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Original Article

Microbiology Section

Correlation of Antibodies against Nuclear Antigen using Immunofluorescence Pattern and Line Immunoassay Profile in Autoimmune Diseases at a Tertiary Care Hospital, Uttarakhand, India

KXITIZA PANDEY[1], SULEKHA NAUTIYAL[2], ARUN PANDEY[3], GEETIKA RANA[4], HIMANSHA PANDEY[5], VIJAY KUMAR KATARIA[6]

ABSTRACT

Introduction: Autoimmunity is a condition characterised by a specific humoral or cell-mediated immune response against constituents of the body's own tissues. The diagnosis of autoimmune diseases (AD) is based on clinical presentation, laboratory diagnosis, and radiological diagnosis. Laboratory diagnosis involves the detection of antibodies directed against nuclear and cytoplasmic components of the cell. The gold standard test to detect antibodies against nuclear antigens is the Immunofluorescence Assay (IFA), which detects the presence of Antinuclear Antibody (ANA) in the serum. Other tests that can be used for ANA detection are Enzyme-Linked Immunosorbent Assay (ELISA), anti-Extractable Nuclear Antigen (anti-ENA), Line Immunoassay (LIA), etc.

Aim: To correlate the results of antibodies against nuclear antigens based on various immunofluorescence patterns and LIA profiles in autoimmune disorders.

Materials and Methods: The present study was a time-bound explorative and comparative cross-sectional study conducted at a teritary care hospital over a period of one year (December 2018 November 2019) in the Department of Medicine in collaboration with the Department of Microbiology, SGRR IM and HS, Dehradun, Uttrakhand, India. The study was carried out on a convenient sample. Out of a total of 178 subjects, 118 suspected cases were included in the study group, and 60 healthy individuals were included in the control group. ANA was detected by IFA using HEp-20-10/liver cell. All the samples that were positive or negative by IFA were further evaluated by LIA. The data obtained were statistically analysed for significance using Stastistical Packages of Social Sciences (SPSS) version 20.0.

Results: The majority of cases in both groups belonged to the 30-50 year age group (69/118 in the study group and 37/60 in the control group). IFA was positive in 49.15% of the samples in the study group and 21.6% in the control group. The most common pattern observed in IFA was nuclear homogeneous. LIA was positive in 45.7% of the cases, with the maximum antibodies detected against dsDNA antigen (double-stranded DNA). In the study group, out of the 49% IFA-positive samples, 40.6% were also positive for antibodies by LIA, and an additional 5% of cases that were negative by IFA were found to be positive by LIA. The statistical strength of correlation between patterns in IFA and bands in LIA is established.

Conclusion: A combination of IFA and LIA can serve as a better tool for early and accurate diagnosis of AD. In the control group, 25% were observed to be positive for ANA using both IFA and/or LIA. Thus, there remains a possibility of such individuals developing ADs in the future.

Keywords: Autoimmunity, Immune profile, Systemic lupus erythematosus

INTRODUCTION

Autoimmunity is a condition characterised by a specific humoral or cell-mediated immune response against constituents of the body's own tissues (self-antigens or auto-antigens) [1]. In 1900, Paul E realised that the immune system could go awry and, instead of reacting against foreign antigens, could focus its attack on self-antigens and termed this condition "horror autotoxicus" [2]. The hallmark of autoimmune disorders generally involves the presence of self-reactive T cells along with the presence of autoantibodies [3]. The overactive immune response to tissues present in the body can be restricted to specific organs, as in Type I diabetes, Graves' disease, primary biliary cirrhosis, autoimmune haemolytic anaemia, or rheumatic heart disease, etc., or it can be systemic or disseminated, as in multiple sclerosis, Systemic Lupus Erythematosus (SLE), Sjogren's syndrome, scleroderma, etc. [4]. Autoimmune disorders may vary in their clinical presentation depending on the organ involved, but all autoimmune disorders go through the three phases of initiation, propagation, and resolution [5]. Various factors closely related to autoimmune disorders are genetic factors like Human Leukocyte Antigens (HLA) gene polymorphism, cytokine and cytokine receptor polymorphism, protein mutation and altered expression, Post Translational Modification (PTM) of proteins, and epitope spreading.

