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Molecular characterization of locus of enterocyte effacement pathogenicity island in shigatoxic *Escherichia coli* isolated from human & cattle in West Bengal, India

Suresh Chandra Das[1], Thandavanaryanalu Ramamurthy[2], Santanu Ghosh[3], Gururaja Perumal Pazhani[2], Tista Sen[1] & Raghubir Singh[1]

[1]Eastern Regional Station, VPH Lab, ICAR-Indian Veterinary Research Institute, [2]Division of Bacteriology, National Institute of Cholera & Enteric Diseases, Kolkata & [3]Molecular Kit Development Division, 3B BlackBio Biotech India Limited, Bhopal, India

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***Background & objectives*: Shigatoxic *Escherichia coli* (STEC) recovered from dairy animals of Kolkata, India, harboured the putative virulence genes; however, the animals did not exhibit clinical symptoms. Similarly, human isolates in this locality also showed variations in degree of symptoms. Hence, this study was designed to know the presence of recognized gene(s) in the locus of enterocyte effacement (LEE) pathogenicity island in these STEC isolates and functional status of the cardinal gene (*eae*) related to pathogenicity.**

***Methods*: Genes were characterized using polymerase chain reaction (PCR) assays, and functional status of cardinal gene (*eae*) was evaluated by fluorescent actin staining (FAS) assay. Variation in *eae* gene was determined by intimin PCR.**

***Results*: Cattle STEC isolates carried 22 genes in LEE pathogenicity island in different frequencies ranging from 5.63 to 47.88 per cent of the isolates. In human isolates, the genes namely *ler*, *escRSTU*, *orf2*, *escC*, *escV*, *orf3* and *tir* that are associated with secretory function, were found to be absent and rest of the genes were present in lower frequency. Further, the cardinal gene (*eae*) responsible for initiation of pathogenesis was in a very low frequency in human (n=2; 10.5%) and cattle (n=11; 15.5%) isolates. None of these *eae*$^+$ STEC isolates from human and cattle revealed positivity in FAS assay.**

***Interpretation & conclusions*: Majority of human STEC isolates lacked the cardinal virulence gene (*eae*), and genes for secretory function that are essential for facilitating pathogenesis. This may partially be attributed to low occurrence of STEC in human clinical diarrhoea in this area. Although a few isolates (11 of 71) from cattle had *eae* gene, they did not express phenotypically. This could be one of the reasons for not appearing of clinical symptoms in the hosts.**

Key words Fluorescent actin staining assay - locus of enterocyte effacement - pathogenicity island - shigatoxic *Escherichia coli*

Shigatoxic *Escherichia coli* (STEC) has been recognized globally as an emerging human diarrhoeal pathogen that can cause a spectrum of illness ranging from watery diarrhoea to haemorrhagic colitis, or even fatal complications such as haemolytic uremic syndrome. Ruminants, particularly cattle, have been found to be the principal reservoir of this organism[1].

Production of one or more shigatoxins (Stx1, Stx2 or variants) has been identified to be the cardinal virulence factor for their pathogenesis, and in addition, the large plasmid (pO157)-borne virulence factors are also presumably associated[1,2]. On binding of STEC to the brush border of host mucosa, pathogenesis facilitates cytoskeletal reorganization and formation of a characteristic histopathological feature, termed the 'attaching and effacement' (A/E) lesion, by subverting the intestinal epithelial cell function[3]. The ability to cause A/E lesions is encoded on a large pathogenicity island termed as locus of enterocyte effacement (LEE) where the *eae* gene (encodes intimin) mediates intimate attachment to the host cell through the intimin receptor (*tir*) which is chaperoned by CesT and translocated into the host cell plasma membrane by the type III system[3,4]. Except enteropathogenic *E. coli* (EPEC) and STEC isolates, LEE is not present in the normal flora of *E. coli* or in any other pathogroups of *E. coli*. In majority of the clinical cases and outbreak incidences, the associated STEC strains carried the LEE and the *eae*[5]; however, a few reports revealed the cases devoid of *eae*[6]. The presence of *eae* in cattle STEC isolates has been reported to vary in different geographical set-up such as France, Spain and Japan[7-10]. Detailed sequence analysis of this gene in STEC isolates from human and different animals revealed that N (5') terminal region is highly conserved whereas C (3') terminal is variable and is responsible for binding to the host enterocytes and intimin receptor (tir)[11,12]. It has been suggested that different intimins may be responsible for different host tissue cell tropism[13].

