

### 3

## REPRODUCTIVE TRACT INFECTIONS

RTIs including STIs and HIV/AIDS are being increasingly recognised as a serious public health problem. RTIs cause suffering for both men and women, but their consequences are far more devastating and widespread among women. Amongst women, RTIs often go undiagnosed and untreated, and when left untreated, they lead to complications such as infertility, pelvic inflammatory disease, ectopic pregnancy, miscarriage, cervical cancer, and an increased risk of HIV transmission. Adolescents too are vulnerable to RTIs due to their ignorance of risk factors, inadequate accessibility to services, and social powerlessness.

A number of studies have been initiated to cover epidemiological, clinical and diagnostic dimensions of RTIs: (i) Estimation of population based information on prevalence of RTIs; (ii) Understanding the mechanism of HIV transmission; (iii) Development and assessment of newer microbicides, including clinical evaluation, which may also be spermicidal.

### 3.1 Association of *Chlamydia trachomatis* Infection and Host Genetic Factors in Manifestation of Reproductive Complications

Principal Investigator: **Jayanti Mania-Pramanik**  
Project Associates: Shilpa Kerkar, Shobha Potdar, Pratibha Mehta  
Project Collaborators: Vinita Salvi, Seth G.S. Medical College and KEM Hospital, Parel, Mumbai  
Duration: 2003-2006

Host genetic diversity i.e., HLA polymorphism is believed to contribute to the spectrum of clinical outcomes in different infections. Allelic variants of the HLA molecules bind and display various self-derived/infection derived peptides with differing affinities to Cytotoxic T Lymphocytes (CTLs), thereby influencing the ultimate immune response. Worldwide, very few reports are available on the role of HLA on reproductive tract infections in the manifestations of reproductive complications. In India we did not have any report on this area of research. The significant observation of our study on *Chlamydia trachomatis* infection was the absence of signs and symptoms of this infection in subjects, though they have different clinical manifestations like infertility and repeated pregnancy loss. Literature also suggested that different serotypes of *C. trachomatis* do not show any relationship on disease manifestations but host immunogenetics might have a role. Hence, our objectives are (i) to establish the association of human leucocytes antigens (HLA class I and class II alleles) and Chlamydia infection in cases with different clinical

manifestations, and (ii) to evaluate the influence of HLA with recurrent spontaneous abortions and infertility.

To study the influence of host genetic diversity, molecular typing of human leucocyte antigen (HLA) has been standardized using high resolution polymerase chain reaction followed by sequence specific oligonucleotide hybridization (PCR/SSOP, Figs. 82, 83).

During the year, 108 subjects were enrolled for the study. Among them 30 were infertile, 23 were with recurrent spontaneous abortion, 25 women had signs and symptoms of lower genital tract infections, 2 did not have any clinical history or present signs and symptom. Twenty-eight women were pregnant of which 5 had current *C. trachomatis* infection (4.63%), while 14.8 percent had antibody against *C. trachomatis*. Other infections like human papilloma virus (8/108, 7.41%), Candida (13/108, 12.04%) and Bacterial vaginosis (13/108, 12.04%) were also detected in these study subjects.

To identify HLA polymorphisms of the community and for purposes of comparison, HLA Class I (A & B) alleles and HLA Class II (DRB1 & DQB1) was analyzed in 9 healthy women, who did not have any history or present signs and symptoms of any systemic diseases. Similarly 23 women with history of recurrent spontaneous abortion and 10 women with infertility were analyzed for HLA Class I and Class II antigens. Phenotype frequency, odd ratio or relative risk factor, etiological factor as well as protective factors were calculated for these alleles in their low resolution form. The expressed alleles were also compared with our own healthy controls and those reported of the same ethnic group. Some alleles have been identified to play a role and will be confirmed in larger samples.

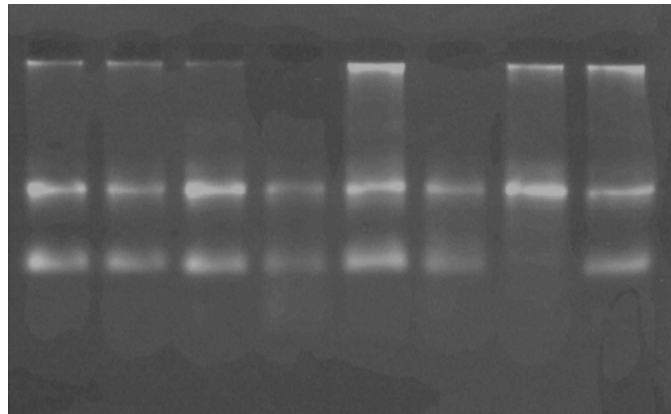


Fig. 82: HLA analysis: Amplification product of PCR.