Autoimmune disorders can also arise due to various environmental factors like infection with certain viruses, bacteria, or mycoplasma. Exposure to Ultraviolet (UV) radiation, particularly UV-B rays, has been linked to a physical insult that results in flare-ups of SLE [6], silica exposure, drug-induced (for example, thiol-containing drugs and sulfonamide derivatives, as well as certain antibiotics and non steroidal anti-inflammatory drugs [7,8]), and molecular mimicry in which foreign antigens, which often differ from their homologous self-antigens in some areas, may have significant structural similarity to self-antigens in other regions, appearing to trigger the onset of some autoimmune disorders in genetically susceptible individuals

[9]. In addition to the above factors, X-chromosome abnormalities and sex hormones such as estrogens and androgens are believed to play a significant role in the sex-based susceptibility to many autoimmune disorders [10,11]. The self-antigens that drive the reaction cannot be eliminated. This problem is amplified by the emergence of new antigenic epitopes as a result of tissue damage and alterations in self-proteins, a phenomenon known as epitope spreading, leading to the propagation of autoimmune disorders [12]. The resolution of autoimmune reactions likely involves the induction and activation of regulatory mechanisms that restore the effector/regulatory balance. The diagnosis of autoimmune disorders is based on clinical presentation, laboratory diagnosis, and radiological diagnosis. Laboratory diagnosis involves the detection of antibodies directed against nuclear and cytoplasmic components of the cell. Lower amounts of these antibodies are seen in healthy individuals, like in pregnancy and the elderly age group, while an increase in titers is seen in patients with autoimmune disorders.

Various tests are available to detect autoantibodies, but the gold standard test for the detection of antibodies against nuclear antigens is IFA. The test detects the presence of ANA in the serum, which adheres to reagent test cells forming distinct fluorescence patterns. IFA uses a combination of two substrates: human epithelial cells (HEp-20/10) and primate liver cells [13]. Other tests that can be used for ANA detection are ELISA, anti-ENA antigen, LIA, flow cytometry, etc. For further confirmation and specification of AD another test used is LIA, which detects ANA using nitrocellulose strips on which specific nuclear antigens are applied parallel and at equal distances. Depending on their specificity, autoantibodies bind to the antigens and are traced by alkaline phosphatase-conjugated anti-human-IgG antibodies, appearing as blue-stained bands on the strips [14].

The present study was conducted to ascertain the prevalence of the burden of autoimmune disorders in patients attending tertiary care hospital. The objective was to correlate the results of antibodies against nuclear antigens based on various immunofluorescence patterns and LIA profiles in autoimmune disorders.

MATERIALS AND METHODS

The present study was a time-bound explorative and comparative cross-sectional study conducted in a tertiary care hospital over a period of one year (December 2018 November 2019) in the Department of Microbiology in collaboration with the Department of Medicine, SGRR IMand HS, Dehradun, Uttrakhand, India. Out of a total of 178 subjects 118 suspected cases were included in the study group, and 60 healthy individuals were included in the control group. Ethical clearance for the study was obtained from the Institutional Ethics Committee (Registration No. ECR/710/Inst/UK/2015/RR-18).

Inclusion criteria: All patients over the age of 18 and of all genders, with clinical features suspicious of an AD, were included in the study group. Patients of all genders and age groups over 18 years, with no clinical features of an AD were included in control group.

Exclusion criteria: Patients with no symptoms suggestive of an AD were excluded from study group and patients of all age groups and genders with clinical features suspicious of an AD or previously treated for an AD were excluded from control group.

Study Procedure

Indirect Immunofluorescence Assay (IF-ANA): A 2 mL serum sample was collected and stored in aliquots at 2-8°C, to be used within 72 hours. ANA was detected by Immunofluorescence assay using IIFT Mosaic: HEp-20-10/liver (monkey) Kit from EUROIMMUN. All samples were processed as per the manufacturer's instructions within the expiry period. The sample to be investigated was diluted 1:100 Phosphate Buffered Saline (PBS)-Tween. Positive and negative controls were included with each test.

ANA Line Immunoassay (LIA): All samples that tested positive or negative by IFA were further evaluated using the EUROIMMUN LIA test. The samples were diluted 1:101 with sample buffer and tested using nitrocellulose test strips coated with 15 antigens, including nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl, Jo-1, centromere protein B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-proteins, and AMA M2, along with the control band.

