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PHENOTYPIC CHARACTERIZATION OF *ESCHERICHIA COLI* DIVERSITY THROUGH PHAGE TYPING

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ABSTRACT

E. coli diversification within their ecological niches was studied phenotypically through phage typing. Three water sites which were ecologically variable (well, river and drainage) were selected for isolation of *E. coli* by multiple tube fermentation technique and identification through biolog system. Three isolates were designated as *E. coli-EG1*, *E. coli-EG2* and *E. coli-EG3* to represent their source from well, river and drainage, respectively. Spot test and plaque assay were adopted for discriminating among such isolates and showed sensitivity for *E. coli-EG1* and *E. coli-EG2*, but quantitatively *E. coli-EG1* exceeded *E. coli-EG2* as, 10^5 and 10^3 PFUs respectively. The image processing and numerical analysis for plaque assay data revealed small variations in mean, median, standard deviation and number of pixels. The last was the most important as it indicated no far extent in sensitivity of *E. coli-EG1*as compared to *E. coli-EG2*. The differential behavior towards phage infection was attributed to mutational effect caused by environmental stress and exerted the role for the generation of new phenotypes which had fundamentally evolutionary impact. From our knowledge a first automated image processing method used for evaluation of plaques number based on scale and rotation invariant analysis of optical transforms was applied here.

Key words: Image processing, Bacteriophage, Bacteria.

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INTRODUCTION

The ability of a phage to infect a particular cell will be dependent on variation in primary surface receptors, which may comprise surface polysaccharides (lipopolysaccharide in Gram negative bacteria, the teichoic acids in Gram positive bacteria), **(Estrela *et al.*, 1991)**, the presence of surface structures such as flagellae or pili **(Merino *et al.*, 1990)**, and the expression of a wide range of different types of cell surface–associated molecules including sugar uptake proteins, membrane protein's layer proteins, and capsular polysaccharide **(Hung *et al.*, 2002).** Even if phage successfully penetrate the cell envelope, replication leading to cell lysis and development of a plaque may be inhibited by a variety of mechanisms, such as the presence of prophage in the cell **(Harvey *et al.*, 1993)**, DNA restriction modification systems **(Frank, 1994)**, and even specific phage inhibition genes **(Chopin *et al.*, 2005)**. These many factors which affect the efficiency of phage replication have lead to the development of phage typing schemes, where the ability of a phage to infect a cell is used as an indicator of biological variation at the cellular level. For phage typing, panels of phage characterized to have a limited host range are chosen and strains are infected with each phage at standard concentration (known as the Routine Test Dilution or RTD). Hence, a bacterial lawn is prepared and samples of the different phage at the RTD spotted onto the surface of the lawn. After incubation, infection is detected by the presence of plaques (zones of clearing) and patterns of susceptibility to individual phage are determined, leading to the characterization of a phage type. These panels of phages have successfully been used by epidemiologists to monitor changes in the predominant organism causing disease in the population and to identify the emergence of new dominant clones. A novel method for estimation of objects (here as plaques) was optical transforms. Fourier spectra analysis has found many applications in various fields as e.g. in signal analysis, optics, statistical analysis or image processing. One of the

basic property of optical Fourier spectrum, it is a shift invariance. Regardless, where the object might be located in the input plane, its spectrum is always located symmetrically on the optical axis. In the case of multiple randomly located objects with the same size and shape, their fourier spectrum contains contributions from each object, involving the phase factors from all possible objects locations. Therefore, the mutual configuration of objects in input plane is reflected in random modulation of initial spectrum, which would be observed for a single object, as a function of transverse spatial coordinates **(Buzalewicz *et al.*, 2010).**

MATERIALS & METHODS

Water samples were collected from well, river and waste waters during March (2010), from subsurface layer of the three sources. Water samples were collected using sterile glass bottles and transported in ice box to be analyzed in the laboratory within 10 hrs.

Isolation of *E. coli* from water:

E. coli detection was done according to **(Pettibone, 1992)**, using multiple tube fermentation technique. Tubes showing gas production with growth is considered a positive indication for presence of *E.coli*. From these tubes several loopfulls were streaked on MacConkey agar plates for testing lactose fermentation ability. The medium was obtained from Difco. USA and consists of (g/liter): peptone; 17.0, proteose peptone; 3.0, lactose; 10.0, bile salts No.3; 1.5, sodium chloride; 5.0, agar; 13.5. neutral red; 0.03. Crystal violet; 0.00 K distilled water; 1.0 liter. On Macconkey agar, the colonies showed pink to red color surrounded by red zone. Red colonies were picked up and purified. The pure colonies were further identified using biolog system.

