European Journal of Biotechnology and Bioscience

www.biosciencejournals.com

E J B B

European Journal of Biotechnology and Bioscience

ISSN: 2321-9122
www.biosciencejournals.com
EJBB 2015; 3 (2): 20-25
Received: 05-02-2015
Accepted: 23-02-2015

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Determination of genetic diversity among antibiotic resistant E. *coli* strains using RAPD molecular markers

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Abstract
Urinary tract infection (UTI) is a common problem in pregnant woman. *Escherichia coli* are known as common microorganism that exists in the intestine tract of human and warm-blooded animals. In the present investigation *E.coli* were isolated on EMB agar from urinary tract infected of pregnant women from hospitals in and around Bangalore. A total of 352 *E.coli* samples were obtained from different patients. Of these 29 samples were found to be resistant to multiple drugs and were used for further investigation for determining phylogenetic Analysis. The DNA isolated was used for RAPD analysis using 10 different primers namely OPV 12, OPA 13, OPA 08, OPR 08, OPU 17, OPA 11, OPU 14, OPD 19, OPS 03, OPA 10 and OPD 20. The RAPD of the samples with the primers showed multiple banding patterns with bands ranging from 5 to 13 bands per sample. The average bands were found to be 8 bands. The band size varied from 100bp - 1500 bp when compared with standard 500bp ladder. The phylogenetic analysis showed that the isolates were mainly of 5 different sub types which varied to each other to a smaller extent.

Keywords: Urinary Tract infection, *E.coli*, RAPD, Phylogenetic Analysis

1. Introduction
Microbial ecology is related to the study of complex microbial communities, such as the micro flora inhabiting the human large intestine. The normal micro flora in the intestine contains an immense number of bacterial species. At any given point of time, many different strains of the same species coexist together. For example, an individual typically harbors one to ten different *E.coli* strains simultaneously [1, 2]. Some of these strains have the capacity to persist in the colonic micro flora for extended periods of time (resident strains), while others are not capable of long term colonization (transient strains) [3, 4].

According to World health organization (WHO) report, approximately 11 million children under the age of five die because of *E.coli*-mediated gastroenteritis. There are wide differences in the prevalence of different categories of diarrheagenic *E.coli* (DEC). These groups include Enteropathogenic *E.coli* (EPEC), Enteroinvasive *E.coli* (EIEC), Enterotoxigenic *E.coli* (ETEC), Enteroaggregative *E.coli* (EAggEC), Diffuse-adhering *E.coli* (DAEC) and Verotoxin-producing *E.coli* (VTEC) or Shiga -toxin producing (STEC) [includes Enterohaemorrhagic *E.coli* (EHEC)]. The incidence of DEC is largely unknown in India as very few laboratories can identify these organisms. ETEC and EPEC are the major bacterial enteric pathogens amongst DEC but the newer increasingly reported DEC in the recent years are the Shiga-like toxin producing *E.coli* (STEC) and entero aggregative *E.coli* (EAggEC) [5, 6].

In many studies of UTIs account for about 10% of office visits by women, and one-third of women will have a UTI at some time during their life. In pregnant women, the incidence of UTI can be as high as 8% [7, 8].

Escherichia coli has been known as one of the most common bacteria found in the intestinal tract of human and warm blooded animals [9]. Most strains of *E.coli* are harmless and as part of the normal intestinal micro flora of man, mammal and birds [10]. Their ability to survive outside the body for longer period of time makes them an ideal indicator organism to test food and environmental samples for fecal contamination [11, 12, 13].

DNA profiling techniques has become one of the important techniques in the present days and many various PCR based techniques have been developed and applied to members of

diverse bacterial genera. These techniques includes enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) [14, 15, 16, 17], random amplified polymorphic DNA-PCR (RAPD-PCR) [18], amplified restriction fragment length polymorphism (AFLP), plasmid profiling and pulsed field gel electrophoresis (PFGE)[19]. These approaches are very helpful to investigate the genetic diversity and epidemiological relationships of the bacteria.

Random Amplified Polymorphic DNA is one of the PCR based technology which is mainly used to distinguish between different strains within same species. In this technique one or few short primers of arbitrary sequence are made to bind under low stringency conditions with various sites on both strands of the template DNA. The PCR reaction yields a series of products with varying size. The band pattern represents a "genetic fingerprint" characterizing a particular bacterial strain [20].

