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Molecular charecterization of multidrug resistance in acinetobacter species

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ABSTRACT

The aim of this study to determine the prevalence of multidrug resistance in *Acinetobacter* species, these are ubiquitous Gram-negative bacteria widespread in nature .They live in soil and water. They utilize a wide variety of carbon and other energy sources and grow well on routine lab media. Originally known as antibiosis, antibiotics were drugs that had action against bacteria. *Acinetobacter* strain which was isolated from blood, pus and urine was collected from different hospital lab. It was further confirmed to be Acinetobacter using various biochemical test and observing the growth in the Mcconkey agar medium .Also it is found that ESBL strains are weak bio film producers, hence spread these organisms are restricted by bio film production. In quantitative assay by observing the OD at 600nm,the rate of bio film production was observed .This results strongly correlates with qualitative assay .Finally the ESBL strain was subjected to plasmid isolated ,separated using Agarose Gel and the gel was documented .The molecular weight was recorded to be 14000 kb by comparing with the marker protein.

Introduction

Nowadays, Anti microbial resistance in growing problem in modern hospitals the increasing severity of illness and compromised immunity of patients treated for cancer and other leads to frequent use of broad spectrum antibiotic agents. There are over 20 species of acinetobacter, though the *aciinetobacter baumannii* accounts for >80% of isolates causing human disease. *Acinetobacter* species are ubiquitous gram negative bacteria wide spread in nature.

Acinetobacter baumannii has emerged as an important nosocomial pathogen (1–5). Hospital outbreaks have been described from various geographic areas (6–9), and this organism has become endemic in some of them. The role of the environmental contamination in the transmission of nosocomial infections in general and in A. *baumannii* infections in particular is well recognized (10, 11).

A. baumannii does not have fastidious growth requirements and is able to grow at various temperatures and pH conditions (12). The versatile organism exploits a variety of both carbon and energy sources.

These properties explain the ability of Acinetobacter species to persist in either moist or dry conditions in the hospital environment, thereby contributing to transmission (13, 14). This hardiness, combined with its intrinsic resistance to many antimicrobial agents, contributes to the organism's fitness and enables it to spread in the hospital setting.

The nosocomial epidemiology of this organism is complex. Villegas and Hartstein reviewed *Acinetobacter* outbreaks occurring from 1977 to 2000 and hypothesized that endemicity, increasing rate, and increasing or new resistance to antimicrobial drugs in a collection of isolates suggest transmission. These authors suggested that transmission should be confirmed by using a discriminatory genotyping test (15).

The importance of genotyping tests is illustrated by outbreaks that were shown by classic epidemiologic methods and were thought to be caused by a single isolate transmitted between patients; however, when molecular typing of the organisms was performed, a more complex situation of multiple unrelated strains causing the increasing rates of infections by *A. baumannii* was discovered (16–17). Almost 25 years ago, researchers observed acquired resistance of *A. baumannii* to antimicrobial drugs commonly used at that time, among them aminopenicillins, ureidopenicillins, first and second-generation cephalosporins, cephamycins, most aminoglycosides, chloramphenicol, and tetracyclines (19).

Since then, strains of *A. baumannii* have also gained resistance to newly developed antimicrobial drugs. Although multidrug-resistant (MDR) *A. baumannii* is rarely found in community isolates, it became prevalent in many hospitals(23). MDR *A. baumannii* has recently been established as a leading nosocomial pathogen in several Israeli hospitals, including our institution (20,21). Several locally contained small outbreaks of MDR *A. baumannii* occurred in our institution during the late 1990s. In 1999, however, the incidence of MDR *A. baumannii* isolation had doubled compared to the previous 2 years, and the organism became endemic in many wards (unpub. data).

The likelihood of isolation of *A. baumannii* from a hospitalized patient is related to temporospatial (extrinsic, ecologic characteristics) factors such as colonizationpressure (22), nurse-to-patient ratio, and other ward characteristics and to individual patient risk factors (characteristics). The current study was designed to examine the occurrence and spread of *A. baumannii* within our institution, as well as to define individual risk factors for isolation of this organism.

Material and methods:

Collection of organism

Eighteen Acinetobacter species were collected from different labs located at Chennai.

Confirmation

The organism were further confirmed as Acinetobacter by performing various biochemical tests such as Oxidase, Catalase, and culturing in Macconkey agar medium.

