The Journal of Poultry Science, 45: 75–81, 2008
http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.45.75

≪**Research Note**≫

A Simple and Quick DNA Extraction Procedure for Rapid Diagnosis of Sex of Chicken and Chicken Embryos

Santosh Haunshi, Arunav Pattanayak, Subhasis Bandyopadhaya, Sharat C. Saxena and Kamal M. Bujarbaruah

ICAR Research Complex for N.E.H. Region, Umroi Road, Umiam-793103 Meghalaya, India

The objective of this study was to develop a simple and quick DNA extraction procedure for rapid diagnosis of sex of chicken and its embryos using multiplex polymerase chain reaction (PCR). Using alkaline method of DNA extraction from whole blood and feather bulb of adults, and tissue samples from embryos, the present study demonstrated that identification of sex by multiplex PCR protocol is simplest, safer, faster and inexpensive. Multiplex PCR was used to amplify W chromosome specific 481–bp fragment in female and 256 bp fragment of 18S ribosomal gene in both male and female chicken. DNA samples were prepared by conventional phenol-chloroform-iso-amyl alcohol (PCI) method, modified PCI method, wizard genomic DNA purification kit and a simple alkaline method from blood samples and feather bulbs of adults and tissue samples of embryos. The protocol successfully identified sex of embryos and White Leghorn (Exotic), indigenous and Vanaraja (Hybrid of exotic and indigenous) varieties of chicken. Sequence comparison of W chromosome specific PCR products amplified from these three varieties showed no difference among them.

Key words: alkaline, chicken, DNA extraction, feather, sexing

J. Poult. Sci., 45: 75–81, 2008

Introduction

Quick and accurate sexing of embryos at early stages of development of chicken embryo is crucial in sex reversal, sex differentiation studies and generation of germ line chimeras. Accurate sexing of chicks of endangered species like jungle fowl and other rare breeds of chicken by non invasive methods is essential not just for captive breeding purpose but also for molecular ecological studies (Itoh *et al.,* 2001). Further, reliable sexing of embryos by morphological differences before 8 d of incubation is not possible (Clinton *et al.,* 2001). Therefore, molecular methods of identification of sex of embryos by karyotyping (Shoffner *et al.,* 1967), southern blot hybridization of W repetitive sequences (Uryu *et al.,* 1989), fluorescent *in situ* hybridization (Klein and Ellendorff, 2000) and more recently PCR based protocols using W chromosome specific DNA sequences have been developed (Petite and Kegelmeyer, 1995; Griffiths *et al.,* 1998; Kahn *et al.,* 1998; Fridolffson and Ellegren, 1999; Clinton *et al.,* 2001).

Normally protocols for extraction of genomic DNA from whole blood and tissue samples of embryos were lengthy, time consuming, expensive and require use of toxic and hazardous materials like phenol, chloroform, iso-amyl alcohol, S.D.S., etc., Various simplified procedures of DNA extraction from different sources viz., blasto-dermal cells (Naito *et al.,* 2003), soft tissues of embryos (Clinton *et al.,* 2001; Minematsu *et al.,* 2004) and amniotic fluid cells (Clinton *et al.,* 2001) have been reported for identification of sex of embryos through PCR. However, the procedures described for extraction of genomic DNA from whole blood and embryonic tissues in earlier studies involved use of digestion buffers, multiple steps of pipetting, centrifugation etc., making them more vulnerable for contamination, time consuming, hazardous and laborious. To our knowledge, there is little information available about non-invasive and safe tissue sampling from chicks and rapid genomic DNA extraction in one step for sexing of chicken and its embryos using multiplex PCR. In this study, we have standardized a safe, quick, inexpensive and single tube alkaline method of DNA extraction from whole blood and feather bulb of adults; and tissue samples of embryos and compared with other three methods for rapid identification of sex of chicken and chicken embryos using multiplex PCR.

Received: January 30, 2007, Accepted: June 22, 2007
Correspondence: Dr. S. Haunshi, Division of Poultry Science, ICAR Research Complex for N.E.H. Region, Umroi Road, Umiam-793103 Meghalaya, India. (E-mail: santosh575@rediffmail.com)

Materials and Methods

1. *Experimental Animals*

White Leghorn (Exotic), indigenous and Vanaraja varieties (Hybrid of exotic and indigenous) of chicken (*G. domesticus*) were used in the present study. Blood samples

were collected aseptically in a sterile syringe from Jugular vein. The sex of these chicken varieties was determined from adult morphology. Fertile eggs were collected and incubated on day of lay at 37±0.5℃ with 70–75% humidity in laboratory incubator. Eggs were taken out from incubator between 5 and 7 d after setting and the embryos along with their associated membranes were kept in sterile Petri dishes. Subsequently, tissue samples of embryos were collected aseptically in sterile microcentrifuge tubes. Feather bulbs from chicks were collected in a sterile microcentrifuge tubes by plucking a single primary feather aseptically. Multiplex PCR was carried out using 60, 27, 57 and 30 DNA samples extracted respectively from alkaline method, conventional PCI method, modified PCI method and Wizard genomic DNA purification kit in equal numbers from whole blood, embryo samples and feather bulbs.

