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Facile Approaches for Microbial DNA Extraction: Comparative Study with Colonies "Picked" from three Different Bacteriological Mediums

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DNA isolation preludes any nucleic acid amplification method but also tend to increase the time duration depending on the procedure adapted. The current work is aimed at swotting simplistic methods involving mechanical shearing, boiling and alkaline lysis for bacterial DNA extraction. Comparative analysis of the DNA yield from bacterium "picked" from three different mediums were also tried to select the optimal practice for productive results. Apart from being an expeditious step, Boiling lysis (BL)was also proved to be efficacious for further analysis using PCR-RFLP.

Key words: DNA extraction, String test, boiling lysis, Selective and differential medium, PCR-RFLP.

The etiology of an infection is of at most importance in clinical practice for proper diagnosis and medication. Preliminary screening of a bacterium involve growth on selective or differential medium followed by battery of phenotypic tests that render a signature profile that are linked to a particular microbe. These conventional methods are time-consuming with results and their interpretation often depending on the subjective judgment and expertise of the personnel involved, time taken for its storage and transport to the testing site. Transient mutational events leading to phase-variable colonies make it further difficult to identify the bacterium, on the basis of morphovars and pigmentation on selective medium[1]. With an ever increasing demand for rapid and reproducible assays, molecular methods especially nucleic acid based amplification tests (NAATS) are now preferred for authenticating the identity of microbes. DNA isolation is a pre-requisite for NAATs with several protocols and kits dedicated to the same. These methods vary with respect to steps and components to bring about lysis, elution, purification and recovery which are decisive for providing sensitivity and resolution[2]. Different steps for isolation can be considered disadvantageous as it increases the time frame required for further assays. This can be circumvented by method of colony PCR, a common choice for screening recombinant plasmids[3] but applicable as an alternative to DNA extraction steps. In spite of number of investigation reporting comparative methods of DNA isolation, a study of the same from different bacteriological medium is missing. The *modus operandii* of the current work was to execute an analogy of three procedures (i) colony PCR (no DNA isolation step) (ii) boiling lysis (mechanical method) (iii) alkaline lysis (chemical method) for obtaining bacterial genomic DNA. These methods were studied against isolates "picked" from two selective (MacConkey Agar: MAC & Eosin Methylene Blue: EMB) and general medium agar (Nutrient: NA) plates (Fig. 1).

To achieve this objective four γ-*enterobacteriaceae* members, *Citrobacter koseri, Enterobacter aerogenes, Escherichia coli* and *Klebsiella pneumonia* were targeted. The

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rationale behind the choice of organism and medium were due to the following reasons:

(i) All the selected strains cause UTI, one of the major nosocomial infection, thus requiring a rapid detection method[4]

(ii) EMB and MAC are common choices of selective medium to screen *E. coli*, the main pathogen for causing UTI[5]

(iii) Phase variation among bacterial strains may result in colony semblance making it difficult to identify the microbe (Fig 2)

(iv) Manual error, incongruous results or new phenotypic trait lead to misinterpretation of the identity of *E. coli* with these strains on biochemical characterisation.

An optimal and standard step for DNA isolation with minimal requirement make it less laborious process thus speeding up the processing and reporting time of molecular assays. The feasibility of the isolated DNA for any further course were checked by identifying bacterial strains by the coupling PCR with Restriction Fragment Length Polymorphism (PCR-RFLP) [6] targeting 23S ribosomal assemblage.

MATERIALS AND METHODS

Strains and culture conditions

C. koseri (MTCC 1658 /ATCC 8090*), E. aerogenes* (MTCC 111 /ATCC 13048), *E. coli* (MTCC 433 /ATCC 15223) and *K. pneumonia* (MTCC 432 /ATCC 33495) were obtained from Microbial type culture collection (MTCC), Institute of Microbial technology (IMTECH, Chandigarh); revived in nutrient broth. The cultures were streaked on NA, EMB and MAC agar (Himedia) respectively and kept for overnight incubation at 37^0C.

