophoresis on 3.0% agarose gel [10,12].

Phenotypic Detection of Toxin Profile and CFs

The detection of LT and ST was carried out by previously described GM₁-ELISA method [8,11]. Six lactose fermenting *E. coli* colonies (half of each) from MacConkey agar plates were inoculated on GM₁-coated micro titer plates containing Luria Bertani broth for 18 h. The culture supernatant was tested for ST using an inhibition ELISA procedure [13] and for LT by using a LT specific monoclonal antibody [11,14]. For ETEC positive samples, the remaining half of each colony was plated onto colonization factor antigen (CFA) agar with and without bile salts and was tested for the expression of CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS14 and CS17 using 13 CF specific monoclonal antibodies by dot-blot immunoassay [8,11]. The isolates were also cultured on Trypticase Soy Agar (TSA) plates and tested for CS21 only [15].

'O'-Serogrouping

ETEC isolates were cultured on blood agar plates containing 5% sheep blood at 37°C. The 'O'-antigenic serogrouping was carried out following standard method using eight commercially available polyvalent and 43 monovalent antisera (Denka Seiken) [16].

Statistical Analysis

Comparisons were carried out where necessary using SigmaStat statistical software (Jandel scientific, San Rafael, Calif. USA). R version 2.14.1 for Chi-square test was used for significance analysis. However, missing data were very minor (<1%), thus excluded from the analysis.

Results

Prevalence of ETEC with Different Toxin Profiles

During 2007–2012 diarrheal specimens (n = 15,152) from patients under the 2% surveillance system were collected and

Table 1. Toxin profile of ETEC strains isolated from diarrheal patients in Bangladesh during two study periods.

Toxin types	Relative distribution of ETEC with different toxin profile (%)		
	1996–1998*	2007–2012	p value
LT	25	27	NS
ST	49	30	0.009
LT/ST	25	43	0.011

*: Qadri *et al* 2000.

NS: Not significant.

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analyzed for the presence of ETEC. The results were compared with those obtained for specimens collected between 1996–1998 (n = 4,662) under the same study. Among the diarrheal specimens tested, ETEC strains were isolated from 14% (1996–1998) and 11% (2007–2012) of the patients of all age groups. During the six year study period, ETEC strains produced, 43% of LT/ST toxin, 27% of LT and 30% of ST toxin type. The isolation rates for ST-ETEC and LT/ST ETEC during 2007–2012 were significantly different from those seen for ETEC isolated between 1996–1998 ($p < 0.05$) (Table 1). Among ST variants, STp was isolated in 5% of patients (Table 2). During the recent study period about 52% of ETEC was isolated from those over five years of age and 48% in younger age groups. The toxin profiles did not differ significantly in the different age groups studied (Table 3).

In patients with co- infections, rotavirus was the most common co pathogen with ETEC (17.2%), followed by *Vibrio cholerae* O1 (16.3%), *Campylobacter* spp (5.7%), *Shigella* spp (2.5%), and *Salmonella* spp (1.0%). Mixed infection together with ETEC and rotavirus occurred mostly in children below five years of age (14.6%) whereas mixtures of ETEC and *Vibrio cholerae* occurred mainly in patients above five years (12.0%).

A comparison was performed to determine if there is an association between the presence of toxin profile and the clinical severity of ETEC diarrhea (Table 4); from 2007 to 2009 we observed ETEC diarrhea of the same frequencies irrespective of toxin profile of the infecting strains. During 2010–2012, however, there was a significant difference ($p = 0.046$) in severity of ETEC diarrhea between strains producing LT only and those producing both LT/ST toxins. According to WHO, severe diarrhea is defined as episodes of diarrhea with fever and

vomiting, requiring intravenous rehydration and needs hospitalization [17].

Colonization Factors

Among the ETEC positive isolates collected between 2007–2012, 49% (n = 812) expressed one or more of the 13 tested CFs as determined by dot blot immunoassay. The predominant CF types were CS5+CS6 (18%), CFA/I (14%), CS7 (12%), CS6 as well as CS17 (10%) and CS14 (9%) (Table 5). ETEC expressing CS5+CS6 (11%), CS7 (8%), and CS17 (3%) were isolated less frequently during the previous 1996–1998 study period.

