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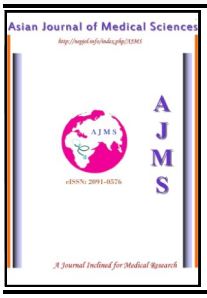

Association of Genetically Related *Escherichia coli* Strains with Ulcerative Colitis - A Preliminary Study

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Abstract

Objective: To explore the genetic relatedness among the *Escherichia coli* isolates recovered from rectal mucosa of patients with Ulcerative Colitis(UC) as well as from non specific diarrhoea patients by using ERIC PCR (whole genome analysis).

Material & Methods: A total of 44 strains of *E coli*, each from patients suffering from UC with exacerbation while on maintenance therapy, were isolated to see if there is any association with specific genotype of *E coli* with the clinical condition. For comparison, 20 strains of *E coli* were also isolated from patients suffering from non specific diarrhoea. These isolates were subjected to ERIC PCR for analysing similarity/ dissimilarity with each other based on the distribution of ERIC sequences in the whole genome of the bacterial species.

Results: The dendrogram prepared on the basis of banding pattern showed that majority of UC patients (39/44, 88.6%) grouped in to one major cluster while second major cluster comprised mostly strains isolated from patients with non specific diarrhoea i.e. controls (17/18, 94.4%). Moreover, in the cluster representing UC patients, a total of 11 strains were observed to be genotypically similar followed by 8 strains by ERIC PCR.

Conclusion: Our results strongly indicate that specific *Escherichia coli* strains may be involved/ associated with UC and its relapse.

***Key Words**: Ulcerative colitis; Escherichia coli; ERIC; PCR*

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disorder of the gastro intestinal tract that mainly affects the large bowel which is a major disorder of inflammatory bowel diseases (IBD). UC has almost similar incidence(6-9 cases per100,000 population) both in USA and India (developed and developing countries respectively).[1,2] Etiologically, UC is suggested to be due to interplay of commensal bacterial flora[3,8] genetic susceptibility[4,5] and autoimmunity[5,6] superimposed on factors viz., diet, stress, smoking, oral contraceptive pills etc. Several clinical and research data suggest the role of bacteria in onset and perpetuation of disease.[8] Positive therapeutic response to antibiotics in patients with UC has been reported.[9] However to date, searches for specific pathogens have been unable to satisfy Koch's postulates. Studies have highlighted the association of *Escherichia coli* with UC. *E. coli* from patients with UC differed from control strains in being more likely to produce hemolysin and necrotoxin and to cause distension of rabbit ileal loops.[10-12] However, these pathogenic differences were seen more consistently in strains following rather than preceding colitis.[13] Patients with UC possess agglutinating antibodies in their sera to a greater number of *E. coli* O antigens and in higher titres than controls.[5] Therefore, it became pertinent to see the genetic aspect of those *E. coli* strains isolated from rectal mucosa of patients with UC. To explore the genetic relatedness among the *E. coli* isolates recovered from rectal mucosa of patients with UC as well as from non specific diarrhoea patients, ERIC PCR (whole genome analysis) was used in the present study.

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2. Material and Methods

2.1. Bacterial strains

A total of 64 *E. coli* strains were isolated from rectal biopsy of patients comprising of 44 UC and 20 non specific diarrhoea (NSD). These *E. coli* strains were predominant growth present along with few other bacterial species. The tissue biopsy was transported to the laboratory in Luria Bertani (LB) broth, then homogenized and cultured on MacConkey and sheep blood agar, incubated at 37^0 C overnight. The strains were identified by typical colony morphology, Gram's staining and by using variety of test substrates for biochemical reaction.

2.2. DNA extraction

Bacterial DNA was isolated using a standard proteinase K and phenol chloroform method.[14] The bacterial colonies underwent DNA extraction in a single batch accompanied by a negative control tube. The tubes containing the DNA were dried at 37°C for 30 min and re-suspended in 50 ml TE buffer (pH 8). The isolated DNA was kept at 4°C till further use.