STATISTICAL ANALYSIS

The observations in present study were analysed using cross tabulation of ANA IFA (gold standard) and ANA profile LIA for the Chi-square test. SPSS software, version 20.0 was used for determining statistical significance.

RESULTS

A total of 178 subjects were included in present study. Of these, 118 cases were clinically suspected of having an autoimmune disorder and were included in the study group, while 60 healthy individuals were included in the control group. The majority of cases in both groups belonged to the 30-50 year age group (69/118 in the study group and 37/60 in the control group). In the current study, out of the total 118 cases in the study group, 58 (49.15%) samples tested positive for IFA, while in the control group, 13/60 (21.6%) samples tested positive for IFA. This difference was statistically significant with a p-value <0.05 [Table/Fig-1].

IFA pattern		Study group n (%)	Control group n (%)
Nuclear	Nuclear homogenous	28 (48.4)	6 (46.1)
	Fine speckled	22 (37.9)	5 (38.5)
	Nuclear dots	1 (1.7)	0
	Centrosome	1 (1.7)	0
Cytoplasmic	Fine speckled	4 (6.9)	2 (15.4)
Mixed pattern	Nuclear and cytoplasmic	1 (1.7)	0
	Nucleolar and cytoplasmic	1 (1.7)	0
Total		58	13

[Table/Fig-1]: Distribution patterns in IFA pattern in study and control groups.

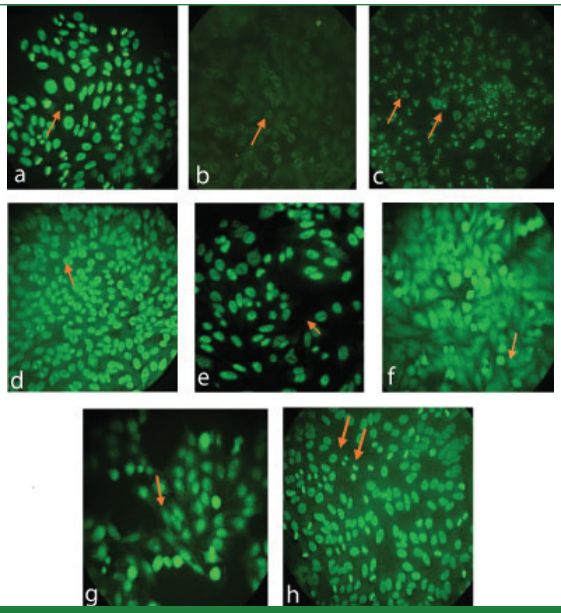
When examining the relationship between IFA positivity, gender, and age group in the study group, it was found that 37/58 (63.8%) positive cases were females. Among the females testing positive by IFA, 25/37 (67.5%) belonged to the 31-50 year age group in the study group. On statistical analysis this value was not significant (p-value >0.05) [Table/Fig-2].

Age group (years)	Male	Female
<30	5	2
30-50	6	25
>50	10	10
Total	21	37

[Table/Fig-2]: Age and gender-wise distribution of IFA positive in study group.

In the present study, the most common nuclear pattern observed in the IFA-positive samples, including both the study and control groups, was nuclear homogeneous 34/71 (47.88%), followed by fine speckled (27/71, 38.02%). The remaining patterns observed are depicted in [Table/Fig-1]. Images of the IFA patterns observed in the current study are shown in [Table/Fig-3a-h].

According to the study protocol, all the samples from the study as well as control groups were tested using both IFA and LIA to detect antibodies against specific antigens for the diagnosis of autoimmune disorders (AD). In the study group, 54/118 (45.7%) of cases tested positive for antibodies by LIA, while 64/118 (54.2%) tested negative. Similarly, in the control group, 6/60 (10%) tested positive and 90% (54/60) tested negative for antibodies by LIA. These differences



[Table/Fig-3]: a-h) Patterns seen in IFA with positive control and negative control.

were found to be statistically significant with a p-value <0.05. When examining the antibodies against various antigens detected by LIA in the study and control groups, it was observed that the maximum antibodies were detected against the dsDNA antigen in both groups (23.4% in the study group and 27.27% in the control group). The other antibodies against various antigens detected by LIA are shown in [Table/Fig-4].

