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Study on the Potential Utility of Phage Therapy and Herbal Extracts for Multidrug Resistant *Atopobium vaginae*

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ABSTRACT

Atopobium vaginae is a facultative anaerobic, Gram-positive bacterium, firstly considered as a normal saprophyte of the healthy vaginal flora but it is now recognized as one of the causal agents of Bacterial Vaginosis (BV). BV is a complex polymicrobial infection of the vagina that perturbs & shifts the vaginal flora from Lactobacilli to opportunistic pathogens that can include Atopobium vaginae. Symptoms of which are, greyish white discharge, pH greater than 4.5, etc. The infection is associated with adverse consequences including late miscarriage, prematurity in high-risk pregnancies and vaginitis in post pubertal women, chorioamnionitis, postpartum endometritis and an increased risk of HIV acquisition. The establishment of polymicrobial biofilm of A. vaginae resists repeated antibiotics & intravaginal antiseptic treatments resulting in its presence in 80-90% of cases of relapse and showing its relevance in BV recurrence after standard treatment with antibiotics. In the present study, isolation and identification of A. vaginae was carried out initially. VITEK 2 system was applied for identification. Demonstration of resistance to Atopobium was found resistant to Augmentin, Amoxicillin, Piperacillin, Ampicillin, Ampicillin/ Sulbactam, Cefotaxime, Ceftriaxone and Furazolidone. which is a global health issue that directs the exploration and attention towards other alternative treatments, viz, plant-derived compounds and phage therapy. This study shows favourable results for the Azadirachta indica extracts, Eucalyptus leaves extracts and for phage therapy of A. vaginae infections. With its host-specific and easy-to-handle advantage, it can emerge as one of the promising therapies in the future to treat infections to overcome bacterial resistance.

Key words: *Atopobium vaginae, bacterial vaginosis, antibiotic resistance, phage therapy, plant-derived compounds.*

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INTRODUCTION

Atopobium vaginae is a facultative anaerobic, Gram-positive bacterium, firstly considered as a normal saprophyte of the healthy vaginal flora but it is now recognized as one of the causal agents of Bacterial Vaginosis (BV) [1] BV is a complex polymicrobial infection of the vagina that shifts the vaginal flora from Lactobacilli to opportunistic pathogens that can include *Atopobium vaginae*. [1,2] Symptoms of which such as greyish white discharge, pH greater than 5, Lecorrhoea., associated with adverse consequences including late miscarriage, prematurity in high-risk pregnancies, vaginitis in post pubertal women, chorioamnionitis, postpartum endometritis and an increased risk of HIV acquisition [1,2,3]

Polymicrobial biofilm of *A. vaginae* resists repeated antibiotics & intravaginal antiseptic treatments. Presence in 80-90% of cases of relapse even after standard treatment with antibiotics [1] High concentration of *A. vaginae* have been recognized as important microbiological markers in detection of infection [4] Multidrug resistance is a global health issue that directs the exploration and attention towards other alternative treatments, viz, antiseptics, probiotics, plant-derived compounds, phage therapy as well as different combination therapies [4][12]

Plant extracts of *Eucalyptus* leaves contain citronellal, isopulegol, and citronellol as the primary constituents that show the antimicrobial activity of aromadendrene. This compound has a reactive exocyclic methylene group and a cyclopropane ring which can alkylate proteins and thereby disturb the conformation of proteins. The compound is highly lipophilic, it may disrupt the fluidity and permeability of Biomembranes. [5] Azadirachta *indica* (neem) shows therapeutic role in health management due to its rich source of various types of ingredients. Leaves contain ingredients such as Nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and amino acid, 7-desacetyl-7-benzoylazadiradione, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione, and nimbiol. [6]

MATERIAL AND METHODS

Isolation and Identification of *Atopobium vaginae*

Isolation of Atopobium vaginae was carried out from the vaginal secretions of patient suffering from leucorrhoea. The bacterial isolate was characterized by Gram's staining, morphological characters and genus specific biochemical tests mentioned in Bergey's Manual of Systematic Bacteriology. [1,3,7,8] The isolate was also verified using automated ® VITEK 2 Compact system.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC was determined for the antibacterial most efficient extracts, using the method of measuring the turbidity in the test tube (CLSI, 2016). Eight sterile test tubes were arranged for each extract in a row. Each potential extract was determined by micro-broth dilution technique. 1 ml of sterile nutrient broth was pipetted into all the tubes. Stock of plant extract of concentration 500 mg/ml was prepared using sterile D/W. Thereafter, 1ml was used for serial dilution of the extract in each tube to obtain concentrations of 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, and 1.95 mg/ml, respectively. One hundred microliters of 10^6 CFU/ml of *A. vaginae* was pipette into each test tube and incubated at 28 °C for 24 hr. Two control tubes were used: nutrient broth inoculated with bacteria was used as a positive control and nutrient broth uninoculated was used as a negative control. The lowest concentration that kills the organisms completely, where no bacterial growth is observed (MBC), was determined by assaying the test tubes resulting from MIC determinations. 20 microliters of the content of each test tube were inoculated by spreading on a solidified nutrient agar plate and then incubated at 28 °C for 24 hrs and observed for bacterial growth. [6,11]