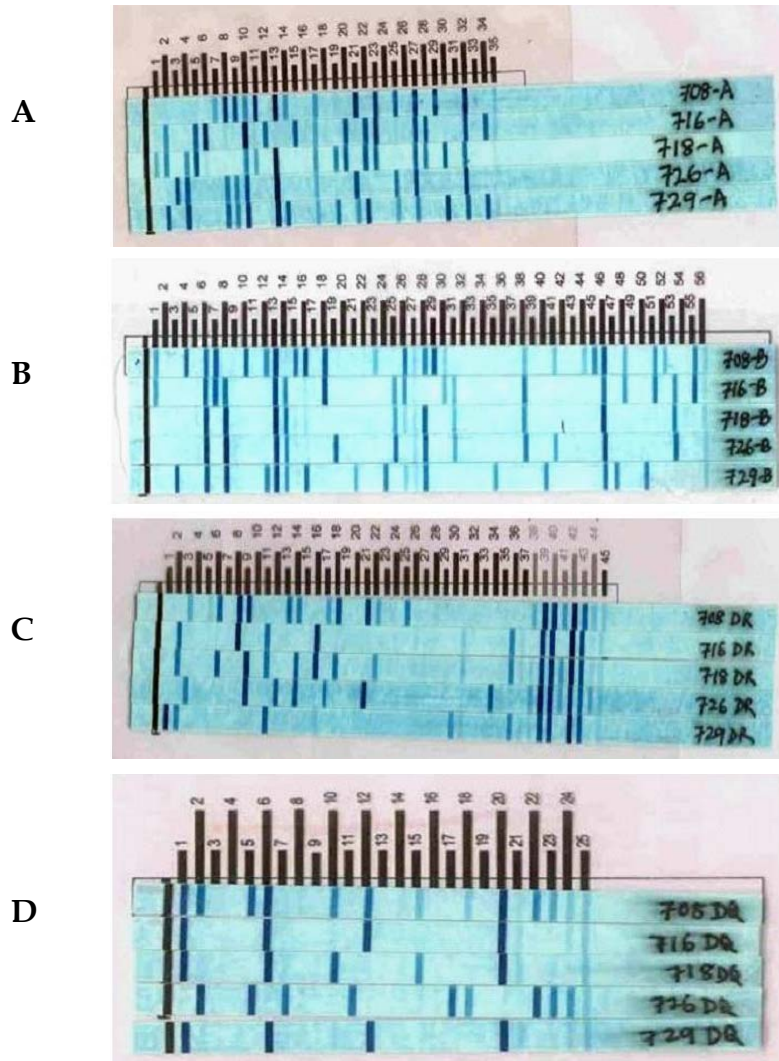


Fig. 83: Hybridization product: (A) HLA-A overlay; (B) HLA-B overlay; (C) HLA-DRB1 overlay; (D) HLA-DQB1 overlay.

### 3.2 Reproductive Tract Infections: Clinical and Microbiological Study in Women *(Partly funded by WHO Budget)*

Principal Investigator: **Kamal Hazari**

Project Associates: Shanta Chitlange, Shubhangi Kalgutkar, Lalita Savardekar, B.N.Mali, Vrushali Palayekar, D. Balaiah, S. Shaikh, Vidya Shenoi, Pramodini Phatak, Dipika Belekar, Rachana Dalvi, A.M. Chandorkar, P. Khopkar, A. Hankare

Duration: 2002-2005

Reproductive Tract Infections (RTIs), especially sexually transmitted infections (STIs) cause a wide spectrum of pathology in women, which includes vaginitis, cervicitis, endometritis, salpingitis, pelvic inflammatory disease (PID), ectopic pregnancy (EP), infertility and also prematurity, stillbirth, conjunctivitis and pneumonia in the neonates. RTIs are a serious concern in the era of HIV since even the non ulcerative STIs increase the risk of HIV transmission by 3-5 folds. It is the burden of asymptomatic disease that is responsible for the frequent and severe or long term morbidity (PID/EP/infertility) and in part for the persistence and spread of STIs in the communities.

The objectives of the study are: (i) to evaluate the relationship between clinical manifestations and microbiological diagnosis of common RTIs (bacterial vaginosis, candidiasis, trichomoniasis and chlamydia trachomatis) in women at low risk for RTIs; and (ii) to evaluate the therapeutic response as assessed by clinical and microbiological tests.