In our previous studies[14,15], it was observed that 87.3 per cent of the STEC isolates (*stx*$^+$) obtained from Kolkata from human and animals did not generate the amplicon for *eae*, the indicator gene for LEE and A/E phenotype in STEC pathogenesis. A pilot polymerase chain reaction (PCR) assay was conducted with these STEC isolates to understand the sequences of genes mainly associated for type III secretory function (left side, left junction, right junction of LEE and *espB*, *espD* and *tir*) flanking to *eae* in the LEE, and the results showed that these genes were not present in most of the *eae*$^+$ STEC isolates[14]. On the contrary, these genes were common in the *eae*$^-$ STEC isolates. Further, the findings with *eae*$^+$ STEC isolates showed that these gene sequences were not constantly present suggesting the presence of incomplete LEE. With this background, the present investigation was designed to know the structural gene sequences of the LEE of the STEC isolates obtained from Kolkata, India, and functional status of the *eae* gene as well as to determine their variants. The findings were compared with the reference strain to relate the importance of LEE genes in pathogenicity of the STEC strains.

Material & Methods

In the present study, a total of 19 and 71 STEC isolates from human diarrhoea stool and cattle faecal samples, respectively, were included. Repository of STEC isolates recovered from human and cattle faecal samples during our earlier study[14] was used in this study. Subsequently, the isolates obtained during 2010-2013 were also included. Human isolates were recovered from stool specimens of diarrhoea patients admitted to the Infectious Diseases Hospital and B. C. Roy Memorial Hospital for children, Kolkata, India, from June 2001 to July 2003 and February 2011 to May 2013. The study was approved by the institutional ethics committee of the National Institute of Cholera and Enteric Diseases (NICED) (ICMR), Kolkata.

Briefly, stool specimens from the diarrhoea patients were collected using a sterile catheter in sterile McCartney bottles; however, rectal swabs were taken from the patients from whom stool could not be obtained and finally introduced in Cary-Blair transport medium. Faecal sample from cattle was collected aseptically by digital rectal retrieval. All the collected samples were transported to the laboratory within four hours of collection and processed immediately following the method adopted in earlier studies[14,15] for isolation and characterization of STEC.

Screening of genes in LEE pathogenicity island: The reference strain (EDL933) was used in the study. The primers for target LEE genes were designed using the software and published EDL 933 sequence (Accession. No. AF071034). The primer sequence for *stx1* and *stx2* gene (EVT1/EVT2 and EVS1/EVC2) was used as mentioned in the earlier study[16]. The LEE genes (*ler*, *orf1*, *escRSTU*, *orf2*, *cesD*, *escC*, *escJ/sep-Z*, *escV*, *escN*, *orf3*, *sepQ*, *espH*, *orf-4*, *sepQ*, *espH*, *orf4*, *tir*, *eae*, *escD*, *sepL, espA, espD, espB, escF* and *espF*) (Table I) were screened by standardizing the PCR

Table I. Detection of genes in locus of enterocyte effacement pathogenicity islands in shigatoxic *Escherichia coli* isolates by polymerase chain reaction assay

Genes	Human (n=19)	Cattle (n=71)	Reference strain (EDL 933)
ler	-	14 (19.71)	+
orf1	1 (5.2)	7 (9.85)	+
escRSTU	-	8 (11.26)	+
orf2	-	7 (9.85)	+
cesD	8 (42.1)	34 (47.88)	+
escC	-	13 (18.30)	+
escJ/sep-Z	1 (5.2)	15 (21.12)	+
escV	-	11 (15.49)	+
escN	1 (5.2)	17 (23.94)	+
orf3	-	18 (25.35)	+
sepQ	1 (5.2)	23 (32.39)	+
espH	5 (26.3)	32 (45.07)	+
orf4	1 (5.2)	14 (19.71)	+
tir	-	4 (5.63)	+
eae	2 (10.5)	11 (15.49)	+
escD	4 (21.05)	28 (39.43)	+
sepL	1 (5.2)	12 (16.90)	+
espA	2 (10.5)	22 (30.98)	+
espD	1 (5.2)	11 (15.49)	+
espB	4 (21.05)	16 (22.53)	+
escF	3 (15.8)	26 (36.61)	+
espF	2 (10.5)	11 (15.49)	+

Values in parentheses are percentages. +, present; -, absent

protocol using the published[16] and designed primer sets (Table II) with reference strain (EDL 933) DNA. The obtained PCR amplicons were visualized in agarose gel electrophoresis using the 1 Kb and 100 bp DNA marker.