Antibiotic Sensitivity Test

The antimicrobial susceptibility test of the isolate was performed. St. Muller-Hinton agar plates were inoculated by maintaining turbidity of 0.5 McFarland standard of the isolate, and antibiotic discs for gram positive bacteria obtained from HI Media Laboratories (Mumbai, India) were placed on the plates as per the guidelines recommended by the Clinical and Laboratory Standards Institute.[8]

Isolation of Bacteriophage

Bacteriophage can be isolated from environment and can be tested against various bacteria as they possess desirable characteristics hence can be used in treatments. [9,10] Media used was TPA, TPB and NB. TPA-(Tryptone Phosphate agar) soft agar-Tryptone -20gm/L, Nacl-5gm/L, Glucose -2gm/L, Na_2HPO_4-2.5gm/L, Agar-1.5%. TPB--(Tryptone Phosphate broth) Tryptone -20gm/L, Nacl-5gm/L, Glucose -2gm/L, Na_2HPO_4-2.5gm/L. NB-(Nutrient Broth)-Peptone -1gm, Meat extract-0.3gm, Nacl-0.5gm, DW-100ml

Procedure for isolation of bacteriophage: Samples were collected from hospital drainage water and sewer water. Processing of the wastewater samples was carried out by homogenization for 2 hours and centrifuged at 3000 rpm for 20 minutes. Supernatant was collected and centrifuged at 5000 rpm for 20 minutes. (In cooling centrifuge). Supernatant was filtered through 0.45-micron millipore syringe filters and the filtrate is then tested for the presence of lytic activity against *Atopobium*. The methods used were Turbidity reduction method [8,9,10] 1mL of chloroform was added in 5mL of processed sewage sample, mixed properly and centrifuged at 3000 rpm for 20 minutes. 1mL supernatant is transferred in 5mL of broth culture of bacteria. The second method was Double agar plate method [6,7,9] where processed samples were inoculated over the surface of nutrient agar plate. Next day the plates were examined for the presence of plaques and lytic activity on the bacterial lawn.

Observations of Clear Plaque Morphology for Phage Lysate Preparation by DAL Method

Where procedure is 500mL of filtrate + 500mL of 6 hours old bacterial culture + 2.4 g/LL MgCl2 solution to enhance the adsorption of plaque over bacterial surface. This was kept on a shaker at a gentle speed for 20 minutes and then added to a test tube containing 2.5 ml of the molten soft agar. It was placed at 45° in a water bath. Mixture was swirled, poured into lactose agar basal plates, allowed to solidify and then incubated at 37°C for 48 hours. The plates were observed at the intervals of 6,12,24 and 48 hours for development of plaques.

Tests to Check Effectiveness of Plant Derived Compounds (Herbal Extracts)

Plant Collection

All the selected plant leaves (Eucalyptus leaves and *Azadirachta indica leaves)* were collected. Plant materials were washed under running tap water and completely air dried for 5 days and then homogenized to fine powder.

Powder Formation

The dried plant leaves were ground finely in a grinder to obtain a homogenous texture.

Extraction using Organic Solvent

Successive extraction of these powders (05 gm) was done with the help of Soxhlet apparatus in ethanol as an organic solvent. [5,6,11] The powdered plant material was extracted with the appropriate volume

(250ml) of solvent at 78°C for 3hrs. The final extract was concentrated by distilling off the solvent by a rotary evaporator. It was stored at 4°C in airtight bottles for further use.

- **Stock Preparation**

Finally, 1 gm of stored plant extracts were dissolved in 1ml sterile distilled water to make the stock for antimicrobial assay. [5,11]

Antibacterial Assay

It was carried out by agar well diffusion method as per CLSI guidelines. One hundred microliters (10^6 CFU/ml or 0.5 McFarland Standard) fresh microbial culture was spread on a nutrient agar plate with non-toxic swab. Four wells of 6-mm diameter were punched off into the agar medium with sterile cork-borer (6 mm) and filled with 40 µl (250 mg/ml) of plant extract by using a micropipette in each well under aseptic conditions. Sterile D/W was used as a negative control. The plates were allowed to stand for 1 hr to allow for pre-diffusion of the extract into the medium. The plates were incubated anaerobically in an upright position at 28 ± 2 °C for 24–48 hrs.