Preparation of virus lysate:

The viruses specific for *E. coli* in collected water samples, were detected according to the method of **(Othman, 1997)**. Erlenmeyer flasks (250 ml) each containing 50 ml of nutrient broth were inoculated with 5 ml of the collected water samples. Mixture of *E. coli* isolates (three isolates

originated from well, river and drainage water and designated as *E. coli– EG1*, *E. coli– EG2* and *E. coli– EG3*,respectively) (1 ml) was added, and the flasks were incubated at the optimum temperature for 72 hrs. Coliphage suspensions were obtained and assayed qualitatively and the data were recorded.

Assaying of *E. coli* viruses:

Coliphages were qualitatively and quantitatively assayed by the spot test and the over layer agar techniques (plaque assay technique) according to the method of **(Othman 1997)**.The data were analyzed using adobe Photoshop, version 9.0.

Turbidity test:

The virus lysates were performed by growing *E. coli* isolates in 10 ml broth to an optical density at 600 nm of approximately 0.2, then $CaCl_2$ was added to final concentration of 10 mM and 10 ml of a solution containing the desired concentration of phage particles was obtained and O.D. 600 over time was measured.

Preparation of coliphage suspension:

The collected samples suspected to contain coliphages were adjusted pH 6.5 with 3N NaOH and centrifuged (6000 rpm for 15 mins). To remove bacterial cell debris; the supernatant was filtered through 0.45 mM pore size syringe filter. The filtrate was added to equal amounts of double strength nutrient broth medium supplemented with 10 mM $CaCL_2$ and inoculated with early lag phase host culture. After incubation at 37 ºC for 24 hrs, the mixture was centrifuged at 3000 rpm for 30min. the enrichment procedure was repeated twice. The suspension obtained from the final enrichment step was filtered, sterilized and tested for the presence of phages.

Isolation and purification of coliphages:

Single plaque assay as described by **(Othman, 1997)** was used to isolate coliphages. Unique single plaques was picked up into nutrient broth medium inoculated with *E. coli* isolates suspension (10^8 cfu/ml) and incubated at 37 ºC. After incubation, the bacteriophage particles were obtained and assayed quantitatively to be sure that all plaques have the same morphological characters.

Coliphages particles were partially purified by the repeating single plaque isolation process twice. The propagated coliphages stock was purified via sedimentation by polyethylene glycol (PEG, 6000) and dextrane sulphate two phase systems. The mixture of phage stock, PEG and dextrane sulphate and NaCL was left to stand overnight at 4 ºC, then the heavily turbid bottom layer was collected and centrifuged at low speed to obtain the remaining interphase layer which contain coliphage particles.

Ultraviolet extinction spectra of purified phages:

To study the spectral properties of coliphage isolates, UV-160A Shimadzu apparatus was used (Dept. Microbiol., Fac. of Agri., Ain Shams Univ.). The absorbance readings were taken at for instance 5 min intervals of the whole range of wavelengths (230 to 300). Data were recorded.

Lytic pattern of coliphage isolates specific for *E. coli* isolates:

Host range of the isolated coliphage for isolated *E. coli* was studied as described by **(Andrejae and Irena, 2003)**. A sample of 0.1 ml of different *E. coli* cultures (10^8 cfu/ml) was mixed with 5ml of soft agar (10%) and poured onto plates containing agar base layer. A liquots (20 µl) of the isolated phages was placed on the over layer. The seeded plates were incubated at 37 ºC for 48 hrs. The observed lysis of the tested *E. coli* isolates was as spots or plaques.

Practical implementation of Fourier transform for evaluation of the plaque forming units:

The Fourier spectral based estimation of plaques count as a novel approach depends on random modulation of initial spectra. The nature of this modulation is associated with the objects (plaques) number and their locations. This can be explained by means of scalar diffraction theory. Presented in this section theoretical consideration will show the correlation between modulation of Fourier spectrum and the object number, since these properties will be used in our approach to determine the number of plaques.