Biochemical assay

Colony morphology and pigmentation were noted for all the isolates. Biochemical characteristics for all the respective strains were marked for Indole production (I), glucose fermentation by Methyl Red (MR) and Voges-Proskauer (VP) test and Citrate utilization (C).[7]

DNA isolation methods

As a pilot study, the task of DNA isolation from *E. coli* strains (ATCC 15223) from NA plates were performed using three methods; mechanical (vigorous vortexing), heat (100^0C) and alkaline treatment (3% Potassium Hydroxide). Isolated DNA were "run" on 0.7% agarose gel and the follow-up of the same with colonies selected from each mediums were repeated. The extracted DNA of these were used as templates for PCR reaction; bands were observed by Agarose Gel electrophoresis (AGE) at 5V/cm on 2% agarose gel stained with0.5mg/ml of Ethidium Bromide (EtBr). Based on the results obtained, a total of five methodological trials were formatted and executed with colonies from all the respective mediums for optimizing the best and effective method for DNA isolation. Discrete colonies were selected and transferred with the wooden tooth pick to respective eppendorf tubes. Trial 1(T1) comprised of directly inserting the selected colonies into the PCR mixture. For trial 2 (T2) and 3 (T3), colonies were dispensed into 10ml of nuclease free water each. T2 were given heat treated in boiling water bath for 10min whereas T3 were vortexed for 30 sec. In Trial 4 and 5, nuclease free water was replaced by 3% KOH. Colonies in T4 were heat treated (as T2) whereas in T5, the colonies were aspirated repeatedly with pipette instead of vortexing. The respective tubes of T2 -T5 were centrifuged at 10,000 rpm for 2 min. 2ml of the supernatant was taken as the DNA aliquot(s) from each for separate PCR reactions.

PCR-RFLP (Molecular assay)

The reaction mixture of 12.5 µl comprised of 2 µl of supernatant of the trails (except for trial 1), 0.5 µM each of forward (FP: 5'-GGCGAAAAGAACCCCGGCGA-3') and reverse primer (RP: 5'-AGGGGTCGACTCACCCTGCC-3') from $23SP1_{880}$ primer set (designed for this experiment, Sigma), 1X of assay buffer with 1.5mM of $MgCl_2$, 800 µM of dNTP mix and 0.5U/ml Taq polymerase (KK5004 KAPA 2G™ Robust PCR kit). The reactions were carried out in a thermal cycler (Bioer) with the program set for 25 cycles wherein each cyclic step comprised of denaturation (95^0C), annealing (66^0C) and extension (72^0C) for 30s each. 3µl of PCR products were restricted using 0.3µl of 5U/ml of *Bfa*I (Fermentaz). The amplicon and restriction digestion products were checked by AGE on 2% and 2.5% agarose gels respectively. Gels were viewed and photographed in Bioimaging system (Syngene).

Confirmatory analysis

The optimal method selected from the trails were rechecked for its reproducibility in 25 isolates each of clinical (UTI samples, Excel

Pathology lab, Vashi, Navi Mumbai), environmental (water samples) and commensal (fecal samples; Humans, Excel pathology lab & Animals: Pet shop Crawford market, Mumbai) origin. Discrete colonies of each isolates on EMB and MAC selected and streaked onto nutrient plates. Both biochemical characterization and molecular analysis were performed; with the chosen method of DNA extraction for latter.

RESULTS

E. coli displayed positive I & MR with negative results in VP and C test, whereas *K. pneumoniae* and *E. aerogenes* these results are reversed. *C. freundii* gave positive MR and C test with negative I and VP test. All the four strains are lactose fermenters (LFs) and appear as pink colonies on MAC with more or less same colony characteristics (Fig 2). *E. coli* strains have reported to exhibits inter-convertible colony forms; epigenetic activity of two surface structures antigen 43 and type 1 fimbriae (Table 1) are credited for such phenomenon[8]. In these instances it becomes difficultto screen and characterise the candidate strain from other LFs solely on the basis of morphology.

E. coli are specifically distinguished from the rest by forming green sheen colonies on EMB; certain isolates with purple, aerogenes type colonies or having limited sheen were confirmed as *E. coli* by molecular assay making this method of screening not as infallible as expected. In the pilot study for DNA extraction, maximum yield was obtained in boiling lysis step (Fig 3a). The PCR reaction with the same set of trials performed across the isolated colonies from respectively mediums showed distinct results. All the methods gave positive amplification with NA colonies indicating successful extraction of DNA. EMB showed no amplification products whereas in MAC only 3% KOH treated colonies gave amplified product (Fig 3b). Characteristic metallic green sheen in EMB are observed due to metachromatic properties of the dyes eosin and methylene blue; and the same could be cited for the negative amplification in EMB as it may have inhibited the polymerase activity

The subsequent additional simplistic approaches comprising of T1 to T5 collaborated the results established in preliminary trials. Amplicons with maximum intensity and minimal noise was obtained for boiling lysis (T2) in both NA (Fig 4a, lane 3) and MAC (Fig 4b, lane 8); whereas in former amplicons were observed also with non-heating step (Fig 4a, lane 2). Recurring trials with NA colonies showed that in few instances bands were observed in KOH method (trial 4) and in some there were no amplification with trial 2 (not shown). Boiling lysis step was chosen as the optimal method of DNA isolation because on their consistent results in multiple trials.