Detection of intimin (eae) variants by PCR assay: The PCR assay was carried out using the published primer sequences (Table II) and methods[17].

Determination of functional status of intimin by fluorescent actin staining (FAS): Functional status of *eae* (intimin gene) in STEC isolates *i.e.* capability for A/E to the susceptible host epithelia was evaluated by assaying the fluorescent actin staining (FAS). The assay was performed adopting the standard published method[18] using the phalloidin dye FITC conjugate and Hep-2 cell line. The EDL933 and E2348/69 were used as positive control and K12 *E. coli* (DH5α) was used as negative control.

Results

A total of 19 human and 71 cattle STEC isolates were screened for the presence of 22 genes in their LEE pathogenicity island. Among human isolates, *cesD* was found positive in eight (42.1%) isolates followed by *espH* [5 (26.3%)], *escD* and *espB* [4 (21.05%) each], *escF* [3 (15.8%)], *eae*, *espA* and *espF* [2 (10.5%) each] and *orf1*, *escJ/sep-Z*, *escN*, *sepQ*, *orf4*, *sepL* and *espD* [1 (10.5%) each]; however, the isolates did not produce amplicon for other seven genes *viz*. *ler*, *escRSTU*, *orf*2, *escC*, *escV*, *orf3* and *tir* (Table I).

In cattle isolates, *cesD* gene was detected most frequently (n=34, 47.88%) followed by *espH* [32 (45.07%)], *escD* [28 (39.43)], esc*F* [26 (36.61%)], sep*Q* [23 (32.39%)], *espA* [22 (30.98%)], *orf3* [18 (25.35%)], *escN* [17 (23.94%)], *espB* [16 (22.53%)], *escJ/sepZ* [15 (21.12%)], *ler* and *orf4* [14 (19.71) each], *escC* [13 (18.30%)], *sepL* [12 (16.90%)], *escV*, *espD* & *espF* [11 (15.49%) each], *eae* [11 (15.49%)], *escRSTU* [8 (11.26%)], *orf1* and *orf2* [7 (9.85%) each] and *tir* [4 (5.63%)] (Table I).

A total of 13 STEC isolates [human (n=2) and animal (n=11)] were found to carry the *eae* gene and were screened by nine different PCR assays for the detection of variants of *eae* (α, β, γ, ε, ξ, η, θ, I and k) (Table III). It was observed that three isolates each were detected to be positive for β and γ variants and four isolates for ε variants. Rest three isolates [13−10 (3+3+4)] remained untypable in the PCR assay. All the *eae*$^{+}$ STEC isolates (n=13) were assessed by FAS for functional expression of their intimin (*eae*); however, none of the isolates was found to be positive for intimin (*eae*) as evident in negative cell adherence.

Discussion

In the present investigation, the data for the presence of genes in LEE of human STEC isolates revealed that seven out of 22 genes in LEE were not detected; moreover, the frequency of the genes present among the STEC human isolates was low. The *eae* gene which is principally responsible for attaching the host epithelium for initiation of pathogenesis was detected in a very limited number of isolates (2 of 19 isolates). Further, FAS assay findings suggested that their functional status was not expressed. The present study findings suggested that presence of *eae* gene, principally responsible for

Table II. The primer sequence and conditions used for detection of different genes in locus of enterocyte effacement pathogenicity island of shigatoxic *Escherichia coli* isolates in this study