Women (n=682) attending the 3 NIRRH family welfare clinics were enrolled for the study. Pregnant women (1), those with concurrent systemic diseases (2) or receiving immunosuppressants or antibiotics (12), known allergy to medicines (1), vaginal bleeding (3) and who were not willing to come for the follow up visits (40) were excluded. In all, 742 women were screened and 60 excluded for the above reasons.

At initial enrollment visit, detailed history of symptoms and signs were recorded, gynaecological examination carried out and the vaginal/cervical samples obtained for wet vaginal microscopy, vaginal pH, Papanicolaou smear and immunofluorescence. Women were treated based on the clinical and laboratory findings. Male partners were also given treatment. The women were followed at 1½ and 3 months after initial visit.

Based on symptoms, signs, bedside tests and laboratory investigations women were grouped as: Group A: These women received treatment at initial visit (definite symptoms, signs and positive bed side tests); Group B: These women received treatment at revisit if any of the test results are positive; and Group C: Control group, with no signs, no symptoms and no positive test results for these infections.

The women in this study, were from a lower middle socio-economic background, married, parous and majority were housewives. These women were at low risk of RTIs since majority of them, 97.7 percent and 95.9 percent of their husbands had single partner relationships. Majority of the women (76%) reported no history of RTIs in the last 12 months.

The prevalence rates were 12.6 percent for bacterial vaginosis, 17 percent for candidiasis, 15 percent for chlamydia trachomatis and 0.2 percent for trichomonas vaginalis. The cure rates after specific treatment were more than 85 percent.

### **3.3 Purification and Characterization of CD4 Independent 160kDa Sperm Receptor for HIV**

Principal Investigator: **A.H. Bandivdekar**

Project Associates: Vijaya Raghavan, Shilpa Velhal,  
Jacinta Pereira, S.D. Rawool  
and R.B. Kadam

Duration: 2002-2006

Human Immunodeficiency Virus (HIV) has been demonstrated to bind and enter into the spermatozoa. HIV infected spermatozoa has been also shown to facilitate the transmission of HIV into urogenital cell as well as the oocyte. However, spermatozoa have been reported to be devoid of the conventional CD4 receptor suggesting the existence of alternate receptors for HIV on the sperm surface. In an attempt to identify the HIV receptor on spermatozoa, a 160kDa HIV binding protein has been identified by Western blot technique using both cell free HIV as well as gp120 HIV envelope glycoprotein (Annual Report 2002-03, p 82). The protein has been further characterized to establish its specificity.

#### **160kDa is different from CD4 receptor**

160kDa protein has been demonstrated to be different from CD4 receptor, as the Western blot analysis of sperm extract using monoclonal antibodies to CD4 and alkaline phosphatase (AP) labelled anti-mouse IgG did not show any reactivity with the protein bands on nitro cellulose (NC) membrane, suggesting

absence of CD4 receptors in sperm extracts. Further it was also observed that pre-incubation of NC membrane with anti-CD4 antibody prior to incubation with gp120 followed by visualization with AP labeled anti-gp120 antibody showed a positive band at 160kDa thereby suggesting that preincubation with anti-CD4 antibodies did not block the reactivity of gp120 to the 160kDa protein band. Moreover, the molecular size of the CD4 receptor is 55kDa, further confirming that the 160kDa protein is independent of CD4 receptors.

### **160kDa is not a glycolipid**

Presence of gp120 binding glycolipid molecule, Galactosyl-alkyl-acylglycero (GalAAG) on human sperm has been proposed as an alternate receptor for HIV. To verify the association of 160kDa protein if any with glycolipids, dot blot analysis of both aqueous and organic phases of sperm extract obtained by fractionation with chloroform/methanol and subsequently with NaCl was carried out. The positive reaction with gp120 was observed only with the aqueous phase and not with organic phase. Western blot analysis of the proteins from aqueous phase also showed reactivity of gp 120 only with the 160kDa protein band indicating that the 160kDa HIV reacting protein is not a glycolipid.

### **Binding inhibition of I<sup>125</sup> labeled gp120 to 160kDa protein**

Receptor binding specificity was also verified by binding inhibition of I<sup>125</sup> labeled gp120 to partially purified 160kDa protein spotted onto a NC membrane. The binding of I<sup>125</sup>-gp120 was displaced in a dose dependent manner by gp120.

