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Densitometric Analysis of Serum Transferrins in Two Air-Breathing Fish (Channidae:Channiformes)

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Abstract: Transferrin (TF) in vertebrates makes a reliable genetic marker system demonstrating extensive polymorphism and heterogeneity at the TF locus in fish species also. The present investigation was conducted on two Indian fish species viz., *Channa punctatus* and *Channa gachua*, to explore the quantitative expression of transferrins, where for the evaluation densitometric analysis of electrophoretically separated serum TF phenotypes was done. Distinct two peaks for heterozygous TF and single peak for homozygous TF was observed in densitographs. There was noticeable incidence of higher densitometric volumes of heterozygous TFs in comparison with the homozygous TFs in both the fish species. Statistically, considering the Percent Coefficient of Variation (%CoV) of the densitometric data, a small range of ~1.17-3.72% was calculated within each TF phenotype in both the populations whereas 17.79 and 24.33% of total divergence was accounted in *C. punctatus* and *C. gachua*, respectively. Further, the resulting significant value ($p < 0.05$) after z-score analysis deduced that irrespective of variations TF phenotypes are expressed quantitatively as per the body requirement when the samples are randomly and normally distributed. This study demonstrates some species-specific electrophoretic and densitometric pattern of TFs and represents a promising and simple biochemical tool for discerning the closely related species.

Key words: Transferrin, phenotypes, polymorphism, electrophoresis, densitometry, *Channa* sps.

INTRODUCTION

Transferrin (TF), a monomeric glycoprotein is structurally folded into two, an N- and a C-lobe, each having one binding site for an iron atom. It occurs in high concentrations ($2-4 \text{ mg mL}^{-1}$) in vertebrate sera playing a central role as transport protein physiologically transporting iron from sites of absorption and heme degradation to those of storage and utilization (Welch, 1990; Cnaani *et al.*, 2002) thereby protecting against iron intoxication. Studies on functional aspects of fish TF, despite being limited, also reveal that it plays an important role in innate immune system (Yano, 1996) and some evidence suggests a correlation between TF levels in sera of fish species and certain clinical conditions (Winter *et al.*, 1980; Hirono and Aoki, 1995). Moreover, TF polymorphism is reported to be

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one of the best appreciated characteristics in most of the vertebrate species where transferrin locus is found to be polymorphic presenting 2 to 13 alleles (Kirpichnikov, 1981).

The analysis of protein itself is the principle source of elucidating its functional and biochemical properties. For determination of plasma protein concentrations different methods like enzyme assay (Marjani *et al.*, 2007), spectrophotometric analysis (Prakash and Shetty, 2008) and nonspecific dye binding methods are commonly used (Harr, 2002). Since electrophoresis directly measures the protein present in a plasma sample, the levels obtained by gel electrophoresis and densitometry are a more accurate and recommended method for measurement of plasma proteins than dye methods which suffer from variability when used in different species due to differences in dye affinity (Harr, 2002). A system of polyacrylamide gel electrophoresis (PAGE) permits rapid and direct comparison of multiple samples of fish plasmas for population studies. The resulting electropherograms are suitable either for enzyme screening or for densitometric scanning after gels are stained for total proteins (Campenhout *et al.*, 2004; Gicking *et al.*, 2004; Ahmad, 2009).

As far as studies representing TF polymorphism are concerned they show applications in breeding and maintenance of stocks, determining gene frequencies, identifying races and genetic structure of natural populations of several fish species (Lacy, 2000; Teixeira *et al.*, 2002, 2008; Nabi *et al.*, 2003; Møller, 2005). Essentially, most of these studies have a focus on electrophoretic surveys within and among different species whilst biochemical and molecular aspects have also been explored (Stratil *et al.*, 1985; Welch, 1990; Nabi *et al.*, 2003, 2007; Jabeen and Hasnain, 2008). Over the years, many experiments are designed to study the relevance and to demonstrate the sensitivity and specificity of transferrin heterogeneity among which densitometry is the most employed one (Görögh *et al.*, 2005; Ahmad *et al.*, 2007).