2. Genomic DNA Extractions

To diagnose the sex of embryos and adults using multiplex PCR, the genomic DNA from the soft tissues of embryos, and whole blood and feather bulb of adult chicken was extracted using alkaline method, conventional PCI method, modified PCI method and wizard genomic DNA purification kit as described below.

2. 1. Alkaline Method

A. Blood samples from adults

Initially, the protocol was standardized by mixing 2 μl of whole blood in 10, 20, 30, 40 and 50 μl of 0.2 N NaOH in a sterile microcentrifuge tubes and then tubes were heated at 75℃ in dry bath for 20 min. Subsequently, 90, 180, 270, 360 and 450 μl of 0.04 M Tris HCl (pH 7.75) was added as neutralization solution to the respective tubes (Rudbek and Dissing, 1998). 1.0 μl of the solution was used as template DNA for multiplex PCR.

B. Tissue samples of embryos

Under this method about 25 mg of soft tissue of embryo was suspended in 40 μl of 0.2 N NaOH in sterile microcentrifuge tube. The tube was heated at 75℃ in dry bath for 20 min. Subsequently, 300 μl of 0.04 M Tris HCl (pH 7.75) was added as neutralization solution. 5.0 μl of the solution was used as template DNA for multiplex PCR.

C. Feather bulb of chicks

In this method, single feather bulb of chick was put in a sterile microcentrifuge tube containing 20 μl of 0.2 N NaOH. The tube was heated at 75℃ in dry bath for 20 min. Subsequently, 180 μl of 0.04 M Tris HCl (pH 7.75) was added as neutralization solution. 5.0 μl of the solution was used as template DNA for multiplex PCR.

2. 2. Conventional PCI Method

A. Blood samples from adults

The genomic DNA was extracted from whole blood of adult chicken using conventional PCI extraction method (Sambrook and Russel, 2001). Briefly, 50 μl of whole blood was suspended in a microcentrifuge tube containing 700 μl of cell lysis buffer (2 M Tris HCl-pH 8.0, 2 M NaCl, 10% SDS and 0.5 M EDTA-pH 8.0), contents were mixed and 100 μg Proteinase–K was added to the mixture, vortexed and incubated in water bath at 37℃ for overnight. Subsequently, the genomic DNA was extracted twice with phenol, twice with phenol–chloroform iso-amyl alcohol (24:24:1) and finally twice with chloroform iso-amyl alcohol (24:1). DNA was pelleted by mixing supernatant DNA solution with to 2-isopropanol solution (Sigma). The pelleted DNA was washed twice with 70% ethanol, air dried and rehydrated in 200 μl nuclease free water (Qiagen, USA). 2.0 μl of the solution was used as template DNA for multiplex PCR.

B. Tissue samples of embryos

The procedure followed to extract genomic DNA from embryo samples was same as that described for blood samples except that 25 mg tissue sample of embryo was suspended in 600 μl of cell lyses buffer, vortexed and incubated at 37℃ for overnight. 2.0 μl of the DNA solution was used for multiplex PCR.

C. Feather bulb of chicks

A single feather bulb was suspended in 600 μl of lysis buffer and 100 μg Proteinase–K in microcentrifuge tube; the tube was vortexed and incubated at 37℃ for overnight. The rest of the procedure was similar to that of blood samples except that the DNA pellet was rehydrated in 25 μl nuclease free water. 2.0 μl of the DNA solution was used for multiplex PCR.

2. 3. Modified PCI Method

A. Blood samples from adults

In this method, about 50 μl of whole blood was mixed with 700 μl of lysis buffer and 100 μg Proteinase–K. The mixture was vortexed and incubated in water bath at 65℃ for 1 h. Then a mixture of tris saturated phenol (pH 7.9) and chloroform–iso-amyl alcohol (24:1) in equal volume was added, gently mixed and centrifuged (14,000 × g for 5 min). The pelleted DNA was washed in 70% ethanol, air dried and rehydrated in 200 μl nuclease free water. 2.0 μl of the DNA solution was used for multiplex PCR.