Marked difference in CF profiles of ETEC isolates during 2007–2012 were observed particularly for strains expressing CS7, CS14 and CS17 (Table 5). About 51% (n = 845) of the CF negative strains identified between 2007–2012 were also tested for presence of genes for CS13, CS15, CS18, CS19, CS20 and CS22 antigens but none of them were positive for any of these colonization factors.

'O'-Serogroups on ETEC Isolates

The most common serogroups on the CF positive ETEC isolates were O115 (16%), O114 (15%), O6 (13%), O25 (9%), O8 (7%) while 3% were non-typeable (NT). We found associations between the presence of CFs and 'O' serogroups of the isolates: CS5+CS6 (O115 and O126), CS7 (O114), CFA/I (O78 and O126), CS17 (O8 and O167) and CS1/CS2+CS3 (O6). Serogroup O1 was detected in the case of CS8 co-express with or without CS6 ETEC strains (Table 6).

Discussion

Our present analysis shows that ETEC was isolated from diarrheal patients of different age groups (from less than 1 month

Table 2. Relative distribution of STh and STp during the study period.

Year	STh	STp	STh/STp ratio
2007	62 (21)	8 (3)	62/8 (7.8)
2008	64 (20)	23 (7)	64/23 (2.8)
2009	68 (24)	16 (6)	68/16 (4.3)
2010	55 (27)	8(4)	55/8 (6.9)
2011	78 (33)	8(3)	78/8 (9.8)
2012	91 (29)	17 (5.4)	91/17 (5.4)
2007–2012	418	80	418/80 (5.2)

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Table 3. Incidence of diarrhea caused by ETEC of different toxin phenotypes in different age groups among 2% of all diarrreal patients seeking care at the icddr,b hospital in Bangladesh during 2007–2012.

Age	Total diarrhea N = 15152 (%) ^b	ETEC toxin phenotypes N (%) ^a			
		LT	ST	LT/ST	Total ETEC
0–5 months	1036 (6.8)	32 (3.0)	32 (3.0)	36 (3.4)	100 (9.6)
6–11 months	3259 (21.5)	113 (3.4)	89 (2.7)	120 (3.6)	322 (9.8)
12–17 months	1535 (10.1)	50 (3.2)	43 (2.8)	46 (2.9)	139 (9.05)
18–23 months	677 (4.5)	23 (3.3)	28 (4.1)	36 (5.3)	87 (12.8)
2–5 years	1113 (7.4)	38 (3.4)	60 (5.3)	52 (4.6)	150 (13.4)
>5 years	7520 (49.7)	186 (2.4)	246 (3.2)	427 (5.6)	859 (11.4)

a/N = Number of cases, % of all diarrreal cases.

b = % of cases in different age groups.

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to over 97 years of age). We observed that ETEC was a major cause of diarrhea not only in those less than five years of age but also in older children and adults. Earlier analyses including the surveillance carried out in the same site have shown that ETEC was more frequently isolated from children than from adults [4,8,18]. In this study, we observed that the relative proportion of ST- only ETEC had decreased significantly compared to that seen in a previous study in Bangladesh [8].

ETEC strains producing ST have previously been found to be more common in children with symptomatic infections where almost 80% were ST or LT/ST expressing strains [19] and also ST has been found to be prevalent in Egypt [18]. The isolation rate of LT/ST ETEC was higher in patients during the study period, which was also the most prevalent type of ETEC in Peru [20]. We also observed that colonization factor negative LT/STp producing ETEC strains were significantly higher in frequency than colonization factor expressing LT/STp ETEC strains. In case of STp- ETEC strains there was no difference in proportion between the CF-positive and CF-negative STp ETEC in our study.

Previous studies in Egypt and Guatemala have shown that there is almost as high frequency of STp compared to STh positive ETEC strains isolated from diarrreal stools of children [5]. However, in Bangladesh, we found STh producing ETEC to be much higher than the STp phenotype. One explanation may be the less prevalent consumption of porcine meat and products in the Bangladeshi settings.