Primer number	Nucleotide sequence (5'-3')	Target sequence	PCR conditions[a]			Amplicon (bp)
			Denaturing Temp (ºC), Time (min)	Annealing Temp (ºC), Time (min)	Extension[a,b] Temp (ºC), Time (sec)	
EVT1*/ EVT2*	CAA CAC TGG ATG ATC TCA G CCC CCT CAA CTG CTA ATA	*stx1*	94, 1	55, 1	72, 60	349
EVS1*/ EVC2*	ATC AGT CGT CAC TCA CTG GT CTG CTG TCA CAG TGA CAA A	*stx2*	94, 1	55, 1	72, 60	110
ler-F *ler*-R	CAG CTC AGT TAT CGT TAT CA CTG ATA AGG TCG CTA ATA GC'	*ler*	94, 1	56, 1	72, 90	440
orf1-F *orf1*-R	GAG ACA TAT CAT CAT TCC TG' GTG AAG GAC ACT GAA GAA GA	*Orf1*	94, 1	57, 1	72, 90	1851
EscRSTU-F *EscRSTU*-R	GAA GGC AAT ACG CAA TGA AA CAG GAA TGA TGA TAT GTC TC	*EscRSTU*	94, 1	56, 1	72, 90	2808
orf2-F *orf2*-R	CGT CTA ACT CTC CTT TTT CC ATT CGT ATT GCG ATA GAC CT	*Orf 2*	94, 1	56, 1	72, 90	1523
Ces-F *Ces*-R	AAA TGA GGC TTT ATG AGC AG GGT ATA GCT GAT GGT AGT TT	*cesD*	94, 1	54, 1	72, 90	487
EscC-F *EscC*-R	CTG ATA TAG GAC GAA TTG TG CTG CTC ATA AAG CCT CAT TT	*escC*	94, 1	56, 1	72, 90	1581
EscJ-F *EscJ*-R	AGG AGA AAG ATC AAT GGA AG GTT CAT ACA TAT TAC CCG TC	*EscJ/sepZ*	94, 1	56, 1	72, 90	1359
EscV-F *EscV*-R	CGT GGG TAT TTT TCC AAT AC GCA GGA TGA CAT CAT GAA TA	*escV*	94, 1	56, 1	72, 90	2064
EscN-F *EscN*-R	ATT CTG TCC AAC ATA CTC AG ACG GTA AAT GAT TTC AGA GC	*escN*	94, 1	56, 1	72, 90	1363
orf3-F *orf3*-R	GGC AAC AAC GGC AAA TAA AA CTA TTC AAA GTG GTT GCC TG	*Orf3*	94, 1	57, 1	72, 90	833
SepQ-F *SepQ*-R	CCC TAT ATA ACG TAT TCC TG GAA ATC TTT CCT GAT GAA GC	*sepQ*	94, 1	56, 1	72, 90	951
EspH-F *EspH*-R	CAG GAA GAA AAC TCA AAT GC GGA ATA CGT TAT ATA GGG AG	*espH*	94, 1	56, 1	72, 90	556
orf4-F *orf4*-R	TAT ACC TGA CAA GGT TAT CC CAT CCA GCG CAG AAA TTA TC	*Orf4*	94, 1	56, 1	72, 90	1298
tir-F *tir*-R	TTA GAC GAA ACG ATG GGA TC ATG CCT ATT GGT AAT CTT GG	*tir*	94, 1	57, 1	72, 90	1676
eae-F *eae*-R	GGGTGGTTATGGAATTATTC TTCTAACTCATTGTGGTGGA	*eae*	94, 1	56, 1	72, 90	2846
escD-F *escD*-R	CAA CCA CCA GGA TGA ATA AA GGG GAA GAG GAT AAG AAA TT	*escD*	94, 1	56, 1	72, 90	1266
sepL-F *sepL*-R	AAC GGA TGT TGC ATT TGA TG ATT ACG TGA GTT TCC AAT GG	*sepL*	94, 1	56, 1	72, 90	1156

Contd...

