Gene transfer in Indian major carps by electroporation

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The rainbow trout growth hormone gene has been successfully transferred into three species of Indian major carps rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*) and catla (*Catla catla*) through electroporated sperm cell. At 0.5 kV/cm (25 μF capacitance, α resistance, and 2 pulses) the gene transfer efficiency was 25% for rohu, 23% for mrigal and 13% for catla. This is the first report on gene transfer in Indian major carps.

TRANSGENIC animals have been produced by introducing foreign DNA into the pronucleus of fertilized eggs; most authors[1,2] have chosen microinjection as the preferred method of transferring the gene. However, the presence of relatively small and invisible nucleus in fish egg surrounded by opaque yolk and tough chorion renders microinjection a difficult task[3,4]. Further, 'egg-microinjection procedures are time-consuming, laborious, species-specific and, in some cases, technically demanding'. Consequently, transgenic fish production using this method is 'slow' and may prove to be a 'bottleneck to the exploration, expansion and realization of the full potential of this powerful technology'[5].

The second best method for transferring transgene into fish egg is electroporation[3]; this method is more suited for fishes with high fecundity. However, the use of electroporation for transferring the transgene into carp eggs encounters other problem. Since the carp eggs are larger (1.8 ± 0.1 mm) than the eggs of medaka (0.9 ± 1 mm)[1], the largest cuvette of the gene pulser II (0.4 cm gap, Catl. No. 165-2088 of the Bio-Rad) can accommodate a maximum of about 50 eggs along with the buffer. Moreover, the eggs of carps, including the three Indian major carps, chosen in the present work are fragile and swell by 2–3 times following fertilization. Owing to swelling it is difficult to remove the eggs from the cuvette after the electroporation. Hence, only a few eggs can be electroporated at the given time and that the difficulty in withdrawal of the electroporated eggs owing to swelling and a high mortality (> 95% even at 150 V/cm) make electroporation technique not adequately suitable for carp eggs.

Consequently, the sperm-mediated (sperm-electroporation) gene transfer technique is the next suitable technique for transferring gene into carp eggs. A series of publications have clearly indicated that sperm cell could be used as a vector to transfer foreign DNA into the embryo[6,7], and the internalization of DNA by sperm cells is enhanced by electroporation[8–10]. Here we report transfer of foreign gene into the eggs of carps through sperm electroporation.

Milt of the three Indian major carps (rohu: *Labeo rohita*; mrigal: *Cirrhinus mrigala*; catla: *Catla catla*) were separately stripped, diluted with Hanks buffer to $4.0–5.0 \times 10^8$ cells/ml. The sperm cell suspension containing 100 μg/ml of linearized pRSVrtGH DNA (rainbow trout growth hormone cDNA fused with the Rous sarcoma virus long terminal repeat)[11] was electroporated at 500 V/cm, 25 μF capacitance, α-resistance and 2 pulses, each pulse lasting for 0.6 ± 0.3 ms. Electroporated sperm cells were subsequently incubated on ice for 5 min and used to fertilize the cognate eggs; fertilized eggs were incubated in water at 25 ± 2°C.

The eggs fertilized with electroporated sperm successfully hatched 16–18 h after fertilization. The yolk sac was reabsorbed and the hatchling commenced feeding three days after hatching. The fry were reared in plastic troughs and fed *ad libitum* on pelleted feed. To know the efficiency of gene transfer into eggs fertilized by electroporated sperm, DNA was extracted from fry (two number of one-week-old individuals of mrigal and rohu, and single individual of two-week old catla) that hatched from the eggs fertilized with the electroporated sperm and analysed by slot blot hybridization. The whole carp juvenile was washed twice with saline and homogenized in extraction buffer [10 mm Tris.HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 20 μg/ml pancreatic RNAase, and 0.5% SDS]. Proteinase K was added (100 μg/ml) and the homogenate was digested at 50°C for 4 h. The digested homogenate was diluted to 500 μl with distilled water and extracted thrice with Tris saturated phenol and twice with chloroform/isoamyl alcohol (24 : 1). DNA was precipitated after adding 0.1 volume of 3 M sodium acetate (pH 5.2) and equal volume of isopropanol. DNA was pelleted at 10 k rpm for 10 min at room temperature, washed with 70% ethanol and air dried. DNA was dissolved in TE buffer checked on a 0.7% agarose gel and quantified by spectrophotometric method.