Broad range PCR (23S $P1_{880}$) with DNA isolated from *C. freundii*, *E. aerogenes* and *K.*

Table 1. Characteristics of Interconvertable colonies of *E. coli* strains

Appearance	Large, flat , frizzy	Small, convex, glossy	Flat, irregular but smooth	Small, convex but frizzy
Ag43/Fim Characteristic	$Ag43^{+}Fim^{-}$ Sediment	$Ag43^{-}Fim^{+}$ Pellicle precipitate	$Ag43^{-}Fim^{-}$ No preference	$Ag43^{+}Fim^{+}$ Pellicle precipitate

Antigen 43 (Ag43) is asurface-displayed auto-transporter whereas type 1 fimbriae are surface adhesives for recognition and colonization [8]

Table 2. Restriction digestion products of 23S P1 880 ampliocns in each strains used in this study

	Citrobacter species	*Enterobacter species*	*E.coli*	*K.pneumoniae*
Bfa I (C/TAG)	475bp,214bp, 189bp	475bp,214bp, 189bp	665bp, 215bp	475bp,214bp, 189bp

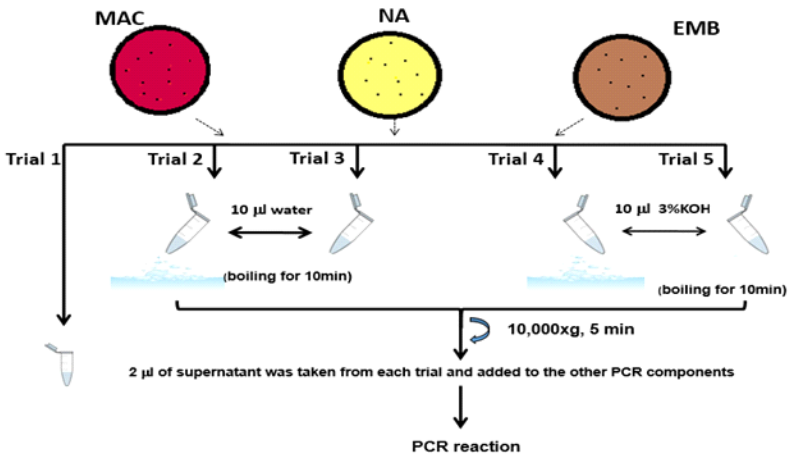

Fig. 1. Different trials for colony PCR from various mediums. Two to three pin head colonies were taken from each medium (MacConkey Agar: MAC; Nutrient agar: NA; Eosin Methylene Blue Agar: EMB)

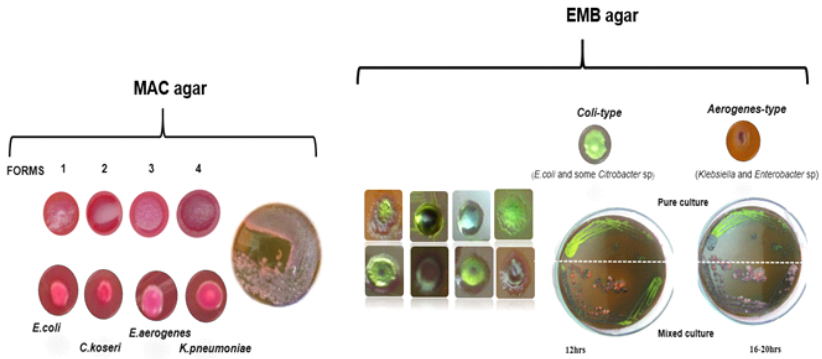

Fig. 2. Different colony morphovars of *E. coli* in MAC (upper row of colonies) and EMB as well as colony resemblance with other Lactose fermenters

Figure 3a: DNA isolation step by mechanical shearing (MS: lane 1), heating (H; lane 2) and KOH treatment (KOH; lane 3).