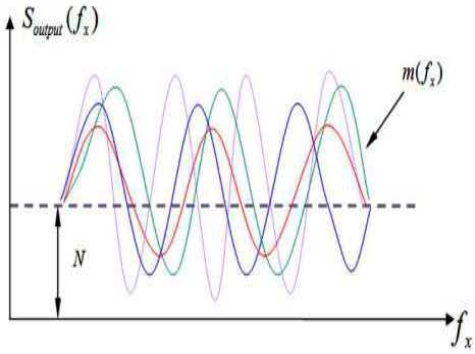

Figure 1. Conception of normalized Fourier spectrum S_{output} along *f*x (*N* is a modulation background correlated with *n*(number of objects), *m(fx)* is a modulation factor in a form of cosines sum associated with the spatial object configuration).(*Cited from* **Buzalewicz *et al.*, 2010).**

As it was already mentioned, it is possible to identify the identical objects by analyzing $S_{output}(fx, fy)$. In order to estimate the modulation background value *N*, we will define it as a mean value of maxima and minima of $S_{output}(fx, fy)$:

$$N \approx \frac{1}{2}\left[S_{output}^{MAX}(f_x, f_y) + S_{output}^{MIN}\left(f_x, f_y\right)\right]$$

RESULTS

The three *E. coli* isolates exhibited the same metabolic fingerprint and identified as *E. coli* species as a result of comparison in biolog database

Isolation and identification of *E.coli*:

Table 1. Results of microplate 96 wells after incubation with the pattern of carbon sources.

No. Symbol	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	+	-	-	+	+	-	+	-	-
B	-	+	+	+	-	+	-	+	-+	+	+	+
C	+	+	+	-	+	+	-	+	-	-	+	+
D	(/)	-	-	-+	-	+	+	-	+	+	-	-
E	+	-	(/)	-	-	+	-	+	-	-	-	+
F	+	-	+	+	+	+	+	+	+	-	+	+
G	-	-	-	-	-	-	-	+	+	-	-	-
H	-	+	+	+	-	-	-	-	+	+	+	+

Key: + = positive result (violet color)
- = negative result (colorless)
(/) = borderline (neither positive nor negative)
±= mismatched negative.

The three isolates of *E. coli* have the same carbon source utilization pattern that they were able to utilize 46 types out of 95 carbon sources and compared through the biolog database and identified as *E. coli*.

Phage typing though spot test and plaque assay:

The three water samples obtained from well, river and waste water from Great Cairo were examined for the presence of virulent phages specific for *E. coli* by determining turbidity, spot test and plaque assay.

Turbidity test:

These samples of water were investigated for the presence of coliphages by the turbidity test. Three *E. coli* isolates were used as an indicator host as in (**Table 2**). Data showed no phage specific for *E. coli-EG3*(Turbidity values in great a proximity with control) and phages were found only in two water samples (well and river) in which turbidity measurements were (1.125) and (1.165) as compared to (0.075) for *E. coli-EG3*.

Table 2. values of turbidity test for the phage lysate against three isolated *E. coli* from water samples.

***E. coli* isolate**	**Control**	**Water sources**		
		Well water	**River water**	**Waste water**
E. coli-EG1	0.050	1.165	1.125	0.052
E. coli-EG2	0075	1.075	1.050	0.075
E. coli-EG3	0.072	0.075	0.065	0.062

Bacteriophage specific for *E. coli* (coliphages) was detected in the water sample using spot test technique, but specificity was restricted to *E. coli-EG1* and *E. coli-EG2* rather than *E. coli-EG3*. Lysis of bacterial cells was demonstrated for those two isolates and plaques count also was evaluated using plaque assay that

showed 10^5 and 10^3 for *E. coli-EG1*and *E. coli-EG2* as shown in the following (**Figure 2**):

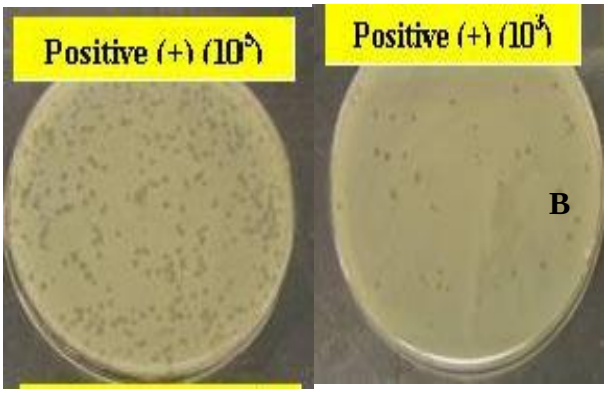

Figure 2. plaque assay illustrating the presence of coliphages through lysis of bacterial lawn that could be seen plaques. Plaques for *E. coli-EG1* (A) and *E. coli-EG2* (B) were shown.

