Chromosomal anomalies screening in the workup of male infertility in Northern Region of India

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ABSTRACT
Infertility is a multifactorial disorder effecting 8% of couples worldwide and among this about half could be traced to the male partner. Different factors contribute to male infertility including various gene defects and chromosomal abnormalities. The incidence of chromosomal aberrations in population is approximately 0.6% whereas infertile men have an 8-to10-fold higher frequency of chromosomal anomalies than fertile men. Hence, there is a need to routinely karyotype infertile men with unexplained infertility. This study deals with the karyotyping of the infertile azoospermia cases for detecting cytogenetic aberrations. We therefore aimed to study the cytogenetic abnormalities in North-Indian population. The present study analysed 412 infertile patients and revealed 5.56% karyotypic abnormalities. 12 cases with 47, XXY showed severe Klinefelter phenotype. In addition to this 11 were with mosaic pattern of Klinefelter with 46, XY/47, XXY were azoospermic. The cytogenetic analysis helps in work-up of cases and assists in counseling for the assisted reproductive technologies. The prevalence of chromosomal anomaly in the present study is in accordance with the global data.

Key words: Chromosome, Karyotyping, Infertility, Azoospermia.

1. INTRODUCTION
Infertility is defined as a failure to conceive a child after 1 year of regular intercourse without contraception (Akgul et al., 2009). It is well reported that it affects 10-12% couples worldwide and male factor accounts for nearly half of the infertility cases (Kleiman et al., 1999).
Genetics play an important role in maintenance of normal spermatogenesis and in about 30% of infertile males; chromosomal abnormalities and gene mutations have been associated with infertility. Chromosomal aberrations interfere with the process of spermatogenesis and the percentage of chromosomal abnormality increases with the decline in sperm concentrations (McLachlan and O’Bryan, 2010). It was approximated that the overall incidence of a chromosomal factor in infertile males ranges between 2% to 8% (Forresta et al., 2002). The prevalence further increases to 20% in azoospermic males, with sex chromosome more commonly involved (Dohle et al., 2002). Aneuploidy is a condition in which the chromosome number of an organism differs from wild type by a small number of chromosomes. These defects involve either numerical or structural aberrations in the sex chromosomes as well as autosomes. Numerical chromosomal anomalies are the most common error resulting in infertility in men (Emery and Carrell, 2006). High incidences of aneuploidy, especially in their sex chromosome were reported in men with nonobstructive azoospermia (Palermo et al., 2002 and Mateizel et al., 2002). Aneuploidy results from non-disjunction of chromosomes during meiosis. The most common chromosomal abnormality reported in infertile males is 47, XXY (Fu et al., 2012) (cumulative 4.9%) which is associated with Klinefelters syndrome, a condition that effects sexual development. Males with klinefelters syndrome have small testis, reduced facial and pubic hair and reduced testosterone level. It affects approximately 1:600 males (Lissitsina et al., 2006). Variants of Klinefelter patients with increasing number of X chromosomes, (48, XXXY) and (49, XXXXY) shift their sexual phenotype to the female side. This suggested an X-chromosome dosage effect on the males gonad development, and established that the balance between X and Y chromosomes is crucial (Vogt, 2004). Other rare chromosomal anomalies include XYY syndrome; 46, XX males and Noonan syndrome. XYY syndrome occurs with the frequency of 0.84/1000 infertile males. They are characterized by tall, azo/o/o/zoospermia and it is known to take place due to paternal meiotic II non-disjunction of the Y chromosome (Robinson and Jacobs, 1999). 46, XX males occur 1 in 20,000 and they lack Y chromosome except SRY. Thus give rise to sterile phenotype. Noonan syndrome has an incidence of 1 in 2,500 and has 46, XY karyotype. Testicular atrophy and cryptorchidism with hormonal imbalance are responsible for this infertile phenotype. Chromosomal translocations are an additional source of aneuploidy (Gianaroli et al., 2002). Break points of genes due to translocations lead to loss of genetic material which results in incorrect genetic message (Carrell, 2008). Robertsonian translocations occur when two acrocentric chromosomes fuse and are the most frequent structural chromosomal abnormalities in humans (Ferlin et al., 2007; Herman & Susman, 1993). The prevalence of Robertsonian translocations is only 0.8% in infertile males however this figure is nine times higher than in the general population. The translocations can result in a variety of sperm production phenotypes from normal spermatogenesis to an inability to produce spermagonia (Forresta et al., 2002). Robertsonian translocations are more common in oligozoospermic and azoospermic men, with rates of 1.6% and 0.09%, respectively (Johnson, 1998 and Meschede et al., 1998). Carriers of Robertsonian translocations may exhibit a normal phenotype but could be infertile because of a lack of gamete production (Ferlin et al., 2007). Present study aims to underscore chromosomal anomalies in non-obstructive Azoospermic infertile males of North-Indian population.