Materials and Methodology:

Isolation of Microorganisms:

Microorganisms were isolated from the urinary tract of pregnant women having intestinal tract infection. The microorganisms were isolated on Nutrient agar medium. The isolates obtained were characterised by Gram's staining and standard Biochemical tests. The microorganisms showing similarity to *E.coli* were then subcultured individually on EMB agar media to confirm the presence of *E.coli*. The presence of *E.coli* was confirmed by the production of Green metallic sheen on EMB agar media. These cultures were then used for further investigation.

DNA Isolation:

The genomic DNA from the samples was isolated by Phenol-chloroform extraction method. The protocol suggested by [21] was followed with some modifications for the isolation of DNA from *E.coli* samples where in 2ml of 24-48 hours culture broth was centrifuged at 10,000 rpm for 10 minutes. The pellet was dissolved with 1ml of CTAB buffer and then the samples were incubated at 60^0C for one hour, 1ml of phenol: chloroform: isoamylalcohol (25:24:1) was added in the vials and vortexed. The samples were then centrifuged at 10,000 rpm for 10 min. Supernatant was transferred to a new eppendorf tube and equal volume of chloroform: isoamyl alcohol mixture (24:1) was added and mixed gently to avoid shearing of the DNA by inverting the tube until the phases are completely mixed and centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube. To this double the volume of chilled ethanol was added and incubated at -20°C for overnight. The tubes were then centrifuged at 10,000 rpm for 10 min and the pellet was air dried. 50 µl of TE buffer was added to the pellet and dissolved. The isolated DNA was then stored at -20 °C for long term until further investigation.

Qualitative and Quantitative estimation of DNA:

DNA quality was assessed according to[21] by using Nanodrop Spectrophotometer (Thermoscientific Nanodrop 1000, USA) at the absorbance ratio of 260 and 280 nm providing a value of 1.8 which determines pure DNA preparation.

The concentration of DNA in the sample was calculated using the given formula:

Concentration of DNA = A260× 50 µg~ dilution factor

Purity of the DNA = A260: A280 ratio = A260 /A280

Quality of DNA fragment was electrophoretically analyzed through 0.8% agarose gel using 1X TAE buffer at 50 V for 45 mints. A 500 base pair ladder (purchased from Chromos biotech) was loaded into the gel as molecular size marker. The gel was visualized by staining with Ethidium bromide (1µl/10ml) and the bands were seen under UV light by using gel documentation system alpha imager hp (Innotech, USA).

Polymerase Chain reaction:

The polymerase chain reaction was carried out for the isolated DNA with RAPD primers. The PCR reaction mixture contained 2.0 µl of DNA, 1.5 µl of 2.5mM dNTP's, 0.3 µl of 3U Taq Polymerase, 2.5 µl of 10x Taq Buffer, 1.0 µl of 5pM primer each (Table 1) and 5.2µl of nuclease free water. The PCR conditions for the primer were standardized and the initial denaturation was carried out at 94^0C for 5 min. Final denaturation at 94^0C for 60 seconds, Annealing temperature was 30^0C for 60 seconds and Extension was at 72^0C for 60 seconds. This cycle was repeated for 30 cycles. The final elongation was carried out at 72^0C for 10 mins. The PCR products were determined on 1.5% agaore gel.

Table 1: Primer Sequence

Primer Name	Primer Sequence
OP V-12	ACCCCCCACT
OP A-13	CAGCACCCAC
OP A-08	GTGACGTAGG
OP R-08	CCCGTTGCCT
OP U-17	ACCTGGGGAG
OP A-11	CAATCGCCGT
OP U-14	TGGGTCCCTC
OP D-19	CTGGGGACTT
OP S-03	CAGAGGTCCC
OP A-10	GTGATCGCAG
OP D-20	ACCCGGTCAC

Data scoring and Dendrogram analysis:

Data on the presence or absence of RAPD bands of identical molecular sizes were used for estimating genetic similarity coefficients. For all pairwise combinations, genetic similarity indices (SI) were calculated following the method of UPGMA. The formula for SI is given as

SI = 2.NAB/ (NA+NB)

where N* is the number of RAPD bands shared in common between individuals A and B, and NA and NB are the total number of bands scored in A and B, respectively.