Antigens	Number of antibodies detected in study group	Number of antibodies detected in control group
RNP/SM	8	0
Sm	5	0
SS-A	16	2
Ro-52	12	2
SS-B	4	0
Scl	8	0
PM-Scl 100	2	0
Jo-1	2	0
CB	3	0
PCNA	4	2
dsDNA	24	3
NUC	4	0
HI	3	0
RIB	3	2
AMA-M2	0	0
Total	98	11

[Table/Fig-4]: Antibodies to various antigens detected in LIA in both study and control group.

In the study group, out of the 49% (58/118) of IFA-positive samples, 40.6% (48/118) were also positive for antibodies by LIA. Additionally, 5% (6/118) of cases that were negative by IFA were found to be positive by LIA [Table/Fig-5].

Parameters	IFA positive	IFA negative	Total
LIA positive	48	6	54
LIA negative	10	54	64
Total	58	60	118

[Table/Fig-5]: Comparative analysis of IFA and LIA in study group.
p-value <0.05: significant

In the control group, out of the 13/60 samples that were IFA-positive, 6.6% (4/60) were also positive for antibodies by LIA. Additionally, 3.3% (2/60) of cases that were negative by IFA were found to be positive by LIA. On statistical analysis, the observations of IFA and LIA results in the study group were found to be significant with a p-value <0.05.

Considering IFA as the gold standard, the Positive Predictive Value (PPV) of LIA in the study group was 88% and the Negative Predictive Value (NPV) was found to be 84%. The sensitivity and specificity of LIA in present study were found to be 82.7% and 90%, respectively. Similarly, in the control group, the PPV of LIA was 66.6% and the NPV was found to be 83.3%. The sensitivity and specificity of LIA in present study were found to be 30.7% and 95.7%, respectively. When studying the correlation between patterns in IFA and bands in LIA, it was observed that the nuclear homogeneous pattern was the most common ANA pattern and it showed an association with dsDNA, nucleosomes, and histones antigens either singly or in combination, along with SS-A/Ro-52, RIB, RNP/Sm, PCNA, CB, and SS-A in some cases [Table/Fig-6].

Patterns in IFA		Antibodies detected in LIA*
Nuclear	Homogenous	dsDNA, HI, NUC, PCNA, CB, Ro-52, SSA, RNP/Sm, Scl, Sm
	Fine speckled	SS-A, SS-B, RIB, RNP/SM, Sm, Ro-52, PCNA, JO-1, HI
Nucleolar		PM-Scl 100
Cytoplasmic	Fine speckled	Ro-52,RIB,Jo-1
Mixed	Nuclear and Cytoplasmic	HI, NU, dsDNA, RNP/SM, Sm, SS-A, Ro-52
	Nucleolar and Cytoplasmic	Scl, PM-Scl 100

[Table/Fig-6]: Correlation of pattern in IFA and antibodies detected in LIA.

Statistical analysis was performed to establish the correlation between the parameters using the Pearson's correlation 2-tailed test [Table/Fig-7]. It was observed that a positive correlation of strong strength was found between the nuclear dots pattern in IFA with the PM-Scl 100 band in LIA, and the nuclear and cytoplasmic mixed pattern with the HI band in LIA. Various other correlations were obtained and are shown in [Table/Fig-8]. In the present study, when comparing the distribution of autoimmune disorders (based on clinical presentation) in the study group with the results of IFA and LIA, it was observed that the most commonly diagnosed cases were for SLE (58.62%), followed by Sjogren's syndrome (18.96%) as shown in [Table/Fig-9].

DISCUSSION

An autoimmune disorder develops when our immune system detects our healthy cells as foreign and attacks them. The presence of ANA in the blood (serum) of patients is an indication of this diseased state of the immune system [15]. When studying the gender distribution of cases suspected of AD was studied, female preponderance of 60% was noted in both the study and control groups. It is well-known that many ADs preferentially affect women more than men. Possible factors contributing to this gender difference include hormonal differences and genetic factors [16,17,18]. In the present study, the majority of cases belonged to the age group of 31-50 years (69/118) in the study group and 37/60 in the control group. This observation was found to be statistically significant with a p-value<0.05. Predominant age groups have been observed in other studies [18-20].