### **Partial Purification of 160kDa Protein**

The protein was then partially purified from human sperm extract by ion exchange chromatography on Mono Q column. The sperm from normal semen samples were separated by swim up and the proteins from pooled sperm samples were solubilized using Triton X-100 as described above. The sperm proteins were then fractionated by ion exchange chromatography on FPLC using a mono Q column HR 10/10. Fig 84 shows the chromatographic separation of the sperm proteins. From six hundred human semen samples approximately about 10 mg of partially purified protein could be obtained. To isolate sufficient amount of homogeneous 160kDa protein, atleast 50 mg of partially purified protein will be further fractionated by chromatofocusing technique.

### **Differential Expression of 160kDa Protein on Sperm**

Sperm from normal semen samples were separated by centrifugation and proteins were solubilized from an aliquot of 50 million sperm with 1 percent Triton X-100 as described above. A 50 µg aliquot of sperm proteins from each

sample as resolved by SDS-PAGE under non reducing conditions and presence of the 160kDa protein band observed in two of the four samples (Fig. 85, Lane 3 and 4). Western blot analysis using gp120 and anti-gp120 antibodies showed reactivity with 160kDa band in the samples showing its presence as detected by SDS-PAGE (Fig. 85, Lane 3 and 4) while the samples devoid of 160kDa band in SDS-PAGE did not show reactivity with any of the protein bands (Fig. 85, Lane 1 and 2).

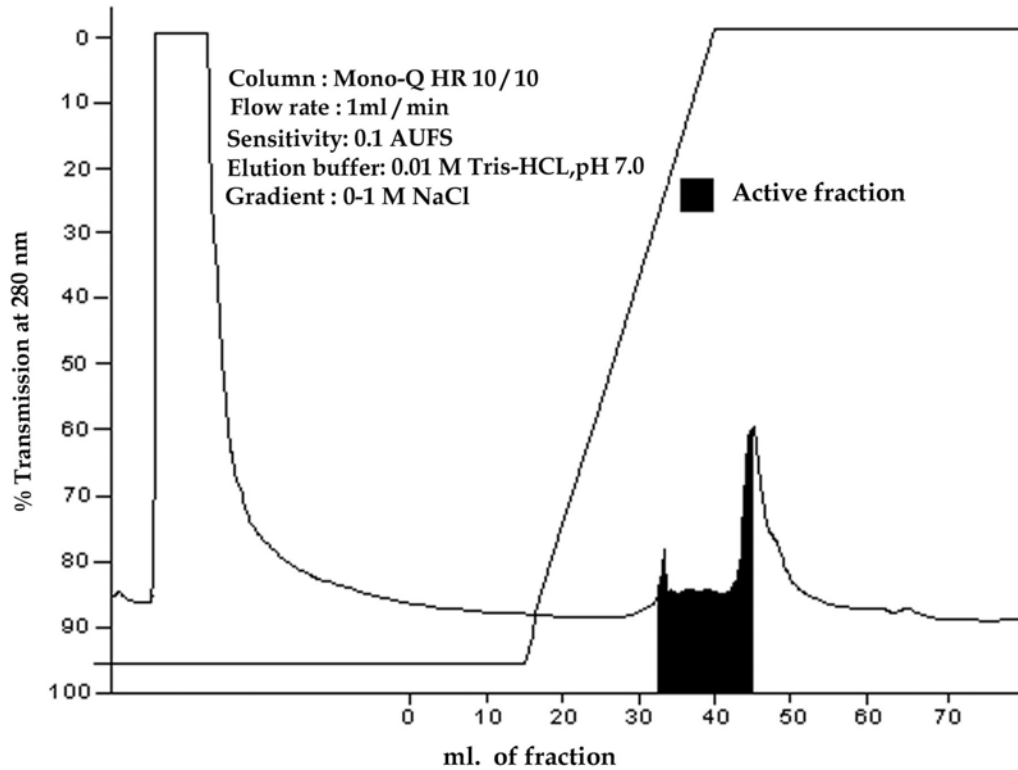


Fig. 84: Partial purification of 160kDa HIV binding protein by ion-exchange chromatography

The reactivity with gp120 was also investigated by incubating an aliquot of 50 million sperms with gp120 and then sequentially with anti-gp120 antibodies and FITC labeled ARGG. The flow cytometric analysis of these samples showed the gp120 reacting peak with higher fluorescence in the case of 160kDa positive samples (Fig. 86, Samples 3 and 4) as compared to that of negative samples (Fig. 86, Samples 1 and 2). Although these preliminary results demonstrate the involvement of 160kDa receptor protein in binding of gp120, it needs to be confirmed using a larger sample size and further substantiated by electron microscopy to demonstrate the involvement of 160kDa protein in HIV binding.