The antibacterial screening was evaluated by measuring the zone of inhibition (mm) [6,11]

RESULTS AND DISCUSSION

1. Isolation of *Atopobium vaginae*

2. Isolation of *Atopobium vaginae* is carried out on MRS medium and on blood agar plates. They are anaerobic, gram-positive rods and positive for acid phosphatase. Their identification was carried out by using VITEK 2

Table:1 Lab. Report of VITEK 2 for Identification of *Atopobium vaginae*

Fig: 1 Antibiotic Sensitivity Tests of *Atopobium vaginae*

Table: 2 Resistance by *Atopobium vaginae* to various antibiotics

where *Atopobium* was found resistant to Augmentin, Amoxicillin, Piperacillin, Ampicillin, Ampicillin/ Sulbactam, Cefotaxime, Ceftriaxone and Furazolidone.

3. Isolation of Bacteriophage for *Atopobium*

Clear plaques were observed on the lawn of *Atopobium.*

Fig:2 Bacteriophage isolation and enumeration

4. Effect of *Azadirachta indica and Eucalyptus* extract on Atopobium

With the use of standard McFarland (0.5) of isolate, Zone of Inhibition was observed against *Atopobium* by using the spread plate technique and was measured for *Eucalyptus* - 3.5cm and for *Azadirachta indica* (Neem)- 3.1cm

Fig: 3. Effect of Azadirachta *indica and Eucalyptus* extract on *Atopobium*

5. MIC and MBC of *Azadirachta indica and Eucalyptus against Atopobium*

The MIC and MBC of *Azadirachta indica* leaves extract against A. vaginae was found to be 125mg/ml and 250mg/ml respectively. The MIC and MBC of *Eucalyptus* leaves extract against A. vaginae was found to be 62.5mg/ml and 125 mg/ml respectively.

CONCLUSION

Atopobium exhibited high to moderate levels of resistance against different classes of antibiotics. The susceptibility data from this study may be worth consideration while implementing empiric treatment strategies. By knowing the antibiogram of A*. vaginae* it might be possible to develop new regimens for the treatment of recurrent bacteria.

Application of phage therapy may emerge as a rescuer to the accelerating crises of antibiotic resistance and prove as asset for bio-controlling of pathogenic agents in medical sciences, after human trials by FDA authorities similarly herbal extracts of *Eucalyptus* and *Azadirachta indica* can be used as an effective alternative for BV treatment. Eucalyptus leaves extract is found more effective than neem leaves against A. vaginae.

ACKNOWLEDGEMENTS

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Table:1 Lab. Report of VITEK 2 for identification of Atopobium vaginae

NCMR,NCCS, Pune Maharashtra(India)

bioMérieux Customer:
System #: VK2C18111

Laboratory Report

Printed Feb 26 2022 10:06 IST
Printed by: Amaraja
Report Version: 3 of 3

Isolate: G FEB 22 150-1 (To be reviewed)
Last Updated Feb 26 2022 10:06 IST By: Amaraja
Card Type: ANC Bar Code: 2441236203507015 Testing Instrument: 000019E1E395 (18111)
Setup Technologist: Dr.Amaraja Joshi(Amaraja)

Bionumber: 2307500610001
Organism Quantity: **Selected Organism: Atopobium vaginae**

Comments:	

McFarland: 3.30 (2.70 - 3.30)

Identification Information	**Card:**	ANC	**Lot Number:**	2441236203	**Expires:** Apr 15, 2022 12:00 IST
	Completed:	Feb 25 2022 22:19 IST	**Status:**	Final	**Analysis Time:** 5.82 hours
Organism Origin	VITEK 2				
Selected Organism	95% Probability		**Atopobium vaginae**		
	Bionumber: 2307500610001				**Confidence:** Very good identification
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					
Atopobium vaginae	SAC(7),PVATE(20),				