B. Tissue samples of embryos

In this method about 25 mg of embryonic tissue was suspended in 600 μl of lysis buffer and 100 μg Proteinase–K. The mixture was vortexed and incubated in water bath at 65℃ for 1 h. Then a mixture of tris saturated phenol (pH 7.9) and chloroform–iso-amyl alcohol (24:1) in equal proportion was added, gently mixed and centrifuged (14,000 × g for 5 min). The pelleted DNA was washed in 70% ethanol, air dried and rehydrated in 200 μl nuclease free water. 2.0 μl of solution was used for the multiplex PCR.

C. Feather bulb of chicks

One feather bulb was suspended in 600 μl of lysis buffer and 100 μg Proteinase–K in microcentrifuge tube; the tube was vortexed and incubated at 65℃ for 1 h. The rest of the procedure was similar to that described for embryo samples under modified PCI method. The DNA pellet was rehydrated in 25 μl nuclease free water. 2.0 μl of the DNA solution was used for multiplex PCR.

2.4. Wizard Method

A. Blood samples from adults

In this method, the genomic DNA was extracted using

Wizard Genomic DNA purification kit as per the protocol described by manufactures (Promega, Madison, WI, USA) for whole blood samples. 1.0 to 5.0 μl of DNA solution was tried for PCR analysis.

B. Tissue samples of embryos

The genomic DNA from tissues of embryos was purified as per the protocol provided by manufacturers for tissue samples. 2.0 μl of template DNA was used for PCR analysis.

C. Feather bulb of chicks

A single feather bulb was suspended in 60 μl chilled nuclei solution, mixed and incubated for 30 min at 65℃. 2.0 μl of RNase was added to the tube, mixed and incubated at 37℃ for 30 min. Subsequently 100 μl of protein precipitation solution was added to the tube, gently mixed, centrifuged (14,000×g for 4 min) and the supernatant solution was transferred to the tube containing 60 μl of room temperature iso-propanol. The DNA was precipitated, washed in 70% ethanol, air dried and rehydrated in DNA rehydration solution at 4℃ for overnight. 5.0 μl of template DNA was used for PCR analysis.

The quality of genomic DNA extracted by all four methods from whole blood, feather samples and embryonic tissues were tested on 0.8% agarose gel electrophoresis in 1×TAE buffer.

3. PCR Primers

3.1. W chromosome primers

W chromosome specific DNA repeats (*Xho*I) sequence of chicken searched from GenBank (NCBI) (Kodama, *et al.,* 1987) was used to design primers to amplify 481 bp single fragment of W chromosome specific sequence from nucleotide position of 135 to 615 using software.

SaC-F primer 5′ TAACACGCTTCACTCACA 3′
SaC-R primer 5′ ATGTTTGGACAGAGGTGC 3′

3.2. Ribosomal Gene Primers

Published primers were used to generate a 256 bp of the 18S ribosomal gene (Clinton *et al.,* 2001) from nucleotide position of 1267 to 1522 in both male and female sex.

18S R-F primer 5′ AGCTCTTTCTCGATTCCGTG 3′
18S R-R primer 5′ GGGTAGACACAAGCTGAGCC 3′

4. Amplification Conditions

PCR reactions were carried out in a volume of 25 μl reaction mixture consisting 12.5 μl Qiagen PCR master mix, 1×Q-solution (Qiagen, USA), 100 ng genomic DNA, 1.6 μM of SaC—F & R primers and 0.25 μM of 18S R-F & R primers. The optimized PCR conditions of 94℃ for 2 min followed by 25 cycles of 94℃ for 5 s, 54℃ for 5 s and 72℃ for 5 s and final extension step of 72℃ for 5 min was used for all reactions. PCR reactions were carried out in programmable Thermal Cycler (Model 2720 Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on 1.5% agarose gel in 1×TAE buffer and bands were visualized under U.V. light after ethidium bromide staining (Sambrook and Russel, 2001). 100 bp DNA ladder (ready to use GeneRuler™ Fermentas-Life Sciences, Glen Burnie, MD, USA) was used for determining the size of amplified products.

5. Sequencing Analysis

The PCR products of W chromosome specific sequence from White leghorn, indigenous and Vanaraja chicken varieties were purified using sample exonuclease shrimp alkaline phosphatase digestion method. Direct sequencing of purified PCR products in both directions was carried out using Big Dye terminator v3.1 (ABI) chemistry in ABI prism 3100 genetic analyzer to ascertain the specificity of primers and to compare the nucleotide sequence between these three varieties of chicken. PCR products were sequenced at Labindia DNA analysis services.