The similarity matrix is calculated by Frequency similarity Index obtained from alpha imager hp gel doc software. Dendrogram for RAPD fragments were constructed by using an un-weighted pair group method of arithmetic mean of UPGMA [22].

Results:

Isolation of Microorganisms:

The microorganisms were isolated from urinary tract infected pregnant women from hospitals in and around Bangalore. The samples were collected and the microorganisms were isolated on nutrient agar, the organisms were then plated on EMB agar for the confirmation of *E.coli*. A total of 352 *E.coli* samples were obtained from different patients. Of these 29 samples were found to be resistant to multiple drugs and were used for further investigation.

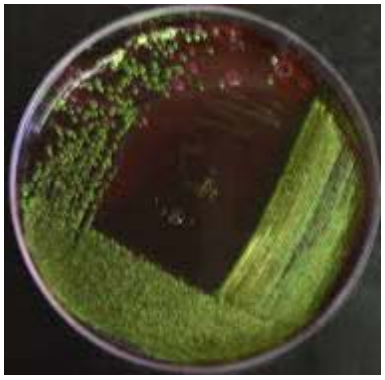

Fig 1: *E. coli* on EMB Agar

DNA Isolation:

The DNA was isolated from all the 29 samples by CTAB buffer and phenol:chloroform method. The qualitative estimation of the DNA on 0.8% agarose gel gave single, sharp and distinct bands devoid of any smear (Figure 2). Thus, genomic DNA of good quality without any degradation was successfully isolated from all the jasmine samples. Here, the quantitative estimation of genomic DNA was done by Thermo Scientific Nanodrop 1000 spectrophotometer. The genomic DNA were obtained in high concentration for all the samples and they showed a good 260/280 ratio (i.e. between 1.8 and 2.0) indicating absence of any protein or RNA contaminants (Figure 3).