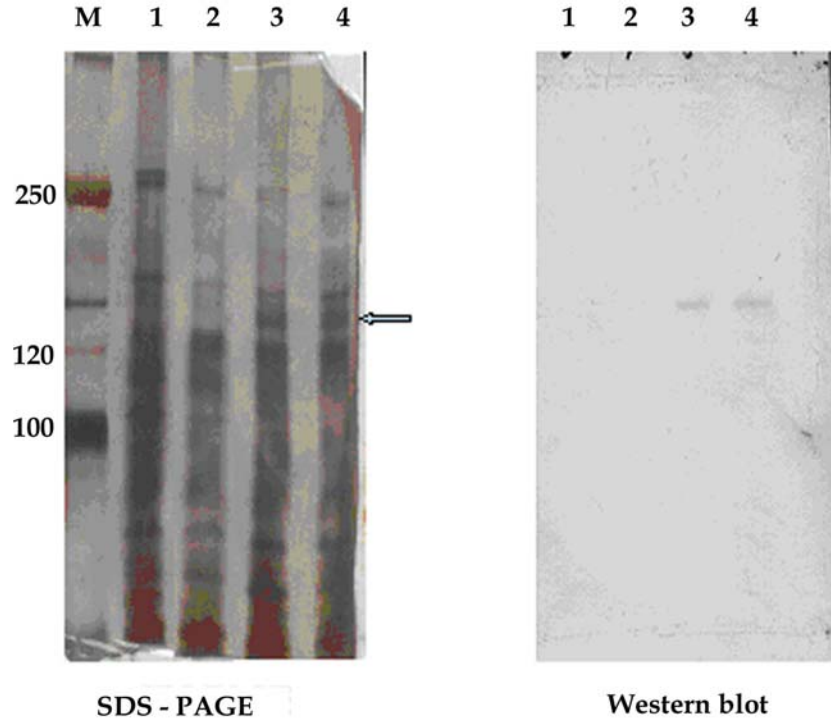


Fig. 85: Differential expression of 160kDa protein on sperm.

### Isolation and Sequencing of cDNA Encoding 160kDa Protein

A human testicular  $\lambda$ ZAP cDNA expression library was immunoscreened using gp120, anti-gp120 antibody and AP labelled ARGG. The positive clones were then isolated and PCR amplified using T3 and T7 primers. The insert size was estimated to be of 1 kb. This was then sequenced using T3 and T7 primers. A partial cDNA sequence has been obtained. Efforts are being made to obtain the full length sequence.

Presence of CD4 independent 165kDa HIV receptor protein on astrocytes has been identified which showed sequence homology with human mannose receptor (hMR) protein. Identity of 160kDa sperm protein and hMR needs to be investigated.

In our attempt to isolate and sequence the full length cDNA encoding 160kDa protein we encountered difficulties in identifying the cDNA due to very weak reaction. In addition, sequence of these positive clones were found to be associated with partial sequence of host genome. This may be due to inadvertent recombination between the cDNA sequence of the protein and the genome of *E. coli* used as a host cell for cloning and plasmid preparation. Similar difficulties have also been reported in full length sequencing of hMR. Use of appropriate competent cells such as STBL has been reported to be suitable to obtain full

length sequence. Hence the full length cDNA sequence will be done using appropriate strategies and also will be checked for the homogeneity of 160kDa protein with hMR.