Qualitative and quantitative characters that discriminate between the three *E. coli* isolates in terms of their sensitivity to coliphages were summarized in the following (**Table 3**):

Table 3. Discrimination between *E. coli* isolates through plaque assay:

***E. coli* isolates**	**Plaque assay**	
	qualitative	**quantitative**
E. coli-EG1	+	10^5
E. coli-EG2	+	10^3
E. coli-EG3	-	-

The binary image (pure black and white) for plaque assay images for both *E. coli* isolates were processed to remove image noises and analyzed numerically via histographic analysis and showed mostly similar values for mean, standard deviation, median and no. of pixels, in contrast to the traditional counting method which showed variation in numbers only regardless of plaque size and intensity which was of exactly great significance as in (**Table 4**). The elementary map, at the same tie showed no great difference in elements gray range or counts as in (**Figure 3**).

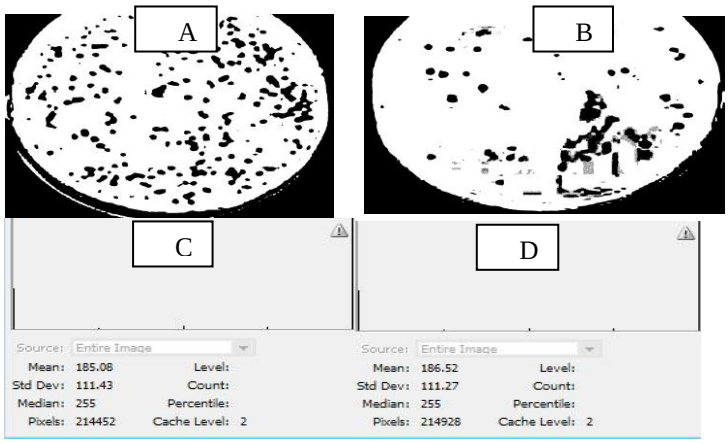

Figure 3. Binary Image processing for plaque assay concerning with *E. coli-EG1*and *E. coli-EG2* (A and B) and histographic analysis (C and D), respectively.

Table (4): Numerical analysis showing variations between *E. coli-EG1*and *E. coli-EG2* during plaque assay:

E. oli isolate	Mean	Standard deviation	Median	Pixels
E.coli-EG1	185.08	111.43	255	214452
E.coli-EG2	186.52	111.27	255	214928

The spectral data recorded from the purified coliphages specific for *E.coli-EG1*and *E.coli-EG2* with discriminating pattern in which spectral values for *E.coli-EG1* exceeded that of *E.coli-EG2* as in table (5).

Table 5. spectral data of coliphage isolates.

Coliphage isolate	Absorbance values					Yield
	A min(nm)	A max(nm)	260 nm	28 nm	260/280	
E. coli-EG1	250	270	0.953	0.725	1.3144	35.5 ng/ml
E. coli-EG2	245	265	0.825	0.672	1.2276	25.75 ng/ml

Image processing guided analysis for plaques count:

The two plaque assay images were analyzed by SIGVIEW software and the spectral data were handled for determining further calculations. From the spectral signals, the following parameters were calculated:

$$S_{output}^{MAX}(f_x, f_y) \quad S_{output}^{MIN}(f_x, f_y)$$

Table 6. Fourier spectral and modulation background values for *E. coli-EG1* and *E. coli-EG2*.

Plaques count for each *E. coli* isolate	$S_{output}^{MAX}(f_x, f_y)$	$S_{output}^{MIN}(f_x, f_y)$	N
E. coli-EG1	360	290	210
E. coli-EG2	60	20	155

The spectral values for *E. coli-EG1* (210) and *E. coli-EG2* (155) were found a great correlation with manual counting (10^5 and 10^3) respectively. Such data were extracted from Fourier spectra as in figure (4) for both bacterial isolates.