Notwithstanding the vastness of the resources and the diversity of fauna, the information on the polymorphism of TFs of fish species inhabiting Indian subcontinent is scarce. Sister species viz., *Channa punctatus* and *Channa gachua* are an important freshwater, food fish of Southeast Asia. They belong to genus *Channa* and are commonly called as snakehead or murrel. They are characterized by their bimodal respiration and are capable of breathing atmospheric oxygen with accessory air breathing organs. Regarding the clinical aspects of TF in these fishes, former studies had shown that under laboratory conditions, *C. gachua* survived longest during ulcerative sickness (Nabi *et al.*, 2000) whereas mixed inoculum of pseudomonads had failed to cause severe symptoms in its sister species, *C. punctatus* (Pradhan and Pal, 1990). From evolutionary perspective, quantitative and qualitative status of several well-identified abundant proteins in lower vertebrates needs explanations.

The present investigation was envisaged as a contribution to the additional information on transferrin phenotypes of *Channa punctatus* and *Channa gachua*, the sister species belonging to the genus *Channa*. The current report deals with the densitometric analysis of electrophoretic patterns of serum TF phenotypes of both *C. punctatus* and *C. gachua*.

MATERIALS AND METHODS

Sample Collection

Samples of *C. punctatus* and *C. gachua* were obtained from local fish markets of Aligarh during the month of May, 2009. Accessory air breathing helped the fish to survive hypoxia during transportation in small water containers. A total of 43 of *C. punctatus* and 32 of *C. gachua* were used for serum collection. To minimize the seasonal and physiological variations (Samantaray and Das, 1995), fishes were procured within a short time span of one month.

Serum Extraction

Blood samples were collected from live fish directly by cardiac puncture using sterilized syringe according to the method mentioned by Nabi *et al.* (2003). The sera from clotted blood was pipetted out and centrifuged at 3,000 rpm for 10 min to sediment out contaminating blood corpuscles. Clear sera were analyzed immediately or were stored at -20°C till further analysis.

Polyacrylamide Gel Electrophoresis (PAGE)

Serum proteins (1 µL of each serum sample) were separated according to Laemmli's (1970) protocol of polyacrylamide gel electrophoresis (PAGE) with the only alteration that SDS was not added to any of the solutions. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) R-250 and destained in 7% acetic acid.

Densitometric Analysis

The gel scans were used for software analyses where densitometric profiles were generated using Scion Imaging software. The quantization of density of all the TF phenotypes in a specific gel lane was done by UviDoc (ver 12.8) software program. The densitometric volumes of each phenotype were taken as their relative percentage values (arbitrary units) assuming them as relative transferrin concentration in that band (Ahmad *et al.*, 2007). For heterozygous phenotypes (two-banded pattern), sum of band densitometric volumes of both TF isoforms was taken as a whole.

Statistical Analysis

Densitometric data was statistically treated (Daniel, 2005) where z-test was applied between the mean TF densitometric volumes of the two populations and the differences were considered significant at $p < 0.05$. Percent Coefficient of Variance (% CoV) was also calculated to distinguish the intra-specific variations.

RESULTS

The results presented in Fig. 1 visibly showed that TF (bands marked in each gel lane), individually appears as one of the most negatively charged protein in a β -globulin region migrating faster than most of the others. Six phenotypes of TF were manifested in sera of *C. punctatus* whereas two were visualized in *C. gachua*. Out of the six TF phenotypes of *C. punctatus*, 13 homozygotes (2 AA, 7 BB, 4 CC) and 30 heterozygotes (13 BC, 9 AB, 8 AC) were found. While in *C. gachua*, there were 6 homozygous BB and 26 heterozygous AB. Moreover, not a single homozygous AA phenotype was observed in *C. gachua*.

The densitometric profiles of the respective gel lanes of TF phenotypes are shown in Fig. 1. The presence of corresponding TF isoforms in the form of two peaks for heterozygotes and one peak for homozygotes can be distinctly visualized in densitographs. In *C. punctatus*, heterozygous TF showed the mean range of 20.73-26.09% densitometric volumes which was distinctly higher than homozygous range of 13.68-16.68%. Similar case was observed in *C. gachua* where TF densitometric volume 17.17% of AB showed the marked increase over the homozygous BB value of 10.81 (Table 1).