For slot blot analysis, 5.0 μg of DNA was blotted onto a Hybond N^+ membrane (Amersham) using a slot blot apparatus (Hoefer) by applying vacuum suction. The membrane was placed on a 3 MM Whatman paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min and transferred to 3 MM Whatman paper containing neutralizing solution [1.5 M NaCl, 0.5 M Tris.HCl (pH 7.2), 0.001 M EDTA] for 2 min. Air-dried membrane was baked at 80°C for 20 min. The membrane was prehybridized without formamide at 65°C following standard protocols[12]. The rtGH cDNA (970 bp) was

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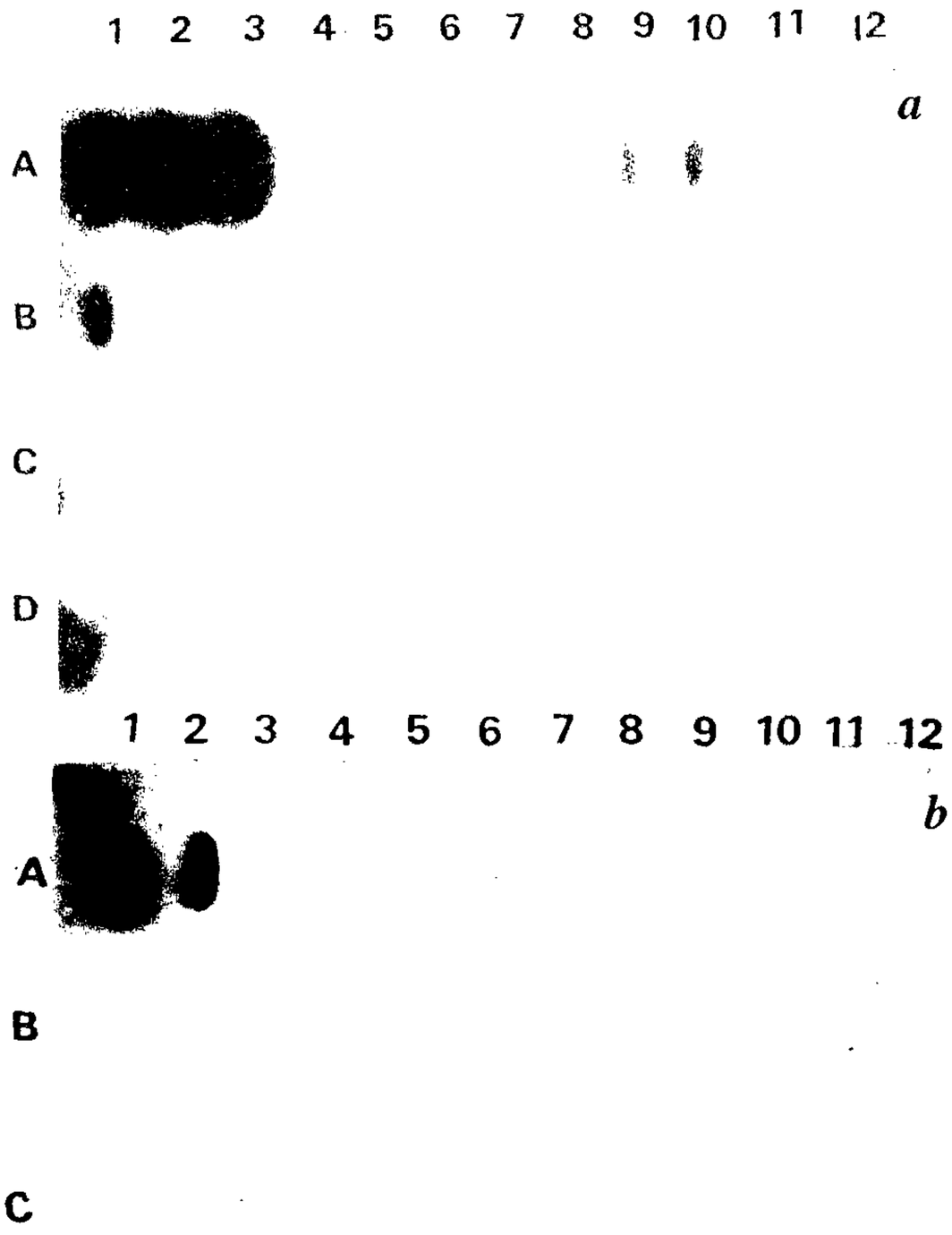