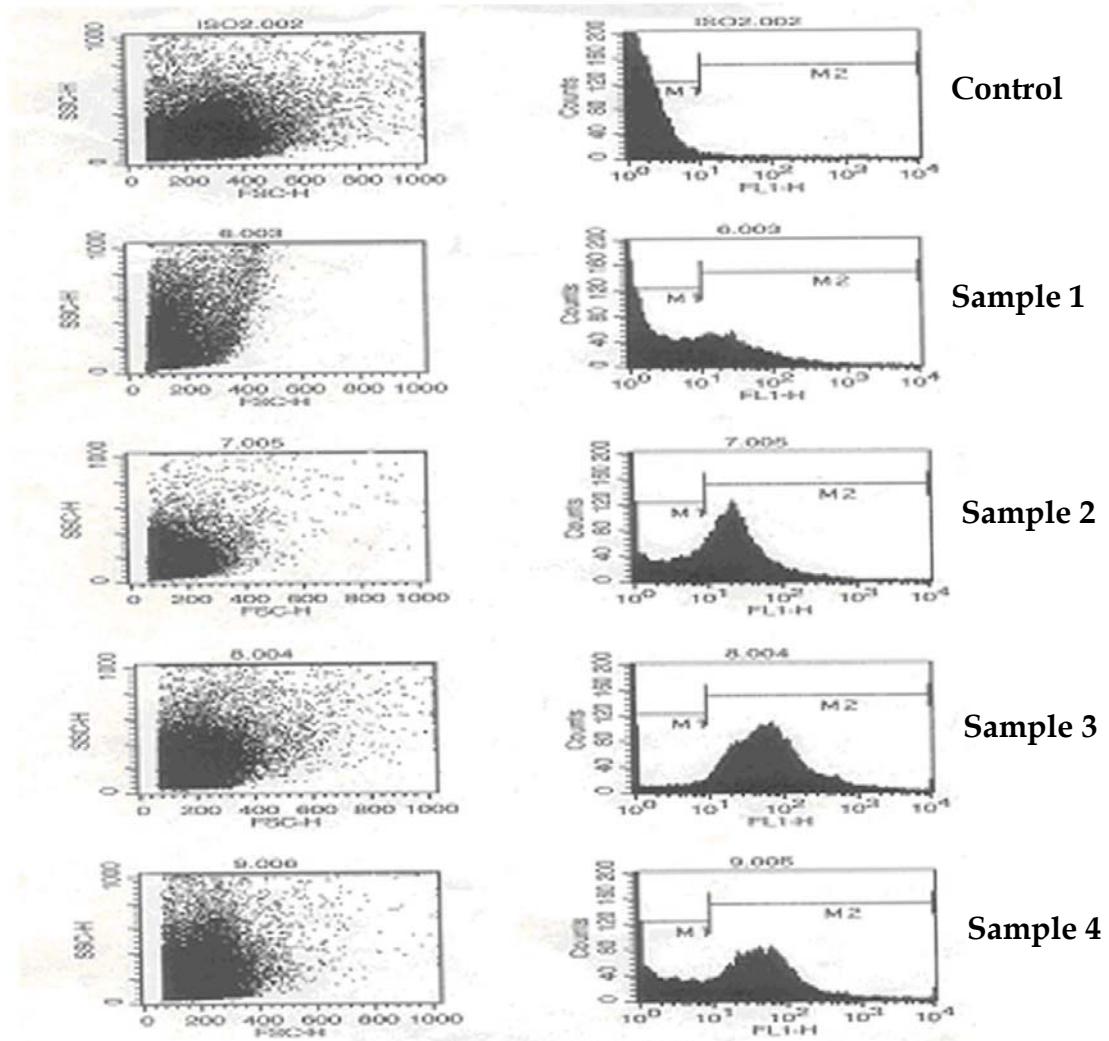


Fig. 86: Differential expression of 160kDa protein by flow cytometric analysis.

### **3.4 Nisin: The Antimicrobial Peptide for the Control of Fertility and Sexually Transmitted Infections** (*Funded by Indian Council of Medical Research*)

Principal Investigator: **K.V.R. Reddy**  
Project Associates: Clara Aranha and Sadhana Gupta  
Collaborators: Sujatha Baveja, Seth G.S. Medical College and KEM Hospital, Parel, Mumbai  
Duration: 2001-2006

Reproductive Tract Infections (RTIs) including Human Immuno Deficiency Virus (HIV) continue to be a serious public health problem worldwide. There exists an urgent need for the development of safe and effective vaginal microbicides that can reduce the transmission of these infections. The objective of this study is to develop dual protective vaginal products having antimicrobial and spermicidal properties.

Nisin, a 34 amino acid peptide (Mol. Wt. 3.4kDa), proven safe food preservative was chosen for this study. In the previous year we have reported that Nisin possesses antibacterial and spermicidal activities and appears to be safe for vaginal use (Annual Report 2003-04, p 117).

#### **Cytokine levels in the vaginal fluid of rabbits**

In continuation of our earlier studies, during the year, studies were initiated to evaluate mild inflammatory reactions if any, after repeated intravaginal administration of high dose of Nisin (50 mg/day/14 consecutive days). Pro-cytokines (IL-1, IL-2, TNF- $\alpha$ , GMSF, IL-8 and IL-10), the markers of tissue inflammation were determined in the vaginal fluid by ELISA (Fig. 87) and in the vaginal tissue by immunohistochemistry. The results indicated no treatment associated increase or decrease in any of these cytokines in the vaginal fluid and in all three regions of the vaginal tissue (ectocervical, endocervical and vaginal epithelium), compared to their respective controls. This confirms our earlier studies on the safety of Nisin in rabbits (Annual Report 2003-04, p 117).