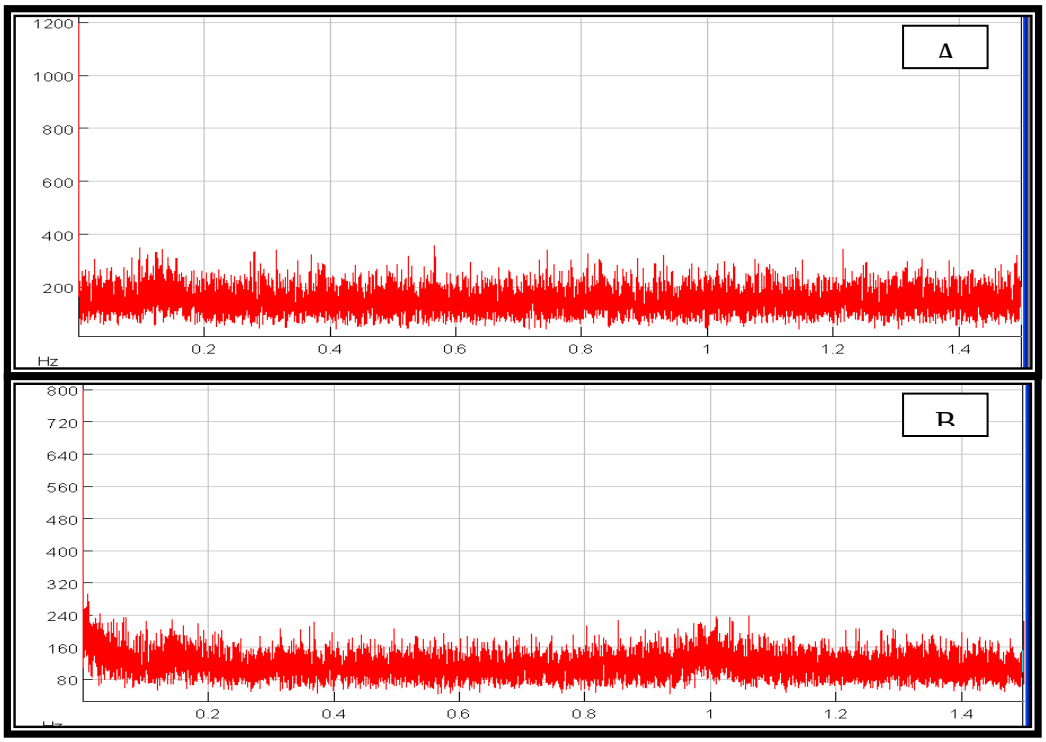

Figure 4. Fourier based spectral analysis for *E. coli-EG1* plaque assay plate (A) and *E. coli-EG2* plate (B).

DISCUSSION

Phage specificity to *E. coli– EG3* showed no specificity for the bacteriophage isolated from water sample, in contrast to *E.coli EG1* and *E. coli EG2* exhibited specificity and susceptibility to phage infection. Altering the specificity surface structures (to which virus attach) by accumulated mutations selected by environmental stress rendered the host isolate *E. coli – EG3* resistant to phage infection. Stability of receptor sites marked *E. coli- EG1* and to some extend *E. coli EG2*, so susceptibility was still attained. The image processing of plaque assay binary images and subsequent histographic analysis showed no marked differences in measurements of mean, median, standard deviation and no. of pixels although plaque counts were in contrast. The pixels represented quantization of cell lysis via viral infection. Based on that, *E. coli- EG1* sensitivity was similar to *E. coli- EG2* because the significance must adopt both size and number, not on the latter only, so the digital image processing considered most reliable in plaques comparison due to size and number adoption. From the previous data, *E. coli–EG2* exhibits a distinct behavior which could be explained in terms of mutations which are transmitted vertically from generation to generation, resulting in a clonal population structure **(Desjardins *et al.*, 1995)**.

Progressive diversification by mutation occurs in a dichotomously branching fashion through the successive accumulation of mutations within lineages, with each new mutant derivative serving as the ancestor for a new branch of the phylogenetic tree. Mutations may be silent (hence neutrally selected) or have functional consequences. Those with functional consequences can be subject to positive, negative, or neutral

selection depending on the nature of the mutation and the particular selective environment **(Boyd and Hartl, 1998)**. Clones with positively selected characteristics will expand, whereas those with negatively selected characteristics will recede or die out altogether unless they encounter a more favorable niche that allows their persistence. Periodic selection resulting from an environmental change that strongly favors a particular clone may lead to the purging of diversity in the species through elimination of less-fit clones; i.e., it may create a clonal sweep **(Gordon, 1997)**. Mutational diversification then resumes from the new starting point. All such events of mutation address the behavior of *E.coli* towards phage infection. Sewage pollution of river water and probability of groundwater pollution was considered as data indicated in sensitivity of *E.coli- EG1*to phage infection **(Azzam, 2009)**.

Practical implementation of Fourier transform for evaluation of plaques number showed that Fourier spectra characteristics are related to the objects number. From our knowledge it is the first automated image processing method used for evaluation of plaques number based on scale and rotation invariant analysis of optical transforms. The performance speed and accuracy of proposed algorithm can be increased by achieving higher contrast between bacteria colonies and agar background, for example by using dyes to color colony or by using appropriate image processing algorithm to obtain binary mask of examined samples on Petri dish, the matter which was applied here.. It should be mentioned as well, that any defect of medium, possible structural and optical non-homogeneities may affect the described above analysis. Such algorithm was used for evaluating bacterial colonies number based on optical transforms **(Buzalewicz *et al.*, 2010)**.

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