Intra-specific differences as calculated %CoV are summarized in Fig. 2. In *C. punctatus*, the %CoV showed the variation between six TF phenotypes where the significant values 1.17, 2.52, 1.58, 2.22, 1.92, 1.41 (AA, BB, CC, BC, AB, AC, respectively) were within the range of 1.17-2.52% accounting for the total divergence of 17.79% within the population. Similarly, the range of 3.23-3.72% in *C. gachua* between two recorded phenotypes showed the total

Table 1: Comparison of percent densitometric volumes of TF phenotypes of *Channa punctatus* and *C. gachua*. (Each data point is the arbitrary unit of average of individual loading)

<i>Channa punctatus</i>		<i>Channa gachua</i>	
TF Phenotypes	Mean values±SD	TF phenotypes	Mean values±SD
AA	13.68±0.16	AA	---
BB	16.68±0.41	BB	10.81±0.35
CC	16.20±0.19	AB	17.17±0.63
BC	22.89±0.51		
AB	20.73±0.40		
AC	26.09±0.37		

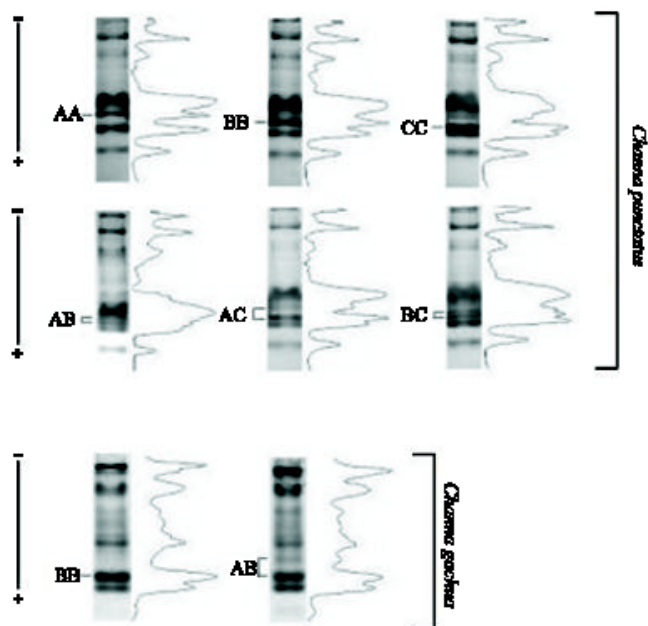


Fig 1: Electrophoresis patterns showing TF phenotypes of *Channa punctatus* and *C. gachua* together with their densitometric tracings. Each record represents a single scan without curve smoothing

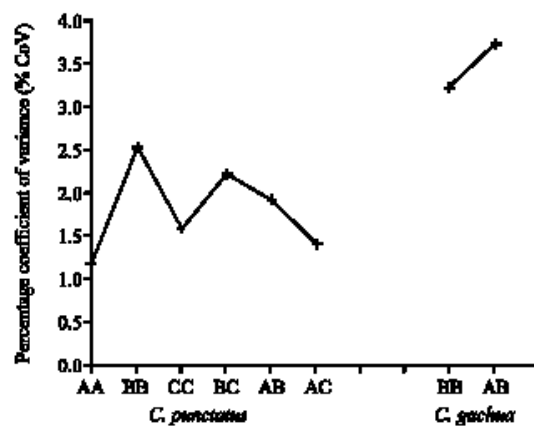


Fig 2: Calculated percent coefficient of variance (%CoV) within each TF phenotype in both the populations of *Channa punctatus* and *C. gachua*

divergence of 24.33% (CoV) within the population where %CoV values were 3.23, 3.72 for BB and AB, respectively. Difference of mean densitometric volumes between the two populations (z) was scored to be equal to 6.16. Results of two tailed P-value was 0.0001 indicating the difference to be extremely significant.

DISCUSSION

Protein markers determined electrophoretically are of considerable importance in a number of aspects of animal breeding and livestock management where variations are generally correlated with the sexual differences, genetic diversity, temperature acclimatization or the seasonal effects. Selective breeding programmes have shown the beneficial effects of increased heterozygosity in polymorphs. The study of transferrins as the genetic marker has been facilitating because of the simplicity of inheritance of its locus and the ease of detection by electrophoresis. Although TF variants represent the polymorphism of a single locus, the data has been extremely useful in discerning the biochemical genetics of fish species also.