2. MATERIALS AND METHODS

Subjects
The present study consists of 412 infertile males (32 ± 4.8 years) who were referred to Male infertility Clinic, Department of Urology, Institute of Medical Sciences, Banaras Hindu University, INDIA, for cytogentic analyses from 2009 to 2014. Patients underwent a standardized clinical and laboratory evaluation. Patients married for a minimum of two years, having unprotected intercourse and absence of any obvious fertility problem in the female partner was considered for the present study. Three semen analyses were carried out after three/four days of sexual abstinence to ascertain their infertility status. The patients were categorized into sub-groups as per WHO 1999 criteria. Questionnaire was maintained for each patient to record details of their lifestyle, habits and family history. Informed consent was obtained from every participant of each group. Patients with obstructive azoospermia, hypogonadism, hypoaandrogenism, chronic diseases, history of pelvic/spinal injuries were excluded. Approval of the University’s ethical committee for research on Human material was obtained.

Blood culturing and chromosomal preparation
Two 15 ml autoclaved culture vials were taken in a laminar flow. 5 ml RPMI (Sigma R6504) media was poured in each of them using sterile pipette tip. This media favors growth of Human lymphoid cells. 500 µl of fetal calf serum (Gibco) was then added in each of them as it contains growth factors and low antibodies. 50 µl of PHA (Gibco) was added to them. 0.3ml (8-9 drops) of freshly collected heparinised blood was added to them. Cultures were incubated in BOD incubator with 5% CO₂ concentration and at 37°C temperature for 70 hours in sterile conditions. About 7.5 µl of colchicines (50 µg/ml) was added to both of the vials and they were again kept into the incubator. After 90 minutes of adding colchicine the cultures were transferred to glass centrifuge tubes. The tubes were then centrifuged at 1200 RPM for 10 minutes at room temperature in a centrifuge. Supernatant was discarded and it was tapped to break the pellet. 1-2 drops of hypotonic maintained at 37°C was added to the pellet and it was tapped to mix. It was done twice and thrice to mix properly. Volume was made to 8 ml, and it was incubated at 37°C for 15 minutes. 2-3 drops of fixative was added and mixed by pipetting. It was then centrifuged at 1200 RPM for 10 minutes at room
temperature. Supernatant was discarded and pellet was mixed by tapping 2-3 times with 3 drops of fixative. Volume was maintained to 8 ml and tubes were incubated at room temperature for 5 minutes. Tubes were again centrifuged at 1200 RPM for 10 minutes at room temperature. Supernatant was discarded carefully and pellet was again resuspended in 8 ml of fixative for washing. Centrifugation was done at 1200 RPM for 10 minutes at room temperature. About 6 mL of upper supernatant layer was discarded and 2 ml was retained. Two or three drops from the left out liquid were dropped over a clean slide from height and flame drying was done. The slides were then stained with Giemsa stain for 10 minutes then washed in distilled water in coupling jars. After slides were dried, they were mounted using DPX and left for drying. Slides were observed in microscope with oil-emersion 100X objective.

G banding of chromosome
Slides made by air drop method were kept for ageing. After aging they were kept in 30 mg/ml trypsin solution made in 0.85% NaCl solution of pH 7.4 for 25 seconds. Immediately they were washed in 1:1 solution of Sodium Hydrogen Phosphate and Potassium Hydrogen phosphate solution. Slides were then washed 2-3 times in distilled water. Slides were then transferred into Giemsa stain and kept for 10 minutes for staining. Slides were then washed in distilled water 2-3 times. They were dried and they were mounted using DPX. After drying they were observed in microscope with oil immersion 100X objective.

Karyotyping
15 metaphase plates were scored in each slide. Selected metaphase plates were karyotyped following the standard human chromosome nomenclature (ISCN 2009) from each individual.