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Characterization of ESBL producing strains using Disk Diffusion Method

In this study, to evaluate the percentage of β lactamase producing organism,18 strains of *Acinnetobacte*r species that was isolated from the blood samples of the patients admitted in the ICU was chosen. All the organisms were subjected to Antibiotic sensitivity tests by Kirby Bauer Disk Diffusion method, to assay the susceptibility and resistant pattern. Penicillin antibiotic of concentration 30µg which is used today for the treatment of β- latamases containing bacterial infection was chosen for the multidrug resistance study. The above mentioned antibiotic disc was placed in the plates containing organisms and incubated for 24hours to view the zone of inhibition.

Qualitative assay for the biofilm formation

The qualitative assay was performed for each species by the tube method.

Quantitative assay for the biofilm formation

The quantitative assay was performed for each species by the microtiter plate method.

Plasmid isolation

The species resistant to ceftazidime (CAZ) 30µg was used to isolate the plasmid by alkalin lysis method.

Determination of molecular weight by agarose gel electrophoresis

The purified plasmid, taken in duplicate, was loaded in lane number 2-6.Amarker DNA of 1Kb ladder was loaded in lane number 1 and it was used to determine the molecular weight of the purified plasmid DNA. The separated plasmid was visualized under UV transilluminator.

Result and Discussion

Acinetobacter species has rapidly become the most common ESBL producing organism, making it difficult to eradicate this organism from the high risk wards such as Intensive Care Units. It is considered to be dangerous species due to its multidrug resistance capacity.

Isolation of Acinetobacter Species:

The Organism which was received from different hospital laboratories located in Chennai was further confirmed by the observation of growth in Maconkey Agar Medium and various Biochemical assays(Table No: 1). The organisms were Gram negative, rod shaped bacteria, Hence the organisms were confirmed to be *Acinetobacter species*. by referring Bergeys Manual of Bacteriology.

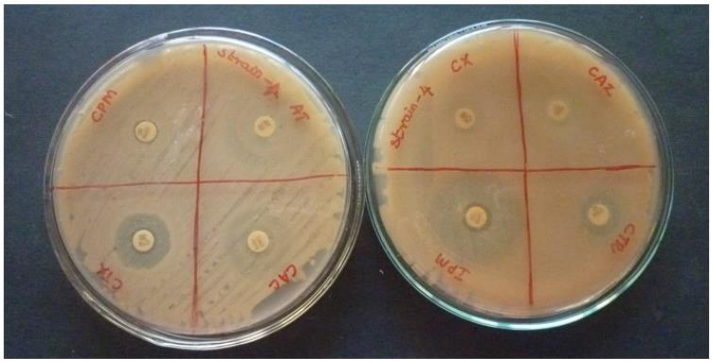
Biochemical identification tests for Acinetobacter

Characterization of ESBL producing Strains using Disc Diffusion Method

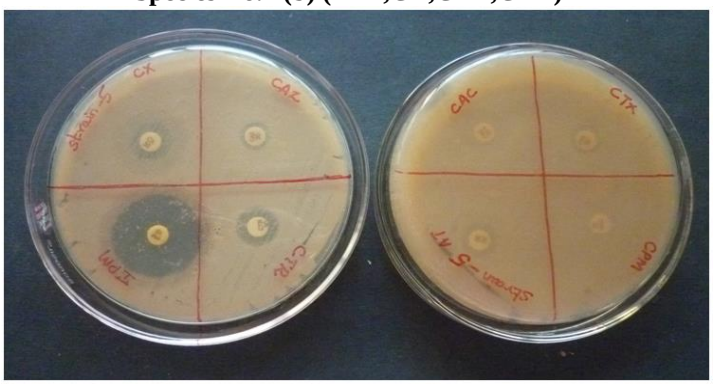
Table No.2 and 3 depicts the data of disc diffusion technique. From the data it is evident that three strains (species No.1, 11, 15) were resistance to Ceftazidime (CAZ) and sensitive to Ceftazimide/ Clavulinic acid (CAC) combinations. Hence these strains are referred to be ESBL strains. These strains has produced the enzyme β lactamases, cleaved the β lactam ring antibiotic CAC, but when the drug was taken in combination the enzyme was not able to cleave the drug and hence no growth of the organism was noticed to certain extent. This finding is consistent with reports of other tertiary care hospitals, giving the evidence of β lactamases (ESBL), can rapidly emerge and establish a condition of endemicity in certain epidemiological settings.