Presently, the comparison of the electrophoretic patterns of serum samples of *Channa punctatus* and *C. gachua* enabled to discriminate clearly between their TF polymorphs (Fig. 1). So obtained species-specific electrophoretic patterns along with their densitometric profiles (Fig. 1) ascertain the polymorphic nature of transferrin locus in the studied species also. At this point, the genetics of *C. punctatus* represents six TF phenotypes AA, BB, CC, AB, BC and AC exhibiting a 3-allele system (Nabi *et al.*, 2003). On the contrary, *C. gachua* TF exhibits a 2-allele polymorphism at the transferrin locus (Sahoo and Khuda-Bukhsh, 1989) representing three phenotypes AA, AB and BB categorizing the TF in a co-dominant biallelic system.

The sera samples extracted from 43 *C. punctatus* and 32 *C. gachua* fish species were subjected to non-SDS polyacrylamide gel electrophoresis. The gel patterns thus obtained were then utilized for generating density profiles and elucidating densitometric data. In native PAGE, the quantitation of the relative amounts of TF isoforms in a sample was done assuming to be extracted in proportion to their relative composition in intact samples. Moreover, the staining of the TF isoforms was considered to be stoichiometric. Under the conditions applied, several quantitative intra- as well as inter-species differences was noticed in TF bands after densitometric analysis of the gel patterns. Most important characteristic of all was the high amount of transferrin in heterozygous condition (Table 1) in both the fish populations.

It has been considered that the synthesis of iron chelators-compounds by certain pathogens is necessary virulence factor for removal of iron from transferrin (Arnold *et al.*, 1977). Studies on coho salmon and steelhead trout stocks have shown that TF genotypes provide resistance to different diseases (Winter *et al.*, 1980). Moreover, allelic variability has been shown to make the stocks less prone to disease, especially in the case of the transferrin loci (Calcagnotto and Toledo-Filho, 2000) where bactericidal properties of transferrins are more strongly expressed in heterozygotes (Hegenauer and Saltman, 1975). These data suggest that heterozygous fish are metabolically more efficient than less active individuals.

Judging by the present results, it appears that the elevated level of heterozygous TF might somehow be inferred to their apparent role in defense mechanism. This could be considered in view of the fact that our earlier study on albumin of *C. gachua* had revealed the relative susceptibility of homozygous albumin phenotype to ulcerative diseases (Jabeen *et al.*, 2001). Also, egg whites of *Phasianus colchicus* containing conalbumin of the heterozygous type show a stronger inhibitory effect on the growth of *Saccharomyces*

cerevisiae than egg whites with conalbumin of the common homozygous type (Lucotte and Kaminski, 1976). According to this hypothesis, importance of heterozygosity of transferrin genotypes remains an important factor in disease resistance. Regardless of the specific mechanism, these data show that genetic variation is an important biological resource to be conserved in captive populations of fishes where disease is likely to be of primary concern. Nevertheless, it would be interesting to account this criterion in detail since many fish species have a high fecundity of protein markers for population studies and therefore considerable opportunity exists for differential survival.

Statistical analysis of the densitometric data also revealed some marked characteristics of TF phenotypes in *C. punctatus* and *C. gachua*. Each phenotype in both the populations demonstrated the divergence (%CoV) within a small range of 1-4% (min-max) (Fig. 2). Also, the densitometric volumes of TFs showed maximum of ~25% of the total variation within both the populations. Irrespective of these variations, the statistical significance ($p < 0.05$) of the study considering inter-specific difference (z-score) helped in concluding that data constitute two independent simple random samples each drawn from a normally distributed population. This independent distribution can be acknowledged, considering the fact, that channid species are reproductively and completely isolated (Banerjee *et al.*, 1988). *C. punctatus* not being a polyploid (Ponniah and John, 1998) might show a different selection mechanism than a polyploid, *C. gachua* where, like polyploid salmonids, a positive selection of beneficial mutants (Ford, 2000) has taken place. On the whole, this helps in attributing, that quantitatively, TF is approximately normally distributed in both the populations where the protein level is expressed as per the body requirement.

Consequently, this is the first ever report presenting the densitometric analysis of TF phenotypes from sera samples of *C. punctatus* and *C. gachua*. The species-specific unique electrophoretic mobility and density along with the similarity in occurrence of higher density of heterozygous TF make transferrins of *C. punctatus* and *C. gachua* a magnificent biochemical tool for elaborate exploration and will be of immense significance in comparing its polymorphic nature in closely related species.

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