3. RESULT
PHA-stimulated whole blood cultures were set up for all the 412 patients. The slides were stained with Giemsa and observed under oil immersion objective of the microscope. From each patient 20 plates were observed and counted for ascertaining the diploid chromosome number for each individual. Other slides were allowed to age for 10 days before being used for G-banding. Selected metaphases were photographed and karyotyped. Numerical abnormalities were taken into consideration for the analysis. Of the total 412 infertile men’s, 5.56% (23/412) were found to have cytogenetic abnormalities. Outcome of chromosomal analysis of 412 patients is as follows: G-banded karyotypes of 12 patient’s revealed chromosome complement of 47, XXY which is a characteristic of Klinefelter syndrome. One of the twelve patients was azoospermic with low testosterone levels, less facial hairs and gynecomastia while other patients were azoospermic and had small testicular volume along with less hair on the body. In addition to this mosaics Klinefelter with 46, XY (Figure 4 a, A)/47, XXY (Figure 2 b, B) were observed in 11 patients. All cases with mosaic karyotype were azoospermic. None of these cases were found to have autosomal abnormalities.

4. DISCUSSION
The incidence of chromosomal aberrations in population is approximately 0.6% (Berger, 1975). On the other hand, karyotype abnormalities are reported in 2%–14% of males presenting infertility (Shi and Martin, 2000). A high percentage of Klinefelter syndrome males are only recognized when they undergo infertility workup. 11% of azoospermia individual’s accounts for non-mosaic form of Klinefelter syndrome and severe oligozoospermia present with mosaic chromosome complement (van Assche et al., 1996). Klinefelter syndrome has variable phenotype and failure to recognize them is due to the misapprehension that all individuals should have classical phenotype like tall, gynecomastia and hypogonadism (McLachlan and O’Bryan, 2010). Studies have shown significant increase in sex chromosome aneuploidy in the sperm of individuals having sex chromosome aneuploidies with higher frequencies reported for non-mosaic individuals with an average of 6% aneuploidy (range: 1%–25%) versus an average of 3% (range: 0–7%) aneuploidy in mosaic 46, XY/47, XXY individuals (Tempest, 2011; Ferlin et al., 2000; Sarrate et al., 2005). Approximately 4% of males undergoing ICSI have chromosomal abnormalities out of which 80% involve sex chromosomes. The major histological representation in KS is of Sertoli cell only and hyalinised tubules (McLachlan et al., 2007), but few areas of spermatogenesis allow the isolation of testicular sperm in 40–69% of non-mosaic KS (Levron et al., 2007; Schiff et al., 2005 and Tournaye et al., 1996). The present study analysed 412 patients and revealed 5.56% (23/412) with 47, XXY including 11 cases with mosaic karyotype. All patients with chromosomal anomalies were azoospermic and they posed one or the other feature of Klinefelter syndrome. The distribution of chromosomal abnormalities detected in the present study showed that Klinefelter’s syndrome (47, XXY) was the most prevalent abnormality and result was in accord with several previously published studies (Fu et al., 2010; Lissitsina et al., 2006; Nakamura et al., 2001; Penna et al., 2001; Rao et al., 2004 and Mohammed et al., 2007).

5. CONCLUSION
Our study did not observed any structural rearrangement, though the chances of ignoring fine level deletion or rearrangement could not be ruled out. The cytogenetic analysis help in infertility work-up of cases and also allow diagnosing them better. The results of cytogenetic analysis assist counseling the patients for the assisted reproductive technologies.

SUMMARY OF RESEARCH
1. Present study aims to underscore chromosomal anomalies in non-obstructive azoospermic infertile males.
2. The present study analysed 242 patients and revealed 6.1% karyotype abnormality.
3. 4/242 patients showed patient showed 47, XXY chromosomal compliment. All cases with 47, XXY showed severe Klinefelter phenotype. In addition to this mosaics Klinefelter with 46, XY/47, XXY were observed in 11 patients. All cases with mosaic karyotype were azoospermic.
4. The cytogenetic analysis helps in infertlity work-up of cases and assists in counselling for the assisted reproductive technologies. The prevalence of chromosomal anomaly is in accordance with the global data.

FUTURE ISSUES
Cytogenetic analysis of infertile patients helps in the counselling in the infertility work up. It help to exclude patients with genetic anomalies.

DISCLOSURE STATEMENT
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CONFLICT OF INTEREST
There is no conflict of interest.

REFERENCES


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**Figure 1**

A G-banded Metaphase plate (a) and a Karyotype (A) made of the plate from an azoospermic infertile male with normal chromosomal compliment 46, XY. G banded metaphase plate (b) and a Karyotype 47, XXY (B) was made from Klinefelter infertile male.