Results

Initially, the multiplex PCR protocol was optimized with new set of primers using genomic DNA extracted from adult male and female chickens belonging to White leghorn, Vanaraja and indigenous varieties. W chromosome specific primers amplified a single 481 bp PCR product only in females whereas 18S ribosomal gene primers generated a 256 bp PCR product both in male and female chicken. The differences in the sizes of the W chromosome specific 481 bp PCR products were observed between predicted ones (481) and migrated ones on agarose gel electrophoresis as shown in Figs. 1–8. Results of multiplex PCR for identification of sex using DNA extracted from different tissues by different procedures are being summarized in Table 1.

1. Alkaline Method

It was evident from Fig. 1 that the multiplex PCR protocol was successful using 1.0 μl of crude DNA solution extracted from whole blood using 0.2 N NaOH and 0.04 M Tris HCl (pH 7.75) solutions with different quantities

Table 1. Results of sexing by multiplex PCR using DNA samples extracted from different tissues by different procedures

Tissue samples	DNA extraction procedure			
	Alkaline method	Conventional PCI method	Modified PCI method	Wizard Kit
Whole blood	+	+	+	—
Embryonic tissue	+	+	+	+
Feather bulb	+	+	+	+
Number of individuals used in each experiment	60	27	57	30

except when 10 μl of 0.2 N NaOH and 90 μl of 0.04 M Tris HCl (pH 7.75) solutions were used. Therefore, under this method subsequent DNA extractions from feather and blood samples were carried out by using 20 μl of 0.2 N NaOH and 180 μl of 0.04 M Tris HCl (pH 7.75) solutions and from embryo tissues using 40 μl of 0.2 N NaOH and 300 μl of 0.04 M Tris HCl (pH 7.75) solutions. Multiplex PCR was successful in accurately sexing the adult chicks and embryos using DNA extracted by alkaline method from whole blood (Fig. 1), feather bulb (Fig. 2) and embryonic tissue samples (Fig. 3). As little as 1 μl of DNA sample extracted from whole blood and 5 μl from that of feather bulb and embryo tissues using alkaline method were adequate for obtaining optimum and reproducible results (Figs. 1, 2 and 3).

Multiplex PCR was robust with DNA samples extracted with alkaline method from embryos and feather samples as compared to the one using DNA extracted from whole blood with alkaline method although the quality of genomic DNA extracted using this method from all three sources revealed smeary background on 0.8% agarose gel electrophoresis indicating shearing of DNA.

2. Conventional PCI Method

Genomic DNA extracted from all three sources viz., whole blood (Fig. 4), feather bulb (Fig. 5) and embryonic tissue samples (Fig. 6) using conventional PCI method worked very well for accurate sexing of adult chicks and embryos by multiplex PCR protocol.

3. Modified PCI Method

Multiplex PCR for identification of sex of embryos and adults using genomic DNA extracted by modified PCI method from whole blood (Fig. 4), feather bulb (Fig. 5) and embryonic tissue samples (Fig. 7) was also successful.

4. Wizard Method

Wizard Genomic DNA purification kit used to extract genomic DNA from whole blood, feather bulb and embryonic tissues. Multiplex PCR was successful with DNA samples extracted from embryonic tissue (Fig. 7) and feather bulb (Fig. 8). However, multiplex PCR was not

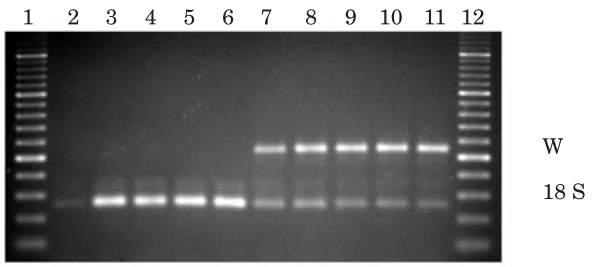

Fig. 1. Multiplex PCR using DNA extracted from whole blood from male (lanes 2 to 6) and female (lanes 7 to 11) chicks using alkaline method. Lane 1 and 12 are M. S. marker. DNA was extracted by mixing 2.0 μl whole blood in 10, 20, 30, 40 and 50 μl of 0.2 N NaOH respectively from lane 2 to 6, and 7 to 11, after mixing samples were heated at 75℃ for 20 min and then neutralized with 90, 180, 270, 360 and 450 μl of 0.04 M Tris HCl (pH 7.75) respectively for corresponding samples. 1.0 μl of crude DNA solution was used as template DNA for multiplex PCR. W and 18 S show W chromosome and 18 S ribosomal gene, respectively.