2.3. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

This method is used for distinguishing intraspecific variations among bacterial strains of the same species using ERIC PCR primers derived from within ERIC sequences.[15] Enterobacterial repetitive intergenic consensus (ERIC) sequences are 127-bp imperfect palindromes that occur in multiple copies in the genomes of enteric bacteria and vibrios. They are distributed randomly in the bacterial species throughout the whole genome. It is speculated that the ERIC sequences might be responsible for gene expression. The primers are designed so that amplification occurs between copies of the ERIC sequence. If the positions of copies vary among different strains, the amplification products provide each strain with a unique fingerprint when run on a gel.

PCR was carried out using forward (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and reverse primers (5′-AAGTAAGTGACTGGGGTGAGCG-3′)[15], in 25µl volume using 10ng of DNA, 1U of Taq polymerase (Bangalore Genie, India), and 10pmoles of each primers (Quiagen Operon, Cologne, Germany), 0.25mM (each) deoxynucleotide triphosphate (MBI, Fermentas) and 1.5 mM $MgCl_2$ in standard PCR buffer. All the amplifications were carried out in a thermal cycler (Biometra, Goettingen, Germany).

2.4. Analysis of PCR amplified products

After PCR, 3 µl of gel loading buffer (0.1% bromophenol blue, 50% glycerol) was mixed with each amplified products, and 15 µl of the mixture was loaded onto 1% agarose gel containing 0.5 µg/µl ethidium bromidein 1x TBE buffer (40 mM Tris-borate; 1 mM EDTA, pH 8.0. DNA ladders 100bp and 1kb (MBI, Fermentas) were used as molecular markers.

The electrophoresis was carried out initially at 250 V for 5 min to allow the samples to enter the gel quickly (to avoid diffusion due to prolonged electrophoresis at 50V) and then at 50V for about 40 min till the dye marker moved at least 3/4th the length of the gel. The DNA bands were visualized under UV transillumination. The photographs were taken using AlphaDigiDoc™ gel documentation system(Alpha Innotech Corporation, CA, USA) to record results and visually compare isolates and thus test for relatedness among different strains (Fig-1). Reproducibility was monitored by comparing the results of ERIC carried out with the same samples on different days.

2.5. Dendrogram construction and genetic relatedness

Dendrogram for cluster analysis of all the isolates were constructed by using NTSYS pc2.0 programme of unweighted pair-group method, arithmetic mean (UPGMA).

A binary table or haplotype matrix for each strain was constructed linearly composing presence (1)/absence (0) data derived from the analysis of the gel. Microsoft Excel program was used for putting strains in the rows and markers in the columns. Haplotype matrix, made in microsoft excel was incorporated in NTSYS pc2.0, SIMINT program was used to compute similarities or dissimilarities in the form of average taxonomic distance which was used to perform sequential, agglomerative, hierarchical, and nested (SHAN) clustering. Dendrogram was constructed from the similarity matrix obtained in the SIMINT program by the unweighted pair group method arithmetic mean (UPGMA).

2.6 Statistical analysis

Fischer's exact test was used to analyse the association between a particular genotype with disease conditions i.e. ulcerative colitis and non specific diarrhoea.

3. Results

Figure-1 shows the gel photograph of the amplified PCR products and quite significantly all the lanes except 2-5

and 26-29 show characteristically a band at 1000bp location which gives a clue to the fact that these band possessing *E.coli* recovered from UC patients may have some common arrangement of ERIC sequences in their genome. On the basis of these bands a dendrogram was constructed which is depicted in figure-2.

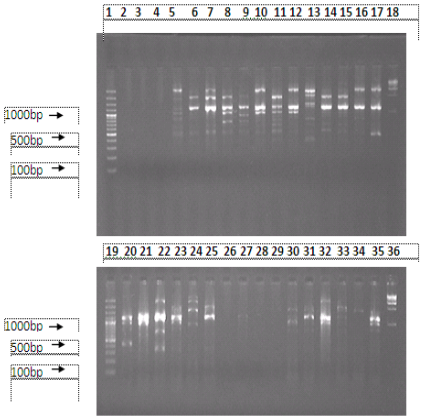

Figure-1: Representative gel showing amplification of *Escherichia coli* genomic sequences by ERIC primers. Lane 1& 19: 100 bp ladder; Lane 18 & 36: 1 kb ladder molecular marker. Lanes 3-17 & 20-35: *Escherichia coli* isolates