Primer number	Nucleotide sequence (5'-3')	Target sequence	PCR conditions[a]			Amplicon (bp)
			Denaturing Temp (ºC), Time (min)	Annealing Temp (ºC), Time (min)	Extension[a,b] Temp (ºC), Time (sec)	
espA-F	TTA AGC ATA GTT ATC TCC GG	*espA*	94, 1	56, 1	72, 90	686
espA-R	GTT ATC GAC TAT AAG GAG GA					
espD-F	CGG AAT TAA CCA TCG TTA CT	*espD*	94, 1	56, 1	72, 90	1208
espD-R	GGT AAA TAA CCG GAG ATA AC					
espB-F	AAA CGT ATC GAC CAT GAT CA	*espB*	94, 1	56, 1	72, 90	982
espB-R	AGA TTG TTA GTG GCC GAA TT					
escF-F	CAC CAG TAT CTT ATT AGC AG	*escF*	94, 1	56, 1	72, 90	331
escF-R	CGG AAT TTG GTT CGT AAT GA					
espF-F	TAG TTG GTT ACC CTT TCT TC	*espF*	94, 1	56, 1	72, 90	789
espF-R	GAG TTA GCC AAG CTT AGA TA					
Primer number	*eae* variants primer sequence					
*eae*F	CCC GAA TTC GGC ACA AGC ATA AGC	*eae* conserved region	94, 1	52, 1	72, 60	863
*eae*R	CCC GGA TCC GTC TCG CCA GTA TTC G					
eae αR	CCC GAA TTC TTA TTT TAC ACA AGT GGC	eae-α	94, 1	55, 1	72, 120	2807
eae βR	CCC GTG ATA CCA GTA CCA ATT ACG GTC	*eae*-β	94, 1	55, 1	72, 120	2287
eae γR	CCC GAA TTC TTA TTC TAC ACA AAC CGC	*eae*-γ	94, 1	48, 1	72, 90*	2792
			94, 1	55, 1	72, 90**	
eae εR	AGC TCA CTC GTA GAT GAC GGC AAG CG	*eae*-ε	94, 1	55, 1	72, 120	2608
eae ξR	AGC TCA CTC GTA GAT GAC GGC AAG CG	*eae*-ξ	94, 1	53, 1	72, 150	2430
eae ηR	TAG ATG ACG GTA AGC GAC	*eae*-η	94, 1	52, 1	72, 150	2590
eae θR	GTT GAT AAC TCC TGA TAT TTT A	*eae*-θ	94, 1	50, 1	72, 150	2686
eae iR	TTT ATC CTG CTC CGT TTG CT	*eae*-i	94, 1	52, 1	72, 150	2685
eae kR	GGC ATT GTT ATC TGT TGT CT	*eae*-k	94, 1	52, 1	72, 150	2769

[a]Before cycle amplification, sample was denatured at 94°C for five minutes. All PCRs consisted of 35 cycles except 30 cycles for *ler*, *ces*, *escJ*, *espH*, *espA*, *escF*, [b]After last cycle, final extension step of seven minutes at 72°C was done; For *stx*1 and *stx*2, published primer sequence was used; the rest the primer set was newly designed using the published sequence (Accession No. AF071034). All PCRs were carried out for for 30 cycles (for *eae*-γ: *3 cycles; **28 cycles). Primer *eae*F was used as forward primer in all PCRs in combination with rest of the reverse primers as mentioned above. Published primer sequence was used.

A/E function, was in a very low frequency and there was an absence of seven genes viz. *ler*, *escRSTU*, *orf2*, *escC*, *escV*, *orf3* and *tir* linked to secretory function in STEC isolates. The present study highlights the frequency of genes in LEE pathogenicity island of STEC isolates; however, screening for the presence and role of other concerned gene(s) and pathogenicity island as carried out in other published studies[18,19] would be contributory to ascertain the detailed reasons for low incidence STEC in clinical diarrhoea in this area.

STEC isolates from cattle possessed the LEE genes in a comparatively high frequency than human isolates including the presence of *eae* gene; however, the hosts (cattle) were apparently healthy and did not show any clinical symptoms. The FAS assay was conducted for these *eae*$^+$ STEC isolates (n=11) from cattle to understand the expression and functional ability (A/E to the host epithelium) of their *eae* gene; however, none of them showed positivity for the cell adherence in this assay. It appeared that the

Table III. Intimin variants of shigatoxic *Escherichia coli* isolates and their attaching and effacement status by fluorescent actin staining (FAS) assay