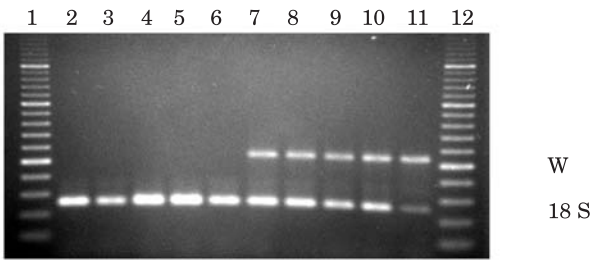

Fig. 2. Multiplex PCR using DNA extracted from single feather follicle in 20 μl of 0.2 N NaOH heated at 75℃ for 20 min and neutralized with 180 μl of 0.04 M Tris HCl (pH 7.75). Lane 1 and 12 are M. S. marker; lane 2 to 6 are feather follicle from males and lane 7 to 11 are feather follicle from female chicks; 5.0 μl of crude DNA solution was used as template DNA for multiplex PCR. See Fig. 1 for explanation of W and 18 S.

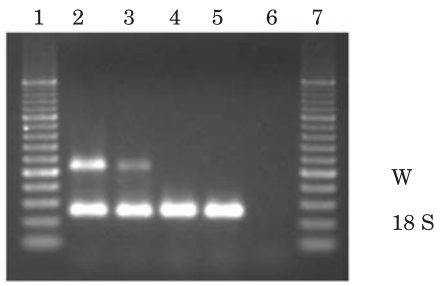

Fig. 3. Multiplex PCR using DNA extracted from embryonic tissue samples using alkaline method, lanes 1 and 7 are M. S. marker. Lanes 2 to 5 are DNA samples of embryos and lane 6 is negative control. 5.0 μl of crude DNA extract was used for multiplex PCR. See Fig. 1 for explanation of W and 18 S.

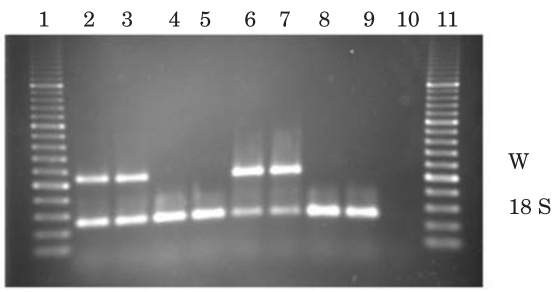

Fig. 4. Multiplex PCR with genomic DNA extracted from whole blood using conventional PCI method (lanes 2 to 5) and modified PCI method (lanes 6 to 9). Lanes 1 and 11 are M. S. marker and lane 10 is negative control. 2.0 μl of template DNA was used for multiplex PCR. See Fig. 1 for explanation of W and 18 S.

robust in sexing of adult chicks using DNA samples extracted from whole blood samples (Fig. 8).

Out of four methods of DNA extraction described above, alkaline method was the simplest and rapid, followed by modified PCI method, Wizard kit and conventional PCI method. Among all methods, alkaline method was most economical followed by modified PCI, conventional PCI and wizard method of DNA extraction (Table 2).

In order to investigate the differences in nucleotide sequence of W chromosome specific PCR products, the single PCR product from all three varieties were sequenced in two directions. The sequences of W chromosome from White leghorn, indigenous and hybrid varieties of chicken as well as from blast searches of NCBI website were found to be identical.

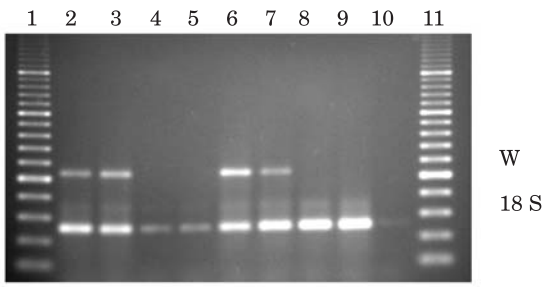

Fig. 5. Multiplex PCR with genomic DNA extracted from single feather bulb using conventional PCI method (lanes 2 to 5) and modified PCI method (lanes 6 to 9). Lanes 1 and 11 are M. S. marker; and lane 10 is negative control. 2.0μl of template DNA was used for multiplex PCR. See Fig. 1 for explanation of W and 18 S.

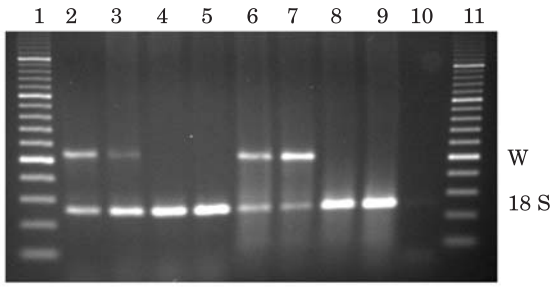

Fig. 7. Multiplex PCR using embryonic DNA samples extracted using Wizard genomic DNA purification kit (lanes 2 to 5) and modified PCI method (lanes 6 to 9). Lanes 1 and 11 are M. S. marker and lane 10 is negative control. 2.0μl of template DNA was used in multiplex PCR. See Fig. 1 for explanation of W and 18 S.