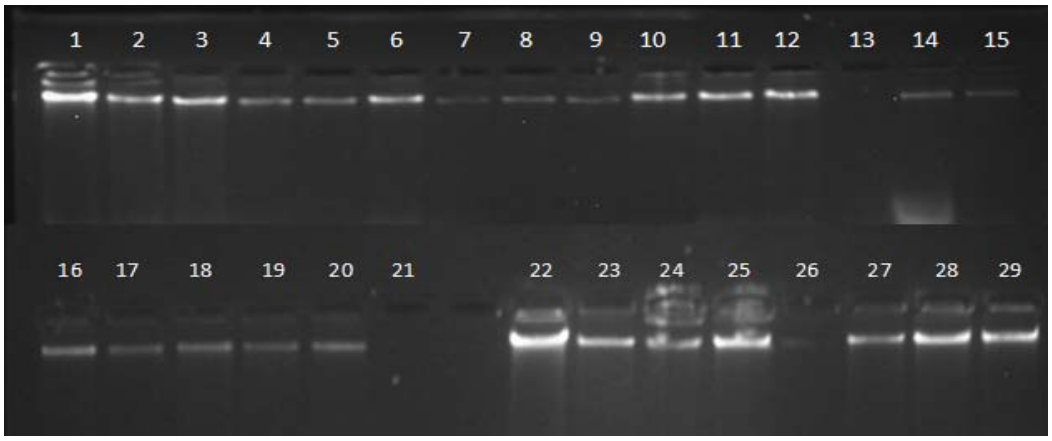

Fig 2: Quantitative Analysis of DNA

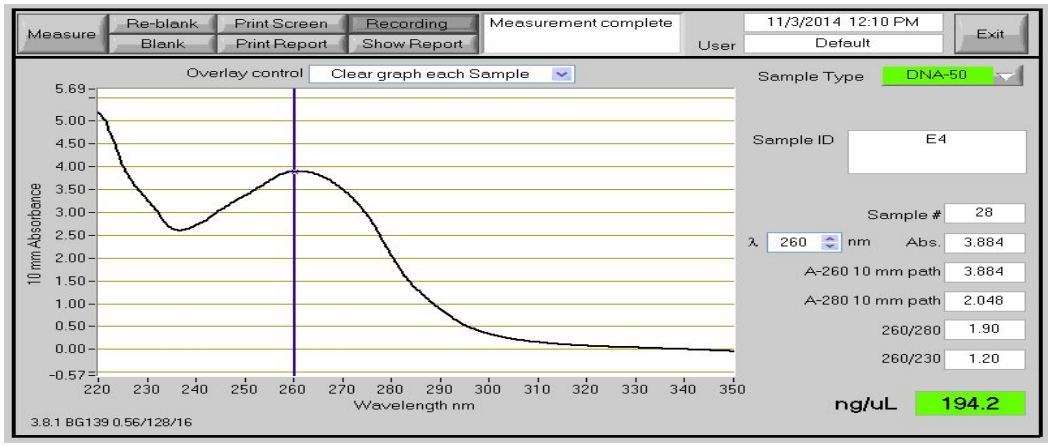

Fig 3: Quantitative Analysis of DNA

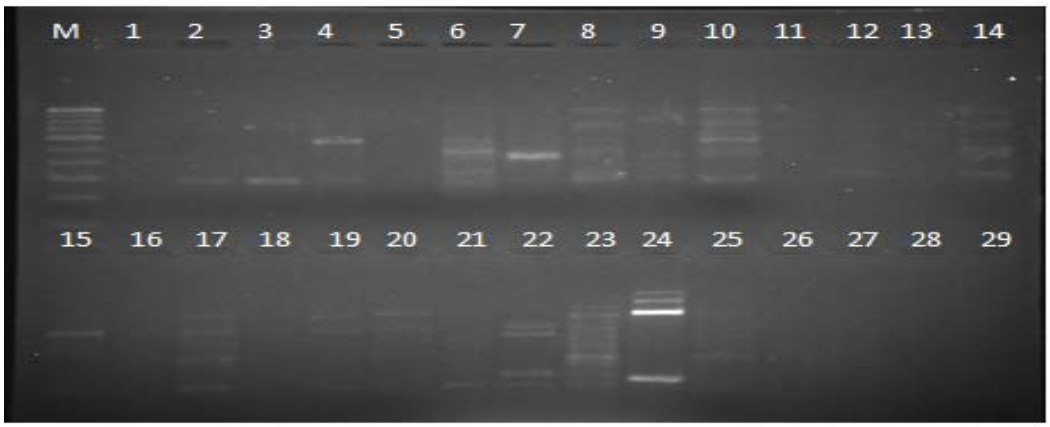

Fig 4: RAPD Analysis with OPD 19

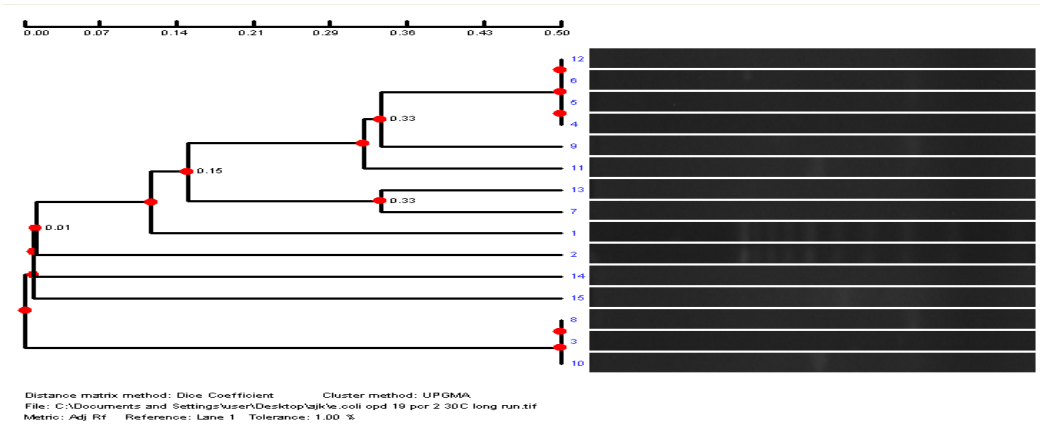

Fig 5: Dendogram with OPD 19

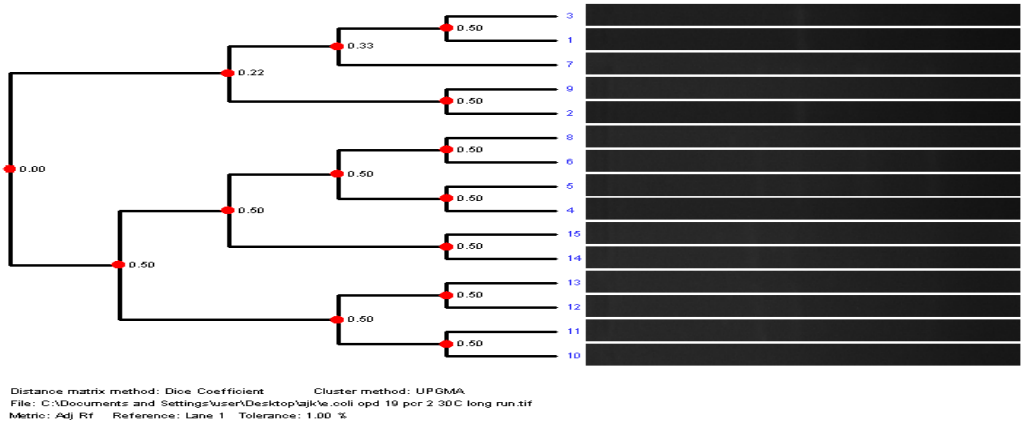

Fig 6: Dendogram with OPD 19

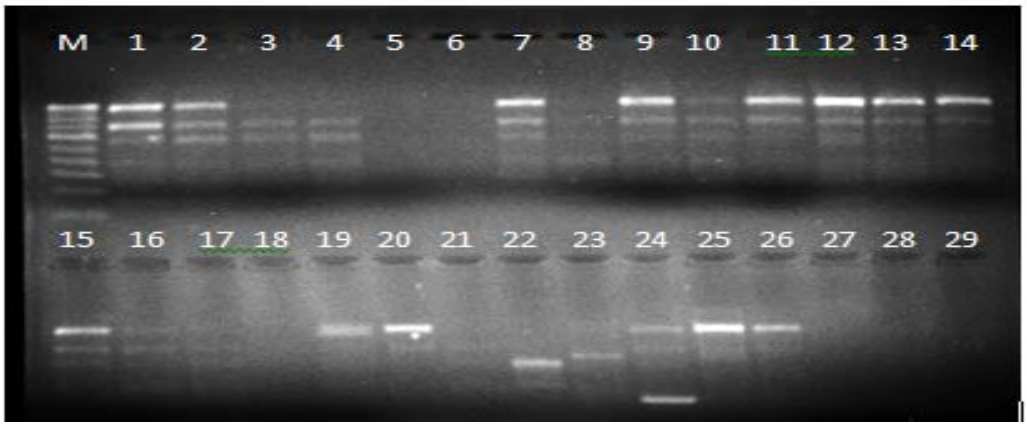

Fig 7: RAPD Analysis with OPU 17

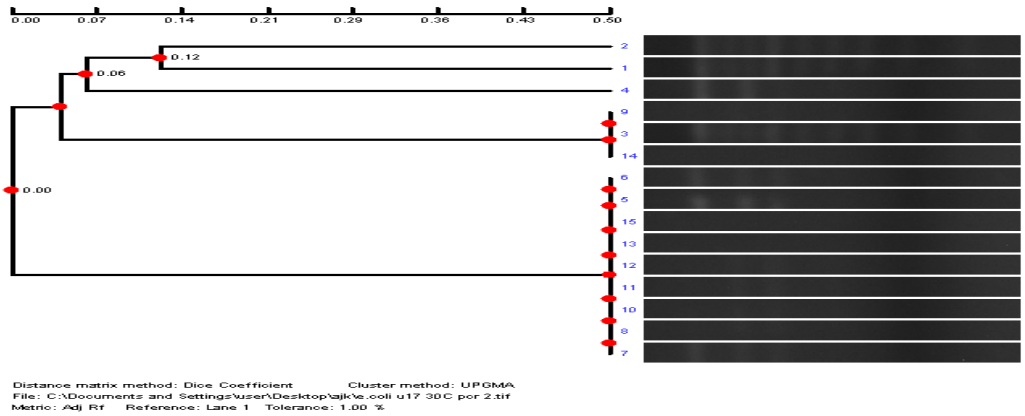

Fig 8: Dendogram with OPU 17

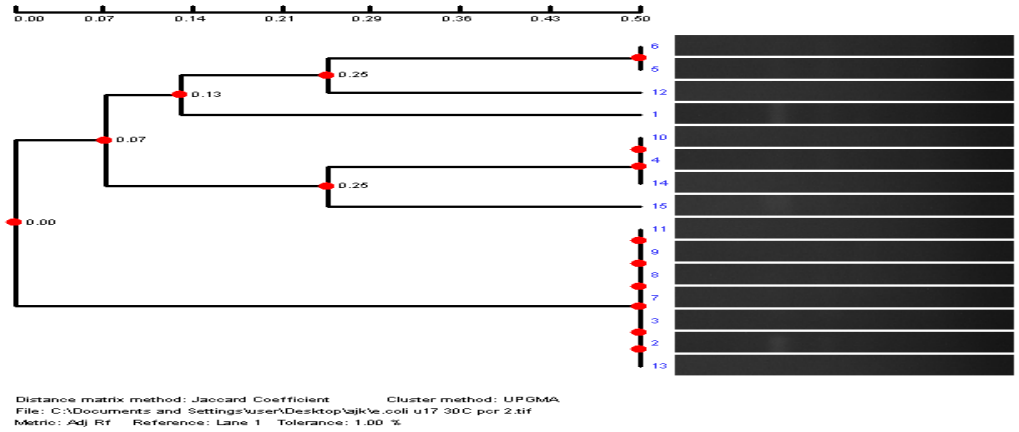

Fig 9: Dendogram with OPU 17

The genetic relatedness among the *E.coli* strains in this study was distinguished by performing RAPD. Figure 4 and 7 shows the RAPD-PCR gel patterns of the *E.coli* strains obtained with primer OPD 19 and OPU 17 respectively. The number of DNA bands produced for a given primer ranged from 1 to 11 with varied molecular sizes ranging from 100 bp - 1500 bp when compared with standard ladder of 500 bp. The possible number of RAPD patterns was estimated by the changes of one or more clear bands or band size. There were 10 RAPD patterns observed using OPD 19 and 8 RAPD patterns with OPU 17. This