Figure 1. *a*, Slot blot analysis of DNA extracted from putative transgenic mrigal, rohu (5.0 µg of genomic DNA from two individuals of one week old in each slot) juveniles hatched from eggs fertilized with electroporated sperm. Lanes A1, A2 and A3: 1.0, 0.5, 0.25 µg pRSVrtGH DNA (positive control); Lanes A4: DNA from Sham electroporated mrigal (negative control); Lanes A5–A12, B1–B12, and C1–C6: DNA from putative transgenic mrigal juveniles; Lane C7: DNA from sham electroporated rohu juvenile (negative control); Lanes C8–C12 and D1–D7: DNA from putative transgenic rohu juveniles. *b*, Slot blot analysis of DNA extracted from putative transgenic catla (5.0 µg of genomic DNA extracted from single individuals of two weeks old in each slot) juveniles hatched from eggs fertilized with electroporated sperm. Lanes A1, A2: 0.5, 0.25 µg pRSVrtGH DNA (positive control); Lane A3: DNA from sham electroporated catla juvenile (negative control); Lanes A4–A12, B1–B12 and C1–C3: DNA from putative transgenic catla juveniles.

purified from pRSVrtGH and 50 ng of DNA was labelled with α^{32}P dATP using random priming method[12]. The membrane was hybridized at 65°C for 12 h and washed stringently as follows: once with 2×SSC – 0.1% SDS, 1×SSC – 0.1% SDS at room temperature and twice with 1×SSC – 0.1% SDS at 65°C (each for 15 min). The membrane was exposed to X-ray film at –70°C and the film was developed after 12 h.

Figure 1 shows positive signals obtained from the DNA samples of mrigal, rohu (Figure 1 *a*) and catla (Figure 1 *b*) putative transgenic juveniles. Of the 26 genomic DNA samples of mrigal subjected to slot blot analysis, three gave strong positive signals and the other three faint signals. Of the 12 rohu samples slot blotted and probed, three gave light positive signals. Analysis of genomic DNA samples from 24 individuals of catla, only 3 slots showed faint positive signal. Clearly, the electroporation conditions used in this study were good enough to ensure gene transfer in the sperm of Indian major carps. The realized gene transfer efficiency was 23%, 25% and 13% for mrigal, rohu and catla, respectively. These values are two times higher than those reported for chinook salmon[9], and nearly 10 times higher than the values reported for common carp, tilapia and African catfish[13] (Table 1); a reason for the low efficiency in these species may be lower concentration of DNA (10 µg/ml; compared to 100 µg/ml in the present study) used[9] in electroporation solution. Although there were variations in the protocol, equipment and fish species used, it is interesting to note that a maximum of 15% efficiency has been observed in zebrafish[8] using a very high DNA concentration (1000 µg/ml). In the case of loach[14], a maximum of 50% transfer efficiency was observed, despite a lower DNA concentration (1 µg/ml) used; this high percentage may be due to a high amplitude (80 kV/cm) used for electroporation. It is known that a high field strength facilitates enhanced uptake of DNA by sperm[9]. The use of such high voltage for sperm electroporation is not possible for the three Indian major carps, as the motility and fertilizability of the sperm of these carps suffered drastically, when subjected to electroporation even at field strength higher than 500 V/cm. The present work shows that for the efficient gene transfer into the sperm cells of carps, electroporation at the field strength of 500 V/cm is adequate.

Table 1. Survival of eggs fertilized with electroporated sperm cells and gene transfer efficiency

Species	Field strength (kV/cm)	Survival (%)	Gene transfer (%)
*Labeo rohita**	0.50	70	25
*Cirrhinus mrigala**	0.50	50	23
*Catla catla**	0.50	65	13
Brachydanio rerio[8]	0.50	73	8
Cyprinus carpio[13]	0.75–2.25	–	3
Oreochromis niloticus[13]	0.75–2.25	–	3
Clarius gariepinus[13]	0.75–2.25	–	4
Oncorhynchus tshawytscha[9]	0.65–1.15	90–95	<10
Misgurnus anguillicaudatus[14]	80.0	61–67	50

*Present study.

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