Figure-2 shows 3 clusters at the similarity level of 25%. The largest cluster (cluster-I) consisted of 42 strains. Many of the strains were genotypically identical by ERIC PCR in this cluster. A total of 11 isolates were found to be identical followed by another group of 8 and 4 and 3 isolates in this cluster. With the exception 3 isolates from patients with non specific diarrhoea, all the 39 (92.8) strains clustered in this group were of UC origin. The number of strains of UC origin were significantly higher than of NSD origin ($p<0.000001$). Further, 2 of the 3 isolate from nonspecific diarrhoea (NSD) cases could be seen on separate branches of the dendrogram while the 3rd one was identical with 2 strains of UC origin (UC 29 and 30). On the other hand, cluster-II was comprised of 18 strains of which 17 strains were of non specific diarrhoea (NSD) origin. A single strain of UC origin (UC22) could be seen in this cluster, but on completely independent branch. In cluster II also the number of strain of NSD origin was significantly higher than those of UC origin (0.000002). Moreover, 8 of the 17 (47.1%) NSD origin strains were found to be identical by ERIC PCR method. Further 3 strains (NSD 12, 13, 20) were identical while 2 pairs (NSD 6, 7 and NSD 14, 16) were also found to have identical banding pattern. Only strains NSD15 and NSD 17 were seen on totally independent branch. Cluster 3 consisted of 4 strains and none of them was identical and all of them were isolated from UC patients.

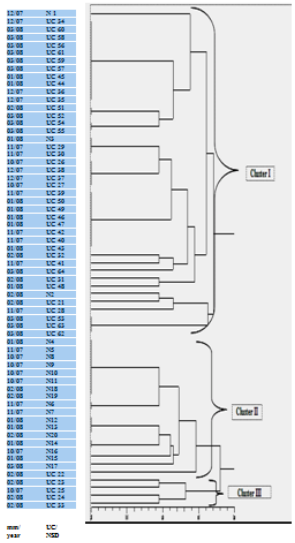

Figure-2: Dendrogram of 64 *Escherichia coli* strains based on ERIC PCR. Strains:1 - 20, Non Specific Diarrhoea; Strains:21- 64, Ulcerative Colitis

4. Discussion

The aim of this preliminary study was to explore the genetic diversity among the *E. coli* isolates recovered from rectal mucosa of patients with ulcerative colitis and patients with non-specific diarrhoea. Majority of the available reports have emphasized on phenotypic characterization of *E. coli* from IBD or on a few of the virulence factors encoding genes.[12,16] Our aim was to assess whether a particular type of strain was associated with UC on the basis of whole genome analysis of *E. coli* isolates by using ERIC PCR.

The efficacy of ERIC PCR based discrimination DI of 0.845 seems to be quite satisfactory than ribotype profiling on the *E. coli* strains isolated from Crohn's disease.[17] Interestingly, 39 (88.6%) of the 44 UC isolates could be placed in single major cluster. Only 3 strains of NSD were seen in this cluster. Further, clustering of 39 UC isolates in one cluster along with presence of 11 genetically identical strains strongly indicates that *E. coli* strains from UC patients are definitely closely related. *E coli* strains from NSD are definitely of different type since 8 of the strains belonging to this group were also identical. The patients yielding the 3 strains of the later origin grouped with UC cluster; need to be followed up whether they are developing any inflammatory bowel disease in future. Therefore, existence of UC specific *E. coli* strains may be considered. Implication of these strains in the disease process and/or relapse may be further affirmed.

The present study indicates existence of *E. coli* strains that may have evolved from same ancestral strains

perhaps by acquisition of additional virulence factors via gene transfer or insertion of pathogenecity island(s) in the bacterial genome. These changes would increase the pathogenecity of some *E. coli* strains leading to the colonization of the intestinal mucosa of patients with ulcerative colitis. As the present ERIC PCR based whole genome analysis cannot throw light on the variations in the specific virulence factor/s in the isolates of UC origin, therefore, various virulence genes and their expression of these *E. coil* strains must be explored extensively. Further work, using molecular techniques for DNA mapping of insertional DNA sequences, different virulence marker genes in *E.coli* strains associated with ulcerative colitis but absent in *those* isolated from healthy individuals is needed to be explained for detection of potentially unknown virulence determinants. Furthermore, 2D proteomic analysis of such *E. coli* strains in terms of their excretory, secretory products and whole bacteria may also provide some clues about virulence protein(s).