patterns of banding indicated that the *E.coli* strains can be differentiated into more groups compared to when using these primers individually. Hence, combined primers provide increased sensitivity towards genetic variations. Some of the *E.coli* strains have indistinguishable RAPD fingerprinting profiles which suggest that they may be closely related or have no genetic differences. Certain isolates did not produce band in this analysis mainly because there were no sequence in the bacterial DNA which is complementary to the sequence of the primer.

DISCUSSION:

Urinary tract infection (UTI) is an extremely common clinical problem. It is important because it may involve the urethra, bladder, uterus, and kidney [23]. UTIs are the commonest infections seen in hospital settings, and the second commonest infections seen in the general population [24].

[25] demonstrated that a history of past urological problems was associated with an increased incidence of UTI in pregnancy. UTI affects all age groups, but women are more susceptible than men, due to short urethra, absence of prostatic secretion, pregnancy and easy contamination of the urinary tract with faecal flora [26].

Several investigations have been carried out on RAPD characterization of *E.coli* strains using a single primer and agarose electrophoresis [27, 28]. The RAPD analysis has proven to be useful in discrimination, characterization and differentiation of the bacterial isolates and grouping them according to similarity. The banding pattern by RAPD is mainly dependent upon the primer and the banding pattern varies with each primer.

Several methods had been used for typing and differentiating *E.coli* isolates. The RAPD-PCR is more sensitive and more cost effective than other molecular methods [29, 30].

[31] carried out Rapid and Simple Determination of the *Escherichia coli* Phylogenetic Group and found that *Escherichia coli* is composed of four main phylogenetic groups (A, B1, B2, and D) and that virulent extra-intestinal strains mainly belong to groups B2 and D.

[32] studied about the Molecular Genetic differentiation of avian *Escherichia coli* by RAPD-PCR using primer 1247 (5/-AAG AGC CCG T-3/) and their RAPD analysis showed that these isolates were grouped into 33 RAPD types and avian isolates were discriminated into 29 genotypes.

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