During the study period between 2007–2012, colonization factors were identified almost in the same proportion of ETEC expressing LT, ST or both LT/ST toxins. However in earlier time

periods, CF positive strains those were mainly observed among ST or LT/ST toxin types whereas LT only strains expressed a CF in low frequencies [8,19]. A low prevalence of CFs on LT- ETEC strains has also been observed in Argentina and Egypt [8,21,22].

During an earlier analysis between 1996–1998 in the icddr,b hospital, CS5+CS6 were the most common CFs; it was also common in the recent period except 2008 and 2012. It was interesting to see that the prevalence of the colonization factor CS7 on ETEC peaked during 2008 which was an epidemic caused during a flood in Dhaka. The CF types expressed by the LT only ETEC were mostly CS7 and CS17, while the LT/ST positive isolates expressed the prevalent CS5+CS6, CS14, CS1+CS3 or CFA/I.

ETEC isolates expressing CS7 and CS17 were previously found only in stools of diarrreal children aged less than 5 years [8]. Our present study shows that, from 2010 to 2012, both CS7 and CS17 as well as CFA/I are higher in isolation amongst younger children. However, CS5+CS6-expressing ETEC were more commonly prevalent in the older children and adults. In the previous study it was also found that some CS7-expressing isolates produced only ST, however during this later surveillance lasting for a six year period, no CS7 expressing ST-ETEC was identified. We found in our present study CS21 associated with the bacteria expressing coli surface antigens CS1/CS2+CS3 and CFA/I which was also observed earlier [8,23].

ETEC can cause severe cholera like disease but also mild and moderate forms of illness. In the period between 2010–2012, there was a significant difference between severity of ETEC diarrhea and toxin profiles which was similar to our previous findings in 1996–1998 [8]. However, we found that in the study period from

Table 4. Relationship between toxin profile and dehydration status of ETEC diarrreal patients during 2007–2012.

Dehydration Status ^a		Toxin profile no. (%)		
		LT	ST	LT/ST
Severe	2007–2009	60 (36.6)	47 (28.7)	57 (34.8)
	2010–2012	22 (17.2)	44 (34.4)	62 (48.4)
Some	2007–2009	82 (34.2)	75 (31.3)	83 (34.6)
	2010–2012	48 (26.8)	55 (30.7)	76 (42.5)

p value for chi-squared test: 0.046 (2010–2012); indicates LT vs LT/ST ETEC for severe cases.

^aDegree of dehydration based on World Health Organization guideline.

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Table 5. Relative distribution of colonization factors (CFs) in ETEC isolates from diarrhea cases in Bangladesh according to year of isolation and association of specific CFs with toxin profile.

CF types	Percent of all CF positive strains distribution by years					Total no. of CFs	
	2007 n = 144 (%)	2008 n = 173 (%)	2009 n = 115 (%)	2010 n = 96 (%)	2011 n = 123 (%)	2012 n = 161 (%)	n = 812 (%) of all CF positives
CFA/II±CS21	17.4	13.9	13.0	12.5	14.6	20.5	127 (15.6)
CS1+CS3±C21	9.7	4.0	6.1	7.3	10.6	9.9	64 (7.9)
CS2+CS3±CS21	5.5	8.0	7.0	7.3	5.7	3.1	49 (6.0)
CS4+CS6	6.9	6.9	2.6	3.1	4.9	5.6	45 (5.5)
CS5+CS6	16.0	19.1	20.9	25.0	18.7	13.7	149 (18.3)
CS6 only	8.3	8.1	14.8	9.4	8.1	9.9	78 (9.6)
CS6±CS8	4.8	1.7	5.2	1.0	1.6	0	20 (2.5)
CS7	13.9	23.7	10.4	12.5	6.5	5.0	101 (12.4)
CS12	2.1	0.6	1.7	0	1.6	1.9	11 (1.4)
CS14	8.3	6.4	6.1	5.2	14.6	14.9	77 (9.5)
CS17	6.3	6.4	8.7	14.6	12.2	14.3	82 (10.0)
CS21 only	0.7	0.6	1.7	2.1	0.8	1.2	9 (1.1)

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Table 6. 'O'-Serogroups on ETEC strains expressing different combinations of toxins and CFs during the study period (2007–2012).