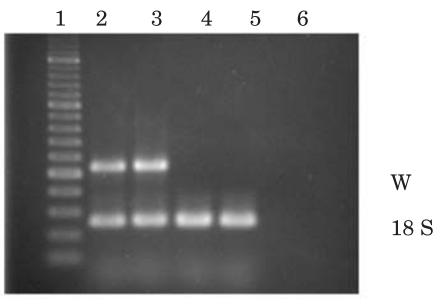

Fig. 6. Multiplex PCR using DNA extracted from embryonic tissue samples using conventional PCI method, lane 1 is M. S. marker. Lanes 2 to 5 are DNA samples of embryos and lane 6 is negative control. 2.0μl of template DNA was used in multiplex PCR. See Fig. 1 for explanation of W and 18 S.

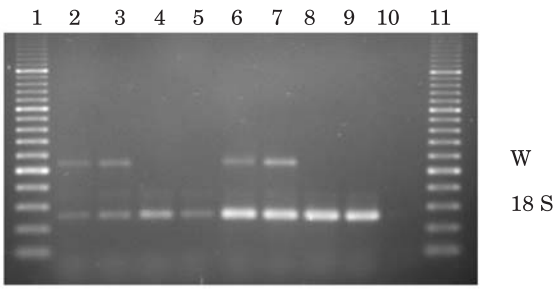

Fig. 8. Multiplex PCR using DNA extracted using Wizard method from whole blood samples (lanes 2 to 5) and feather bulbs (lanes 6 to 9). Lane 1 and 11 are M. S. marker, and lane 10 is negative control. 5.0μl of template DNA was used for multiplex PCR. See Fig. 1 for explanation of W and 18 S.

Table 2. Comparison of different DNA purification methods

S. No.	Parameters	Alkaline method	Conventional PCI method	Modified PCI method	Wizard method
1	Duration	20 min	14 h	1.5 h	1.5 h
2	Centrifugation	No	Yes	Yes	Yes
3	Single tube method	Yes	No	No	No
4	Quantity of sample used	2.0μl blood/ 25 mg tissue/ 1 feather bulb	50μl blood/ 25 mg tissue/ 1 feather bulb	50μl blood/ 25 mg tissue/ 1 feather bulb	50μl blood/ 25 mg tissue/ 1 feather bulb
5	DNA quality on 0.8% agarose gel electrophoresis	Smeared	Intact	Intact	Intact with smear background
6	Safety	Safe	Hazardous	Hazardous	Safe
7	Cost/sample (US $)	0.022	2.430	0.890	3.080

Discussion

The rapid isolation of good quality DNA for genetic analysis, sex diagnosis or any other application has become one of the major concerns for DNA based techniques, especially when large number of samples must be processed. Conventional PCI and other commercial DNA purification kits are the preferred methods for high quality genomic DNA extraction. However, there are some difficulties with these procedures, since conventional PCI method is labour intensive, requires longer period of time, and involves handling of toxic and hazardous chemicals like phenol, chloroform, S.D.S., etc. On the other hand commercial DNA purification kits are expensive.

The present study demonstrated that DNA samples extracted from all three sources by simple alkaline method of DNA extraction, modified PCI method, conventional PCI method and Wizard kit worked well in identifying the sex by multiplex PCR except DNA samples prepared from whole blood sample using Wizard genomic DNA purification kit. Inability to reliably sex chicks using DNA extracted by Wizard kit from whole blood is due to poor results of multiplex PCR to amplify both the bands more efficiently that might have been due to the presence of proteins and haemoglobin which have interfered with extraction of DNA and/or with the multiplex PCR resulting in weak PCR products (Fig. 8). However, this method was successful in sexing of embryos (Fig. 7) and chicks using tissues although multiplex PCR was not robust with DNA extracted from feather samples (Fig. 8).

Similarly multiplex PCR protocol for sexing was robust using DNA samples extracted by alkaline method from feather and embryo samples as compared to the whole blood samples. It was evident from the finding in this study that the multiplex PCR was successful when just 1.0 μl of template DNA extracted from whole blood was used in the multiplex PCR and more than 1.0 μl of template DNA solution had inhibited successful amplification of both the bands in female sex. Template DNA samples extracted from sources like embryos and feather samples seems to have little or no PCR inhibitors as compared to the DNA sample extracted from whole blood where presence of traces of PCR inhibitors like hemoglobin and proteins might have interfered with the efficient multiplex PCR when used in more than 1.0 μl of template DNA solution.