#### **Effect of pH on Nisin solubility**

In the previous year, several Nisin gel formulations using various gelling agents and their combinations have been prepared and tested for their contraceptive efficacy in rabbits (Annual Report, 2003-04, p 117). Of the several gels prepared, it was observed that, out of ten animals administered with 5mg Nisin in 0.5 percent carbopol, eight animals conceived. This could be due to delay in the release of Nisin from the gel formulation. Therefore, during the year,

studies were extended to develop improved gel formulations with faster release of Nisin. Buffers with varying pH (2, 3, 4, 5, 6, 7 and 8) were prepared and a known concentration of Nisin was added to each buffer. The effect of Nisin at different pH was tested against various organisms. The growth of organisms was inhibited maximally at pH 4, indicating that Nisin is highly soluble at pH 4 [Figs. 88(I), 88(II) and Table 8].

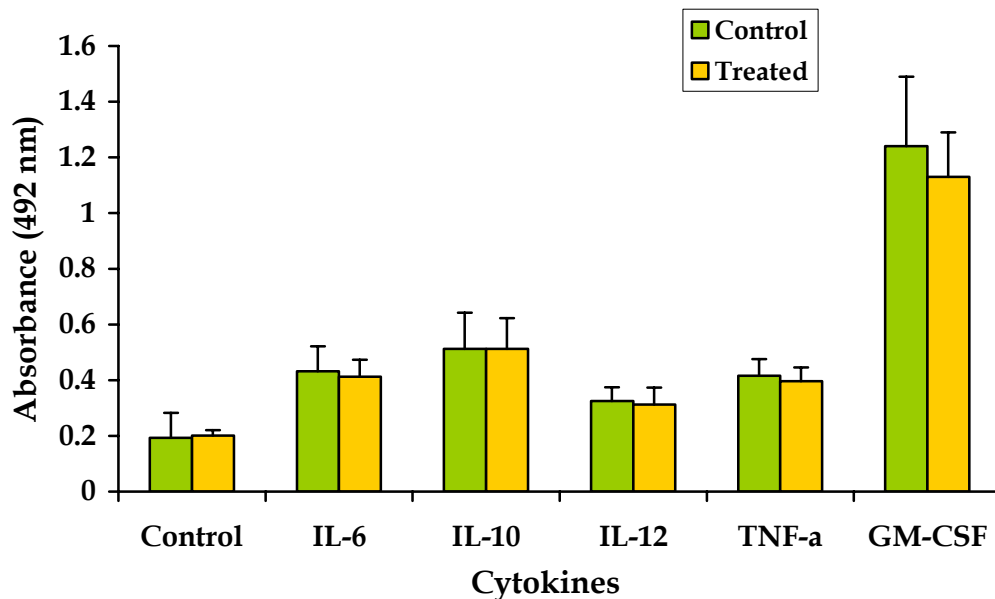


Fig. 87: Determination of pro-inflammatory cytokines in the vaginal fluid of rabbit by ELISA

### Time kill kinetics of Nisin gel formulation

Nisin gel was further tested to determine time-dependent inhibition of bacterial growth. The exponentially growing cultures (*E. coli*, *S. aureus*, *B. subtilis*, *S. hemolyticus*, *P. aeruginosa* and *C. krusei*) were incubated with the Nisin gel for different time-intervals (0, 5, 10, 15, 20, 25, 30 min) and plated on nutrient agar. The number of colony forming units was determined after incubation at 37°C for 24 hr. Maximum inhibition in the growth of organisms (except *P. aeruginosa*) was observed after 20 min.

### Contraceptive efficacy of Nisin gel formulation

1 mg of Nisin in 1 percent polycarbophil gel showed complete inhibition of sperm motility *in vitro*. The *in vivo* studies with this gel were conducted in rabbits. 2 ml of gel was administered vaginally to three groups of rabbits (3 animals/group) and allowed to mate with proven fertile bucks after 15 min.

(group 1), 30 min. (group 2) and 45 min. (group 3) following application. These studies are under progress.