Major Toxin Profiles	Colonization factors (CFs)	'O' Serogroup	
		Major	Minor
ST, LT/ST	CFA/I ± CS21 (61)	O78 (9), O126 (12), NT (15), OR (5), O? (10)	O128 (3), O8 (2), O114 (1), O15 (1), O6 (1), O55 (1), O44 (1)
ST, LT/ST	CS1+ CS3 ± CS21 (42)	O6 (34), NT (7)	O? (1)
ST, LT/ST	CS2+ CS3 ± CS21 (29)	O6 (26)	O115 (1), O2 (1), NT (1)
ST, LT/ST	CS4+ CS6 (24)	O25 (23)	NT (1)
ST, LT/ST	CS5+ CS6 (100)	O115 (87)	O126 (3), O167 (2), O169 (1), O20 (1), O27 (1), NT (2), OR (2), O? (1)
LT, ST, LT/ST	CS6 (46)	O159 (6), OR (7), NT (14),	O167 (3), O25 (3), O27 (2), O18 (2), O115 (1), O151 (1), O148 (2), O78 (1), O20 (1), O169 (1), O? (2),
LT	CS6+ CS8 (14)	O25 (11)	O159 (1), O158 (1), O1 (1)
LT	CS7 (64)	O114 (60)	O115 (1), O86 (1), NT (1), OR (1)
LT	CS8 (2)	O1 (2)	-
LT, LT/ST	CS12 (7)	O18 (5)	NT (2)
LT/ST	CS14 (27)	NT (21)	O6 (2), O8 (1), O86a (1), O78 (1), O167 (1)
LT	CS17 (48)	O8 (36),	O167 (4), O6 (1), O153 (1), O20 (1), NT (3), OR (1), O? (1)
ST	CS21 (4)	O6 (3)	O128 (1)
LT, ST, LT/ST	Negative (130)	O8 (7), O114 (6), O20 (5), O153 (5), O25 (5), O15 (5), OR (5), O? (15), NT (59)	O18 (4), O27 (3), O1 (2), O86a (3), O159 (2), O78 (2), O6 (1), O128 R(1),

NT = Not Typeable.

OR = Rough strains.

O? = Polyvalent positive, mono valent negative.

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2007 to 2009, there was no relation to the toxin type with the severity of diarrhea. In recent observation in Bolivia there was also no difference between severity of ETEC diarrhea and toxin profile on ETEC isolates [24].

The usefulness of 'O' serogroup antigens in ETEC vaccine formulation has been limited due to the large variation of 'O' serogroups, with over 80 serogroups identified among clinical ETEC isolates [25]. However, before the characterization of CFs, some 'O' serogroups such as O6, O8, O78, O128 and O153 have been shown to commonly associated with ETEC strains [26]. Similar association between 'O' groups and CF types were seen in most cases in the present study. Our results suggest that the shift observed in CF profile over time was also noted for ETEC strains in Bangladesh over the study period. Although there have been some shifts in CF antigenic types over time and it seems that the majority of ETEC still express the major CFs i.e. CS1-CS6 [2,3] with occasional outbreaks of the closely related CS7, CS14 and CS17 all of which belong to the CFA/I group of antigens [27].

One of the limitations of our study was that we screened only the ETEC positive isolates for 13 of the 25 CFs that are known to exist and are common [2], using specific monoclonal antibodies. Six more CFs (CS13, CS15, CS18, CS19, CS20, CS22) were tested by using specific primers. However, neither monoclonal

antibodies nor specific primers were available in our lab for detection of any remaining CFs that may have been present.

Vaccine formulation and development therefore should be based on the prevailing CF phenotypes of ETEC strains. We feel that the formulation of an effective cocktail for designing a multivalent ETEC vaccine should include CFs being isolated in high frequencies in recent studies in Bangladesh (e.g. CS7, CS17) and elsewhere [28].

Supporting Information

Checklist S1 STROBE checklist. (DOC)

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Author Contributions

Conceived and designed the experiments: YAB ASGF AC AMS FQ. Performed the experiments: NIB NJ. Analyzed the data: YAB NIB NJ ASGF. Contributed reagents/materials/analysis tools: YAB ASGF AC AMS FQ. Wrote the paper: YAB NIB ASGF AMS FQ.

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