Isolate number	Intimin (*eae*$^+$)	Intimin (*eae*$^+$) variants									FAS assay
		α	β	γ	ε	ζ	η	θ	i	κ	
STEC eaeH1*	-	-	+	-	-	-	-	-	-	-	-
STEC eaeH2*		--	-	-	+	-	-	-	-	-	-
STEC eae1	+	-	-	-	-	-	-	-	-	-	-
STEC eae2	+	-	-	+	-	-	-	-	-	-	-
STEC eae3	+	-	-	-	-	-	-	-	-	-	-
STEC eae4	+	-	-	-	+	-	-	-	-	-	-
STEC eae5	+	-	-	-	-	-	-	-	-	-	-
STEC eae6	+	-	+	-	-	-	-	-	-	-	-
STEC eae7	+	-	+	-	-	-	-	-	-	-	-
STEC eae8	+	-	-	-	+	-	-	-	-	-	-
STEC eae9	+	-	-	+	-	-	-	-	-	-	-
STEC eae10	+	-	-	+	-	-	-	-	-	-	-
STEC eae11	-	-	-	-	+	-	-	-	-	-	-
Positive control	+	-	-	+	-	-	-	-	-	-	+

*STEC *eae*H1 and STEC *eae*H2 from human stool; rest STEC *eae* isolates were from cattle source. STEC, shigatoxic *Escherichia coli*. +, positive; -, negative

cattle harboured this organism having the important virulence gene-like *eae* for establishing pathogenesis but did not express.

The *eae*$^+$ STEC isolates from human and animal were tallied with history of samples and found that the concerned two human cases had history of loose stool; however, the cattle were apparently healthy. This observation generated two ideas. First, these *eae*$^+$ isolates might have variation in their *eae* sequence and as a result could not bind to the host enterocyte. Alternatively, their *eae* gene is not expressed for functional ability. In earlier study[20], heterogeneity in C'(3) terminal of *eae* sequence in STEC isolates from different sources was reported. This idea prompted us to explore the possibility of *eae* variants in these STEC isolates, for *eae* (intimin) variants *(*α, β, γ, ε, ξ, η, θ, I and k), and to evaluate the status of expression of this gene by FAS. It was observed that three isolates each were detected to be positive for β and γ variants and four isolates for ε variants. Rest three isolates [13−10 (3+3+4)] remained untypable with the used primer sets. It suggests that these three isolates may possess *eae* variants that differ from the, so far, recognized *eae* variants. To understand the functional expression of their intimin (*eae*), all the *eae*$^+$ STEC isolates were assessed by FAS; however, all were found negative as evaluated by cell adherence in FAS.

The activation of LEE pathogenicity island is regulated at various levels, including transcriptional and post-transcriptional regulators located both inside and outside of the pathogenicity island[21-23]. Several molecules are involved in a complex network of regulation, including mechanisms such as quorum sensing and temporal control of LEE genes transcription and translation[23], by the chemical signalling system depending on the factors available in the environment of host epithelium and bacteria (*e.g*. stressed epithelia and fucose sugar)[24]. LEE island carries the regulator gene (*ler*) for its own expression and type III secretory system that infuses the effector molecules to the host cell leading to A/E lesions enterocytes[24]. Promotion of LEE expression is associated with signal inducer (QseC and QseE) produced by bacteria. On passing through the steps of activation and phosphorylation, these components end at the FusKR complex (fucose sugar repressor) that determines the LEE expression[25]. Further study on this aspect may supplement information on the pathogenesis with STEC of this area.

In conclusion, the study findings suggested that all the genes in LEE island in STEC isolates of human and animal origin in this study area were not constantly present; particularly, human isolates were carrying 15 of these genes in low frequency

and seven genes were lacking. Besides, frequency of cardinal gene (*eae*) was very low in human and in moderate frequency among cattle isolates; however, the phenotypic expression of the gene was not evident. These findings may relate to the low incidence of this organism in clinical diarrhoea in this area as well as the apparently healthy carrier in cattle. Further study on the screening of other gene(s) and pathogenicity island including the FusKR complex that determines the aspect of LEE expression and its regulation may be supportive to address the reasons for such low incidence of STEC in clinical diarrhoea and its silent carrier status in animals in this area.

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***Conflicts of Interest*:** None.

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Reprint requests: Dr Suresh Chandra Das, ICAR-Indian Veterinary Research Institute, Eastern Regional Station, 37, Belgachia Road, Kolkata 700 037, West Bengal, India
e-mail: dasivriers@gmail.com