Biochemical Details

4	dGAL	-	5	LeuA	+	6	ELLM	-	7	PheA	+	8	ProA	+	10	PyrA	-
11	dCEL	-	13	TyrA	-	15	APPA	-	18	dGLU	+	20	dMNE	+	22	dMAL	+
28	SAC	+	30	ARB	-	33	NAG	+	34	BGLUi	-	36	URE	-	37	BGURi	-
39	BGALi	-	41	AARA	-	42	AGALi	-	43	BMAN	-	44	ARG	+	45	PVATE	+
51	MTE	+	53	ESC	-	54	BdFUC	-	55	BNAGi	-	56	AMANi	-	57	AIFUC	-
59	PHOS	-	60	IARA	-	61	dRIB2	-	62	OPS	-	63	AARAF	-	64	dXYL	-
	GRAM	+		MORPH	-		AERO	-									

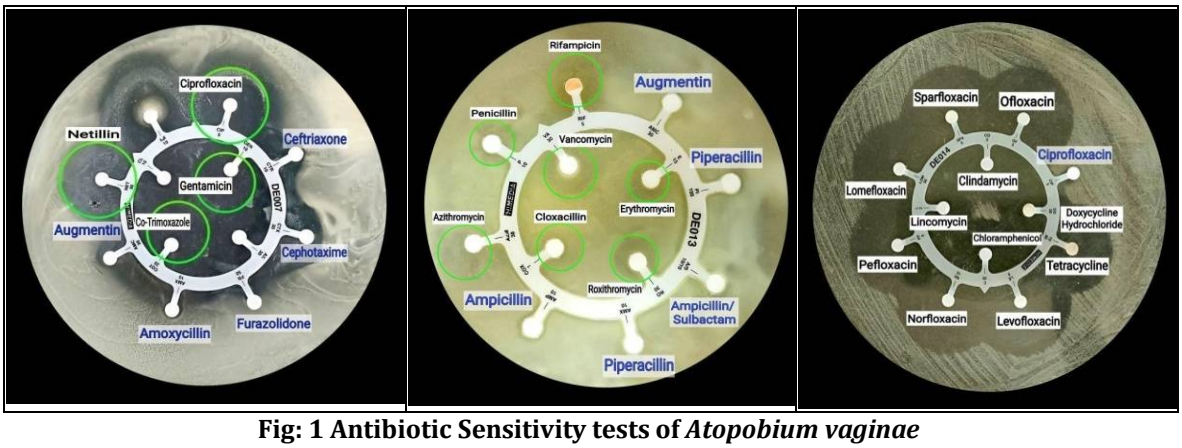

Fig: 1 Antibiotic Sensitivity tests of *Atopobium vaginae*

The result obtained are represented in the table 2

Table: 2 Resistance by Atopobium vaginae to Various Antibiotics

RESISTANT	Symbols	Concentration mcg	SENSITIVE	Symbols	Concentration mcg	Zones of inhibition (cm)
Augmentin	AMC	30	Tetracycline	TE	30	2.9
Amoxicillin	AMX	10	Vancomycin	VA	30	2.0
Piperacillin	PI	100	Penicillin	P	10	1.8
Ampicillin	AMP	10	Azithromycin	AZM	30	2.1
Ampicillin/ Sulbactam	A/S	10	Erythromycin	E	15	1.6
Cefotaxime	CTX	30	Cloxacillin	COX	1	1.7
Ceftriaxone	CTR	10	Roxithromycin	RO	30	1.7
Furazolidone	FR	50	Lomefloxacin	LOM	5	3.5
			Clindamycin	CD	2	3.0
			Gentamicin	GEN	10	3.0
			Chloramphenicol	C	30	2.8
			Rifampicin	RIF	5	1.9
			Netillin	NET	30	3.1
			Sparfloxacin	SPX	5	3.2

Where resistance = the zone of inhibition is measured as less than 0.3cm.

Isolation of Bacteriophage for Atopobium: Clear plaques were observed on the lawn of Atopobium.

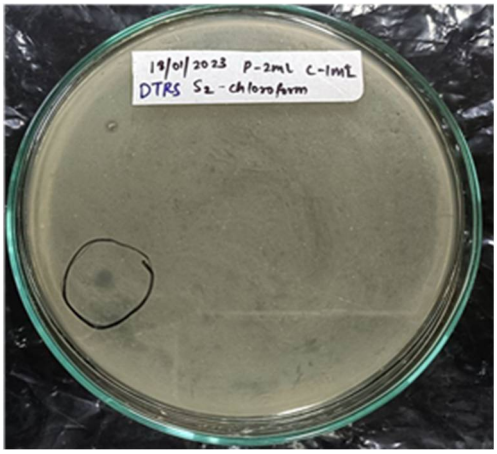

Fig: 2 Bacteriophage isolation and enumeration

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