In a study by Madhavi LB et al., maximum IFA positivity was observed in the age group of 31-50 years [20], and in a study by Sodani S et al., the most common age group for IFA positivity was 41-60 years (33.78%) [19]. In the current study, 49.15% of samples were found to be positive for IFA, while in the control group, 21.6% of samples were positive for IFA. Similar rates of IFA positivity have been observed in studies by Raman S et al., (35.02%), Sodani S et al., (43.08%), and Begum J et al., (36%) [21,19,22]. The result of IFA positivity will depend on the selection of autoimmune cases. When there is a strong clinical suspicion, the rate of positivity will be high.

Parameters	Nuclear homogenous	Nuclear fine speckled	Nuclear dots	Centrosomal	Cytoplasmic fine speckled	Mixed nuclear and cytoplasmic	Mixed nucleolar and cytoplasmic
RNP/SM	WC*	NC	NC	NC	NC	MC**	NC
Sm	NC	WC*	NC	NC	NC	MC**	NC
SS-A	WC	MC**	NC	NC	NC	WC**	NC
Ro-52	NC	WC	NC	NC	MC**	WC**	NC
SS-B	NC	WC*	NC	NC	NC	NC	NC
Scl	WC**	WC	NC	NC	NC	NC	NC
PM-Scl 100	NC	NC	SC**	NC	WC	NC	NC
Jo-1	NC	WC	NC	NC	SC**	NC	NC
CB	NC	NC	NC	NC	NC	NC	NC
PCNA	WC**	NC	NC	NC	NC	NC	NC
dsDNA	MC**	NC	NC	NC	NC	WC*	NC
NUC	WC	NC	NC	NC	NC	MC**	NC
HI	NC	NC	NC	NC	NC	SC**	NC
RIB	NC	WC	NC	NC	WC*	NC	NC
AMA-M2	NC	NC	NC	NC	NC	NC	NC

[Table/Fig-7]: Correlation of pattern in IFA and bands in LIA.
WC: Weak correlation; MC: Medium correlation; SC: Strong correlation; NC: No correlation; **: Correlation is significant at 0.01 level; *: Correlation is significant at 0.05 level

Positive correlation	Patterns observed in IFA	Antibodies in LIA
Strong strength	Nuclear dots	PM-Scl100
	Nuclear and Cytoplasmic mixed	HI
Medium strength	Nuclear homogeneous	dsDNA
	Nuclear fine speckled	SS-A
	Cytoplasmic fine speckled	RO-52
	Mixed nuclear and cytoplasmic	RNP/SM, Sm, NUC
Weak	Nuclear homogenous	RNP/SM, SS-A, Scl, PCNA, NUC
	Nuclear fine speckled	Sm, Ro-52, SS-B, Scl, Scl, Jo-1, RIB
	Cytoplasmic fine speckled	PM-Scl 100, Jo-1, RIB
	Mixed nuclear and cytoplasmic pattern	SS-A/Ro-52,dsDNA

[Table/Fig-8]: Correlation of pattern in IFA and bands in LIA.

Autoimmune disease	n (%)
SLE	34 (58.62%)
Sjogren's syndrome	11 (18.96%)
Systemic sclerosis	5 (8.62%)
Polymyositis	4 (6.89%)
Mixed connective tissue disorder	3 (5.17%)
CREST syndrome	1 (1.72%)
Total	58

[Table/Fig-9]: Autoimmune Diseases (AD) found in study group based on the results of IFA and LIA.
CREST: Calcinosis, Raynaud phenomenon, Esophageal dysmotility, Sclerodactyly, and Telangiectasia

In the present study, the nuclear patterns in IFA-positive samples showed that the nuclear homogeneous pattern (47.88%) was the most prominent. Similar results have been observed in various studies [5,23,24-26] conducted by other researchers. For example, Wendy S et al., found that the most common IFA pattern was nuclear homogeneous (45.5%), followed by fine speckled (35.6%) [27]. Sarojini R et al., also observed that the most common IFA pattern was nuclear homogeneous (52.6%), followed by fine speckled (40.3%) [21]. A comparison of the results of various studies with the present study is provided in [Table/Fig-10].