5. References

1. Ardizzone S. Ulcerative colitis. Orphanet encyclopedia. September 2003 :http://www.orpha.net/data/patho/GB/uk-UC.pdf.
2. Khosla SK, Girdhar NK, Lal S. Epidemiology of UC in Hospital and select general population of Northern India. JAPI 1986; 34:405-7 PMid:3771475
3. Scaldaferri F, Fiocchi C. Inflammatory bowel disease: progress and current concepts of etiopathogenesis. J Dig Dis2007; 8:171-8. http://dx.doi.org/10.1111/j.1751-2980.2007.00310.x PMid:17970872
4. Farrel RJ, LaMont TJ. Microbial factors in inflammatory bowel disease. Gastroenterol Clin Nor. Am: pp. 2002; 41-62.
5. Tabaqchali S, O'Donoghue DP, Bettleheim KA. *Escherichia coli* antibodies in patients with inflammatory bowel disease. Gut 1978; 19:108-3. http://dx.doi.org/10.1136/gut.19.2.108 PMid:344155 PMCid:1411820
6. Farmer RG, Michener WM. Association of inflammatory bowel disease in families. Front Gastrointest Res1986; 11: 17-26.
7. Orholm M, Iselius L, Sorensen TI. Munkholm P, Langholz E, Binder V. Investigation of inheritance of chronic Inflammatory Bowel diseases by complex segregation analysis. BMJ 1993; 306: 20-24. http://dx.doi.org/10.1136/bmj.306.6869.20 PMid:8435571 PMCid:1676367
8. Sartor RB. Enteric microflora in IBD: Pathogens or commensals? Inflamm Bowel Dis1997; 3:230-5. http://dx.doi.org/10.1002/ibd.3780030309
9. Rahimi R, NikfarS, Rezaie A, Abdollahi M. Meta- analysis of antibiotic therapy for active ulcerative colitis. Dig Dis Sci 2007; 52: 2920-5. http://dx.doi.org/10.1007/s10620-007-9760-1 PMid:17415632
10. Von Wulffen H, Russman H, Karch H. Verocytotoxin-producing *Escherichia coli* O2: H5 isolated from patients with ulcerative colitis. Lancet1989; I: 1449-0. http://dx.doi.org/10.1016/S0140-6736(89)90151-7
11. Burke DA. *Escherichia coli* and ulcerative colitis. J R Soc Med1997; 90:612-7.PMid:9496273 PMCid:1296671
12. Giaffer MH, Holdsworth CD, Duerden BI. Virulence properties of *Escherichia coli* strains isolated from patients with inflammatory bowel disease and infectious diarrhoea. Gut1992; 33: 646-50. http://dx.doi.org/10.1136/gut.33.5.646 PMid:1612481 PMCid:1379295
13. Cooke EM, Ewins S, Hywell-Jones J, Lennard-Jones JE. Properties of strains of *Escherichia coli* carried in different phases of ulcerative colitis.Gut1974; 15:143-146. http://dx.doi.org/10.1136/gut.15.2.143 PMid:4595080 PMCid:1412904
14. Ho SA, Hoyle JA, Lewis FA, Seker AD, Cross D, Mapstone N P, Dixon M F, Wyatt J I, Tompkins DS, Taylor GR, Quirke P. Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. J Clin Microbiol 1991; 29:2543-9.PMid:1723072 PMCid:270370
15. Versalovic J, KoeuthT, Lupski JR. Distribution of repetitive DNA sequence in Eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 1991; 19: 6823?6831. http://dx.doi.org/10.1093/nar/19.24.6823 PMid:1762913 PMCid:329316
16. Burke DA, Axon ATR. Adhesive Escherichia coli in inflammatory bowel disease and infective diarrhoea. BMJ1988; 297: 107-14. http://dx.doi.org/10.1136/bmj.297.6641.102 PMid:3044496 PMCid:1833778
17. Masseret E, Boudeau J, Colombel JF, Neut C, Desreumaux P, Joly B, Cortot A, Darfeuille-Michaud A. Genetically related Escherichia coli strains associated with Crohn?s disease. Gut 2001; 48: 320-5. http://dx.doi.org/10.1136/gut.48.3.320 PMid:11171820 PMCid:1760134