Also, from the result, it is able to view that species No.6 was completely resistant (N Z) to the antibiotic Imipenum (IPM). And the strains (12, 14, and 16) were also considered to be resistant even though little zone formation was seen. This is predicted that, the carbapenase enzyme produced by the organism has inactivated the Antibiotic Imipenum. **Similar findings were reported by Patrice Nordamn *et* al, 2008.**

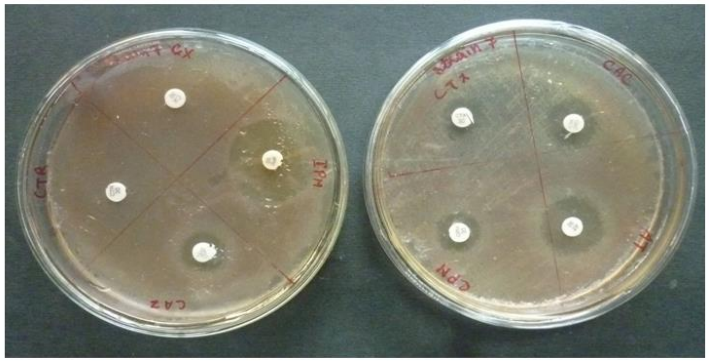
Figures No.1-4 Antimicrobial zones of each Acinetobacter species against 8 antibiotics



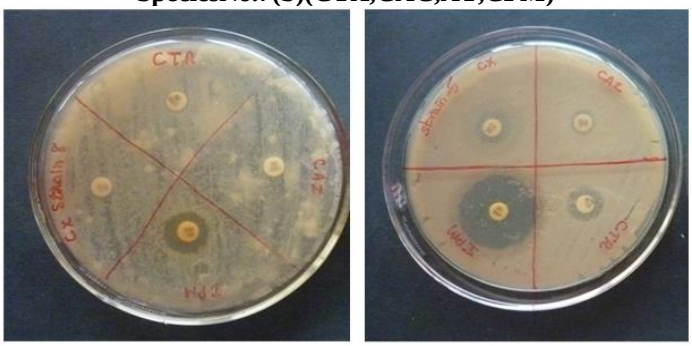
Species No.4(a) (CPM,AT,CAC,CTX)
Species No.4 (b) (IPM,CX,CAZ,CTR)



Species No.5 (a) (CPM,AT,CAC,CTX
Species No. 5(b) (IPM,CX,CAZ,CTR)



Species No. 7 (a) (IPM,CX,CAZ,CTR
SpeciesNo.7(b)(CTX,CAC,AT,CPM)



Species No. 8 (a) (IPM,CX,CAZ,CTR)
Species No. 5(b) (IPM,CX,CAZ,CTR)

By analyzing the percentage of susceptibility and resistance to the antibiotics which is used presently for the treatment of Acinetobacterenlightens the idea of prevalence of the infectious strains today in India.

Susceptibility represents that the antibiotic are active and inhibits the growth of the organism, hence different size formations are seen and the standard limit of zone formation if 14mm. hence, if the zone formation is greater than 14mm it is considered to be susceptibility.

Intermediate resistance is categorized as if the zone formation is in the border line i.e,12-14mm size.

Resistance represents endangered it means that the antibiotics losses its ability to kill the organism, and hence these organisms grow till very closer to the antibiotic disk, and the standard of resistance is considered to be no zone formation or below 12mm size.

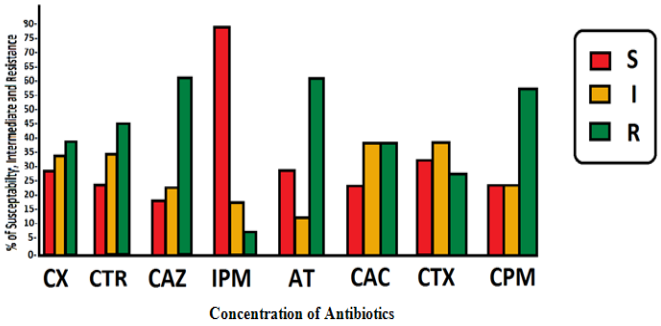


Table No: 3 and Fig No: 2 depict the Percentage of Susceptibility, Intermediate resistance and Resistance to different antibiotics. From the data, it is clear that 61.1% of the strains was highly resistant to the antibiotic CAZ, AT which is one of the antibiotics used today for the treatment of infectious Acinetobacter. If this situation prevales, it is difficult to treat the infections and hence new antibiotics has to be discovered.Also, it is evident that 77.7% of the strains were sensitive to the antibiotic Imipenum, one of the antibiotic used in the treatment of species producing carbapenase ,which is difficult to treat.

Biofilm formation:

A bio film is a key pathogenic feature in Acinetobacter infections. A bio film is a congregation of microbial cells surrounded by extracellular polymeric substance matrix. It has two important properties- increased synthesis of Exo polysaccharide (EPS) and development of Antibiotic resistance. The increased production of Exo polysaccharide creates a protective environment that leads to difficulty in Antibiotic penetration. This develops resistance.