In the study group, 45.7% (54/118) of cases were positive for antibodies by LIA, while in the control group, 10% (6/60) of cases were positive for antibodies by LIA. Similar to the IFA results, there was also a female preponderance in the LIA-positive cases. When studying the antibodies against various antigens detected by LIA in the study and

Name of the author	Year of the study	Comparative results	Percentage positivity detected
Velammal P et al., [28]	2018	IFA - Positive LIA- Negative	27%
		IFA- Negative LIA- Positive	22%
Jabeen B et al., [29]	2018	IFA - Positive LIA- Negative	10%
		IFA- Negative LIA- Positive	6.6%
Present study	2019	IFA - Positive LIA- Negative	8.5%
		IFA- Negative LIA- Positive	5%

[Table/Fig-10]: Comparative results of various studies done.

control groups, it was observed that the maximum antibodies were detected against the dsDNA antigen in both the study group (23.4% i.e., 23/98) and the control group (27.27% i.e., 3/11).

In cases where IFA was negative and LIA was positive, the range of positivity was between 5-22%. In the present study, 8.5% of cases were IFA positive and LIA negative, and 5% of cases were IFA negative and LIA positive. This observation highlights the importance of considering the clinical presentation of an individual, as symptomatic cases in the study group increased the PPV and sensitivity of LIA in the diagnosis of AD. Because of high sensitivity of IFA is considered the gold standard for screening autoimmune disorders due to its high sensitivity [5,23,24]. Discrepancies between the results of IFA and LIA can be explained by the fact that the LIA kit used in the present study contains only 15 common antigens, and rare autoantibodies may be missed if the kit does not include specific antigenic substrates. Negative immunofluorescence in IFA of LIA-positive samples may be due to the low concentration of autoantigens [5]. The correlation between various patterns in IFA and antigens in LIA is shown in [Table/Fig-11]. These observations indicate that the screening test, IFA, can provide predictions for the antibodies against the detected antigens, but the accuracy of these predictions decreases as the correlation parameters move from medium correlation to no correlation.

Strength of association	Coefficient, r, Positive negative	
Weak	0.1 to 0.3	-0.1 to -0.3
Medium	0.3 to 0.5	-0.3 to -0.5
Strong	0.5 to 1.0	-0.5 to -1.0

[Table/Fig-11]: Interpretation of correlation factor for Table-7.

Limitation(s)

The LIA kit used in this study comprised only 15 specific antigens, thus not representing all the rare antigens that might have tested positive with IFA. This is one of the limitations of present study. The correlation between IFA patterns and LIA antibodies can be reanalysed in future studies after selecting LIA kits with a wider range of antigen bands. However, the correlation between the IFA patterns and the antibodies against antigens detected in LIA has not been statistically analysed in any of the published studies we reviewed. Therefore, the present study is exceptional in this regard, which is a positive point.

CONCLUSION(S)

Antibodies against nuclear antigens are a hallmark of autoimmune diseases (ADs). ANA IFA is considered the "gold standard" test for screening and detecting autoantibodies, which can be further confirmed using the LIA test. In the present study, it was observed that many cases were missed by IFA but detected by LIA. Therefore, a combination of IFA and LIA can serve as a better tool for the early and accurate diagnosis of AD. In the serum samples of healthy asymptomatic individuals, which constituted the control group of this study, a few samples were observed as positive for ANA using both IFA and/or LIA. Thus, there remains a possibility of such individuals developing ADs in the future. Counseling of such individuals is recommended for future follow-ups. As present study was hospital-based, it does not provide a true representation of the problem in the general population. Therefore, it is recommended to screen the general population with a larger sample size, using the current study as a baseline.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Microbiology, SGRR IM and HS, Dehradun, Uttrakhand, India.
2. Professor and Head, Department of Microbiology, SGRR IM and HS, Dehradun, Uttrakhand, India.
3. Assistant Professor, Department of Internal Medicine, GDMC, Dehradun, Uttrakhand, India.
4. Associate Professor, Department of Microbiology, SGRR IM and HS, Dehradun, Uttrakhand, India.
5. Senior Resident, Department of Pathology, VMMC and Safdarjung Hospital, New Delhi, India.
6. Ex-Professor and Head, Department of Microbiology, SGRR IM and HS, Dehradun, Uttrakhand, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Kxitiza Pandey,
505/440 East Patel Nagar-248001, Dehradun, Uttrakhand, India.
E-mail: kxitiza89@gmail.com

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