Efficiency of DNA extraction differed according to the source of DNA and method of DNA extraction. Conventional PCI method seems to be the most efficient in extracting good quality DNA from all the three sources particularly from whole blood and embryo samples. Modified PCI method of DNA extraction also provided equally good results although with the single step of PCI extraction and with less incubation time (1 h). DNA quality on 0.8% agarose gel electrophoresis revealed that both conventional PCI method and modified PCI method of genomic DNA extraction from whole blood and embryo samples yielded good quality DNA without smeary background whereas genomic DNA extracted by both the methods from feather samples revealed smeary background. On the other hand wizard method of genomic DNA extraction from embryos and feather samples yielded good quality DNA with smeary background but it was difficult to extract genomic DNA from whole blood samples with a quality that is good enough for successful multiplex PCR. Genomic DNA extracted using alkaline method from whole blood, feather and embryo samples revealed smeary background without intact band indicating shearing of genomic DNA.

In the present study, the differences in the sizes of the W chromosome specific PCR products were observed between predicted ones (481 bp) and migrated ones on agarose gel electrophoresis (Figs. 1–8). It was assumed that slow mobility of PCR products derived from W chromosome, contrary to their actual size, was due to the occurrence of DNA curvatures/bent due to arrangement of nucleotides as described previously (Kodama *et al.,* 1987; Saitosh *et al.,* 1991; Suka *et al.,* 1993).

The findings of the present study are in agreement with the results of Minematsu *et al.* (2004) who observed that the PCI method and proteinase methods of DNA extraction were more effective than the ammonium method in eliminating proteins that influence the extraction efficiency of DNA from whole blood and embryonic tissues. It was found in their study also that PCI method and proteinase method were practical means of extracting DNA for reliable amplification of single band of 276 bp of W chromosome as compared to ammonia method. However, PCI method takes a relatively long period of time and is an expensive method. While these disadvantages could be overcome by following modified PCI method but this method still involves use of toxic and hazardous chemical reagents although both the methods produced consistently good results.

Results of sexing by multiplex PCR using DNA extracted by alkaline method were consistent, reproducible and comparable with those using DNA samples extracted by conventional and modified PCI methods from all three sources (Table 1). Out of four methods described for DNA purification, alkaline method of DNA extraction was found to be rapid (20 min) as compared to other methods. This method had significantly reduced the time required for DNA extraction (Table 2). The method was economical (Table 2) as compared to any other methods used in the present study, as only NaOH, Tris HCl buffer and a single microcentrifuge tube were required for extraction of DNA. In addition, no hazardous materials were used in the alkaline method and more over, alkaline method of DNA extraction is a single step procedure, which obviates the need for use of multiple tubes and multiple pipetting steps, and hence there are less chances of contamination. These advantages of the alkaline method would make it ideal method of DNA extraction for high throughput sex diagnosis and genetic analysis studies us-

ing multiplex PCR and fluorescent technology.

Feathers moulted or non moulted have recently become favoured as non-invasive samples for genetic research, especially for mitochondrial DNA analysis (Horwath *et al.,* 2005), diagnosis of infectious diseases like Marek's disease virus and Avian leucosis virus through PCR (Davidson and Borenshtain, 2002) and sexing of birds (Grant, 2001; Malgo *et al.,* 2002). There appears to be no information on use of feather bulbs for DNA extraction using alkaline method for PCR based sexing in chickens. To our knowledge, this is the first study where alkaline method of DNA extraction from different tissues like embryos, whole blood and feather bulbs of chicken was used for successful sexing using multiplex PCR and the results of sex diagnosis were same whether and whole blood or feather bulbs of chicks were used as source of DNA for multiplex PCR.

W chromosome specific *Xho*I repeat sequence used to design primers in this study and earlier studies (Clinton *et al.,* 2001) are conserved in female genome of *Gallus gallus domesticus* and of three jungle fowl species (*Gallus galus, Gallus soneratti* and *Gallus various*) (Tone *et al.,* 1984). Since the protocol of PCR based sexing using alkaline method of DNA extraction from feather bulbs is accurate, rapid, inexpensive and non invasive, it could be useful for large scale sex typing of chicks of Red Jungle fowl using feather samples collected from the wild nesting sites by multiplex PCR.

In conclusion, extraction of genomic DNA using alkaline method from embryonic tissue, whole blood and feather bulb for sex diagnosis by multiplex PCR is simplest, rapid, safe and more economical than any other methods described in this study as well as in previous studies. Further, extraction of genomic DNA from feather samples from any of the four methods for rapid diagnosis of sex through multiplex PCR in chicken is also non-invasive.