Qualitative assay:

Qualitative assay gives the preliminary idea, whether the strains are capable of producing biofilm are not.

Figure No. 5(a),5(b) shows the visualized picture of qualitative tube method of biofilm. In the qualitative assay for bio film production, the isolates were classified as strong bio film producing, moderately biofilm producing and weekly biofilm producing (non bio film producing).

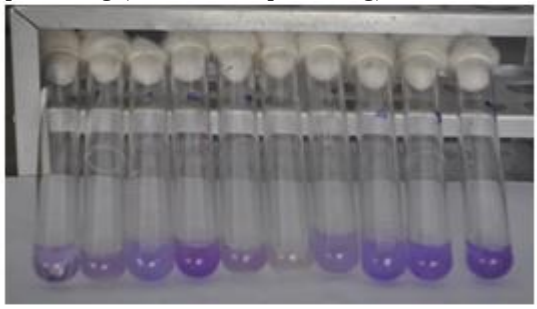


Figure No.5(a) shows the biofilm formation by the tube method. (Control, species-1, Species-2, Species-3, Species-4, Species-5, Species-6, Species-8, Species-9, Species-10)

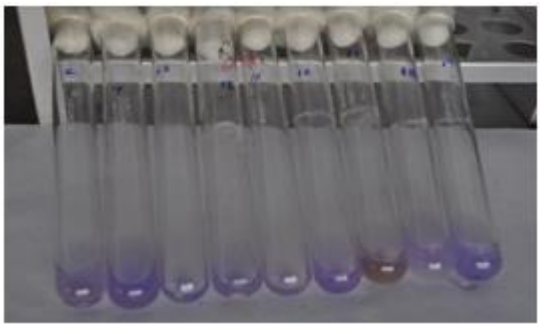


Figure No.5(b) Shows the biofilm formation by the tube method (control,species-7,species-12,species-13,species-15,species-16,species-17,species-18, species-19)

Of the eighteen isolates (Species No. 3,7,8,9,10,), 27.7% of the organisms were categorized under strong biofilm producers since they show a thick blue ring at the liquid air interface.

(Species No.2, 6, 11, 14, 16,) 27.5% were categorized under moderately biofilm producers since the blue ring which was formed was likely thin comparative to the strong producers.

(Species No. 1, 4,5,12, 13, 15, 17, 18) 44.4% were categorized under non bio film producers since a very light tinch of blue appearance was seen in the tube. The present investigation correlates with the findings of **SrinivasaRao *et* al .**

Quantitative Assay:

Figure No.4 and table No.5 postulates the picture and the data of the confirmatory test for the bio film production. The readings which were gained strongly correlates with the Qualitative assay. These observations were confirmed when cell attachment to plastic was quantified by relating the total cell mass to the stain retained by the attached cells as described in Methods. It is interesting to note that the*Acinetobacter* cells form denser aggregates at the liquid–air interface than on the side and bottom surfaces of the tubes and plates. Furthermore, the biofilm grows upwards from the liquid–air interface onto the walls of the plate , a phenomenon that is not caused by the movement of the broth, When the biofilm formation of the organisms was correlated with the resistant capacity, it is seen that, ESBL producing strains such as (1, 11, 15) were weak and moderate biofilm producers.

Figure No 6. Quantitve biofilm formation by microtitre method

Hence, even though these strains are considered to be the resistant strains and ESBL producers their capability of producing biofilm is less and hence the spread of these strains can be controlled. Also species No.6, which is considered to be Imipenum resistant, is also found to be a Moderate biofilm producer. The present study go hand in hand with Maria A.Mussi *et*.al., 2010.

Table 1.

Species No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
GRAM STRIN	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
GLUCOSE	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
LACTOSE	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SUCROSE	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
CAESIN	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
UREASE	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
STARCH	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
MR	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
VP	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
INDOLE	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CITRATE	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
OXIDASE	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
GELATIN	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
CATALASE	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Table 2:(a)&(b) Antibiotic Zone for each *Acinetobacter species* using Disc Diffusion Technique

Antibiotic	Species1	Species2	Species3	Species4	Species5	Species6	Species7	Species8
CX	N Z	12mm	6 mm	7mm	12mm	15mm	10mm	25mm
CTR	N Z	34mm	N Z	12mm	15mm	7mm	N Z	20mm
CAZ	10mm	28mm	N Z	N Z	14mm	N Z	N Z	20mm
IPM	26mm	14mm	22mm	16mm	25mm	N Z	15mm	29mm
AT	18mm	32mm	N Z	N Z	N Z	N Z	10mm	20mm
CAC	14mm	12mm	12mm	N Z	N Z	N Z	10mm	20mm
CTX	10mm	32mm	12mm	N Z	16mm	N Z	25mm	20mm
CPM	12mm	20mm	N Z	N Z	N Z	N Z	15mm	22mm