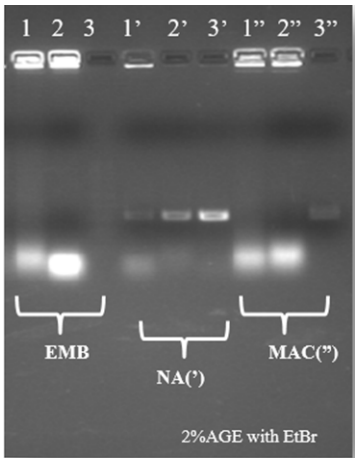

Fig. 3(b). PCR amplification with DNA obtained from mechanical shearing (1, 1',1''), boiling lysis or heating method (2, 2',2'') and 3% KOH treatment (3, 3',3'') of colonies from respective medium

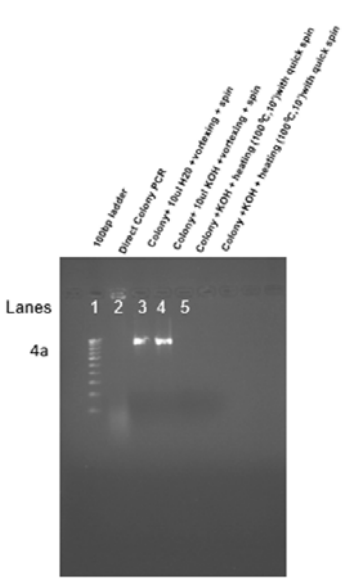

Fig. 4 (a): PCR amplification with DNA obtained with all the five trials from colonies selected from NA plates.

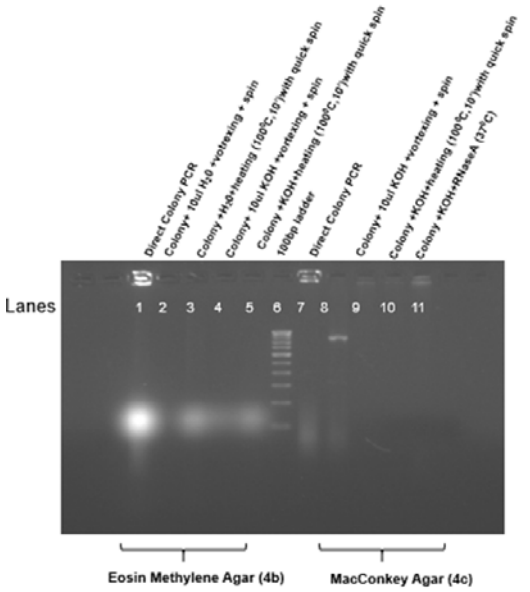

Fig. 4 (b & c): PCR amplification with DNA obtained with all the five trials from colonies selected from EMB (4b) and MAC (4c).

pneumonia (non-candidate strains, colony morphovars) using the quick boiling method showed successful amplification. Restriction analysis of the amplicons with *Bfa*I showed unique pattern in *E. coli* thus enabling the differentiation of the candidate from the rest (Table 2). This method was successfully replicated wherein 18, 21 and 25 isolates from a lot of 25 each from clinical, commensal and environmental isolates were proved as *E. coli* with PCR-RFLP.

DISCUSSION

This study was intended only in analysing a simplest yet fastest technique of DNA isolation. Confirming the identity of organism with molecular assay method was a collateral strategy to prove the duplicability of the proposed method. Routine DNA isolation from bacterial strains mostly involve repeated phenol: chloroform: isoamyl steps culminating with ethanol precipitation[9]. Apart from being arduous process the same requires two hours per reaction; other approaches involving kit methods guaranteeing rapidness and not cost-effective. The simple step of boiling causes disruption of bacterial cell wall and proteins thus allowing the DNA to emerge out from the membrane confinement. No further added steps or treatments are required for the same. KOH treatment degrades the cell membrane, the logic behind is based on the principle of "string" test that allows the differentiation between Gram negative and Gram positive bacteria[10] due to the difference in thickness of peptidoglycan layer. In the current work even though the sticky lump mass of DNA was visually noted, very few isolates that includes both standard and screened showed positive amplification results. As this method was confined only to Gram negative organism(s) the same was not further analysed.

The current work also demonstrates an alternate method of employing PCR-RFLP targeting 23S rRNA, the largest ribosomal segment for bacterial identification. In the routine molecular analysis, amplification with universal primer (especially 16S rDNA) followed by sequencing and analysis of contigs against the data pool in genome repositories is carried out[11]. A number of detriments ranging from impure culture to sequencing errors and presence of chimeric

sequence prevent in authenticating the bacterial identity[12]. The process of PCR-RFLP not only validates the organism based on their distinct restriction profile but also reduce the cost fact involved in the routine methods. This particular methodology is a corollary to the optimal DNA isolation step of boiling lysis, as a measure of its reproducibility and efficacy.

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