Acknowledgments

This study was supported by grant in aid from Science and Engineering Research Council (SERC) fast track scheme for young scientists, Department of Science and Technology, Ministry of Science and Technology, Government of India (Grant No. SR/FTP/L-97/2003). Some of the laboratory facilities extended to carry out this study by divisions of Animal Production and Animal Health are gratefully acknowledged.

References

Clinton M, Haines L, Belloir B and McBride D. Sexing chick embryos: a rapid and simple protocol. British Poultry Science, 42: 134–138. 2001.

Davidson I and Borenshtain R. The feather tips of commercial chickens are a favourable source of DNA for the amplification of Marek's disease virus and avian leukosis virus, subgroup. Journal of Avian Pathology, 3: 237–240. 2002.

Fridolffson AK and Ellegren H. A simple and universal method for molecular sexing of non-ratitae birds. Journal of Avian Biology, 30: 116–121. 1999.

Grant A. DNA sexing of brown kiwi (*Apteryx mantelli*) from feather samples. DOC Science Internal series 13. Department of conservation, Wellington, pp. 16. 2001.

Griffiths R, Double MC, Orr K and Dawson JG. A DNA test to sex most birds. Molecular Ecology, 7: 1071–1075. 1998.

Horwath MB, Martinez-Cruz B, Negro JJ, Kalmar L and Godoy JA. An overlooked DNA source for non-invasive genetic analysis in birds. Journal of Avian Biology, 36: 84–88. 2005.

Itoh Y, Suzuki M, Ogawa A, Munechika I, Murata K and Mizuno S. Identification of the sex of a wide range of carinatae birds by PCR using primer sets selected from chicken EE0.6 and its related sequences. Journal of Heredity, 92: 315–321. 2001.

Kahn NW, St John J and Quinn TW. Chromosome specific intron size differences in the Avian CHD gene provide an efficient method for sex identification in birds. The Auk, 115: 1074–1078. 1998.

Klein S and Ellendorff F. Identification of Xho1 repetitive sequences on autosomes in addition to the W chromosome in chickens and its relevance for sex diagnosis. Animal Genetics, 31: 104–109. 2000.

Kodama H, Saitoh H, Tone M, Kuhara S, Sakaki Y and Mizuno S. Nucleotide sequences and unusual electrophoretic behaviour of the W chromosome-specific repeating DNA units of the domestic fowl. Chromosoma, 96: 18–25. 1987.

Malgo W Jr, Fanco HM, Matheucci E Jr, Medaglia A and Silva FH. Large scale sex typing of ostrich using DNA extracted from feathers. BMC Biotechnology, 2: 19. 2002.

Minematsu T, Sugiyama M, Tohama Y, Tajima A and Kanai Y. Simplified DNA extraction methods for sexing chick embryos. Journal of Poultry Science, 41: 147–154. 2004.

Naito M, Sano A, Kasashima T, Nakamichi H, Haumi T, Matsubara Y and Kuwana T. A simple method for sexing chicken embryos at stage X. Journal of Poultry Science, 40: 226–230. 2003.

Petite JN and Kegelmeyer AE. Rapid sex determination of chick embryos using the polymerase chain reaction. Animal Biotechnology, 6: 119–130. 1995.

Rudbek L and Dissing J. Rapid simple alkaline extraction of human genomic DNA from whole blood, buccal epithelial cells, semen and forensic stains for PCR. Biotechniques, 25: 588–592. 1998.

Saitoh Y, Saitoh H, Ohtomo K and Mizuno S. Occupancy of the majority of DNA in the chicken W chromosome by bent-repetitive sequences. Chromosome, 101: 32–40. 1991.

Sambrook J and Russel DW. Molecular cloning: a laboratory manual, 3rd Edn., Cold Spring Harbor Laboratory Press. New York. 2001.

Shoffner RN, Krishan A, Halden GJ, Bammi RK and Otis JS. Avian Chromosome methodology. Poultry Science, 46: 333–344. 1967.

Suka N, Shinohara Y, Saitoh Y, Saitoh H, Ohtomo K, Harata M, Shpigelman E and Mizuno S. W-heterochromatin of chicken; its unusual DNA components, late replication, and chromatin structure. Genetica, 88: 93–105. 1993.

Tone M, Sakaki Y, Hashiguchi T and Mizuno S. Genus specificity and extensive methylation of the W chromosomespecific DNA sequences from the domestic fowl, *Gallus gallus domesticus.* Chromosoma, 89: 228–237. 1984.

Uryu N, Nagata Y, Ito K, Saitoh H and Mizuno S. Determination of the sex of chickens by biotin-labelled deoxyribonucleic acid probe. Poultry Science, 68: 850–853. 1989.