Antibiotic	Species 9	Species 10	Species 11	Species 12	Species 13	Species 14	Species 15	Species 16	Species 17	Species 18
Cx	N Z	17mm	17mm	N Z	12mm	N Z	N Z	N Z	N Z	24mm
CTR	13mm	9mm	17mm	12mm	12mm	N Z	N Z	N Z	N Z	N Z
CAZ	9mm	N Z	N Z	N Z	9mm	N Z	10mm	N Z	N Z	N Z
IPM	37mm	17mm	25mm	8mm	22mm	9mm	26mm	12mm	16mm	28mm
AT	17mm	N Z	N Z	N Z	12mm	N Z	18mm	N Z	N Z	N Z
CAC	N Z	N Z	24mm	8mm	N Z	N Z	14mm	9mm	9mm	8mm
CTX	N Z	24mm	12mm	24mm	8mm	N Z	10mm	9mm	N Z	8mm
CPM	N Z	N Z	28mm	9mm	N Z	N Z	12mm	9mm	N Z	N Z

Table No.3: Percentage of Susceptability, Intermediate and Resistance to different antibiotics

ANTIBIOTICS	SUSCEPTIBLE STRAINS(%)	INTERMEDIATE STRAINS	RESISTANT STRAINS
CX	27.7%	33.3%	38.8%
CTR	22.2%	33.3%	44.4%
CAZ	16.6%	22.2%	61.11%
IPM	77.7%	16.6%	5.5%
AT	27.7%	11.1%	61.1%
CAC	22.2%	38.8%	38.8%
CTX	33.3%	38.8%	27.7%
CPM	22.2%	22.2%	55.5%

Table No.5: Presence of bio films rated as Weak, Moderate and Strong

Species No. 1	Weak
Species No.2	Moderate
Species No.3	Strong
Species No.4	Weak
Species No.5	Weak
Species No.6	Moderate
Species No.7	Strong
Species No.8	Strong
Species No.9	Strong
Species No.10	Strong
Species No.11	Moderate
Species No.12	Weak
Species No.13	Weak
Species No.14	Moderate
Species No.15	Weak
Species No.16	Moderate
Species No.17	Weak
Species No.18.	Weak

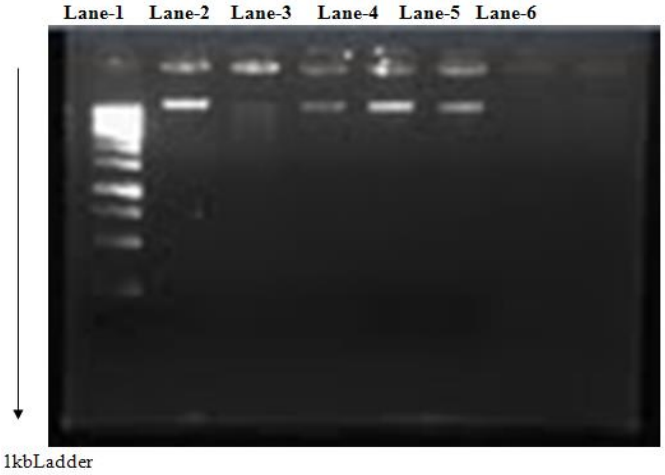
Table No.5: Quantitative bio film formation by microtitre method

Acinetobacter Strains	O.D 640nm
Control	0.051
Species 1	0.1925
Species 2	0.107
Species 3	0.5015
Species 4	0.062
Species5	0.115
Species 6	0.1852
Species 7	0.3485
Species 8	0.5415
Species 9	0.3385
Species10	0.5825
Species11	0.192
Species12	0.0623
Species13	0.0825
Species14	0.1905
Species15	0.062
Species16	0.115
Species17	0.1925
Species18	0.250

Plasmid isolation and determination of its molecular weight:

Fig No:7 picturizes the documented gel for the plasmid isolated and separated. The organism which was considered to be the ESBL producers was subjected for plasmid isolation and separated using Agarose Gel Electrophoresis.

In the figure Lane 1 represents the marker of molecular weight ranging from14000kb-67000kb .In the lanes 2,4 and 6the plasmid which was isolated from strains 1,11,15 was separated. The molecular weight which corresponding to the marker was calculated to be14000kb. The present findings is supported by Karishma R. Pardesi *et.*al.,*2007*



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