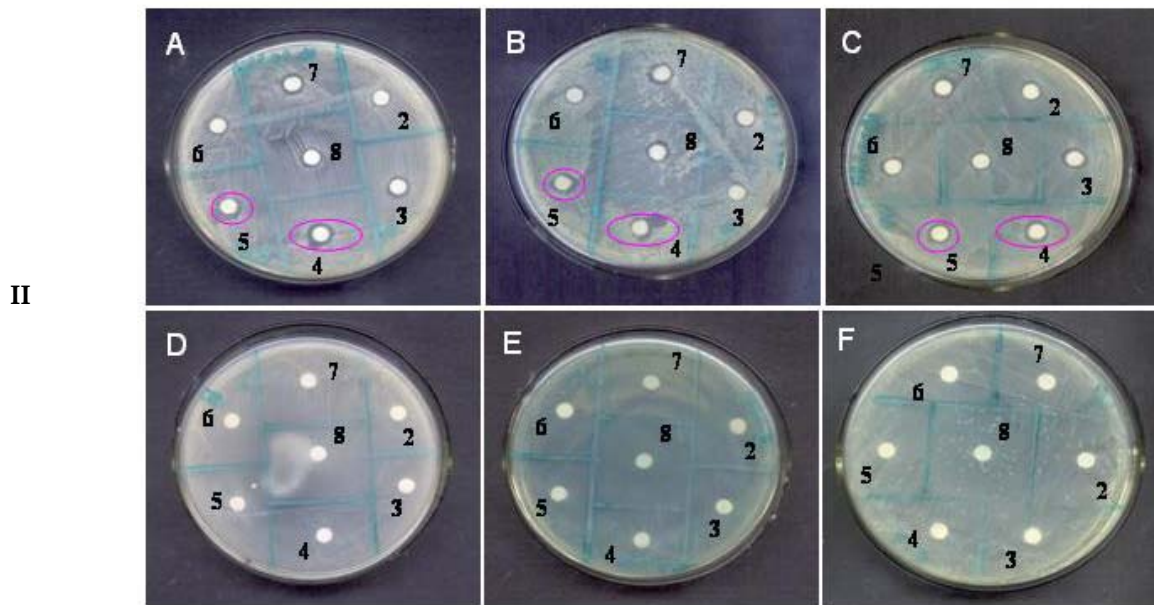
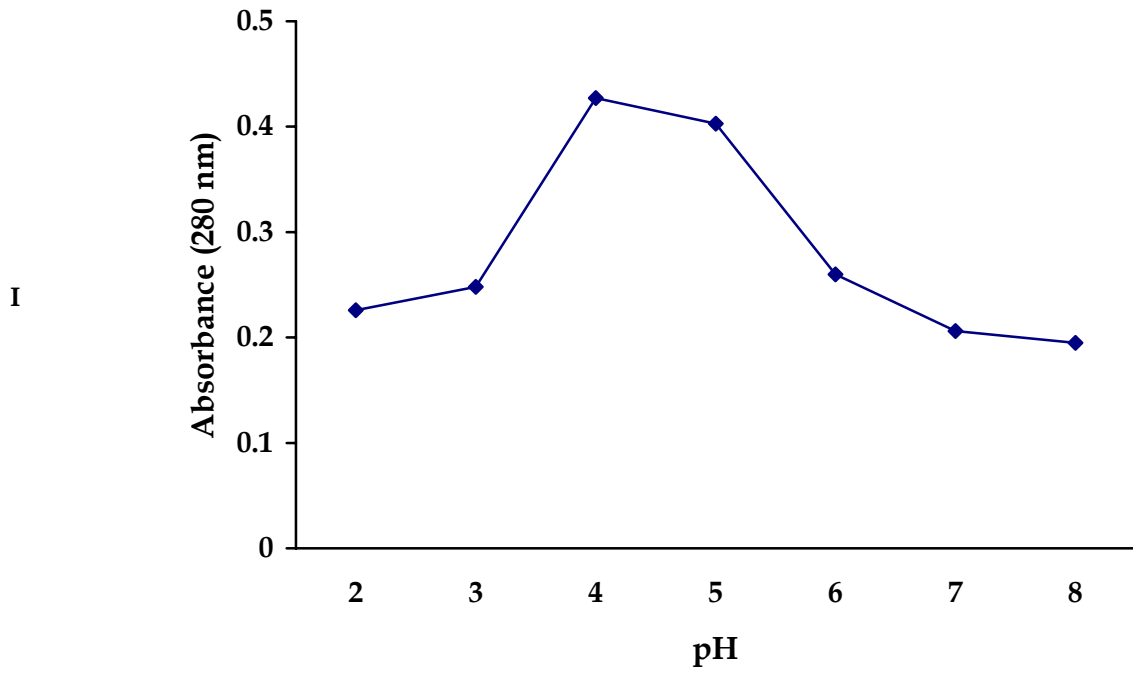


Fig. 88: (I): Effect of pH on the solubility of Nisin; (II): Effect of pH on the growth of various organisms such as *E. coli* (A), *S. aureus* (B), and *S. hemolyticus* (C, D, E and F) are respective controls.

Table 8: Effect of Nisin on the inhibition of growth of various organisms

Organisms	Zone of inhibition (mm)						
	Different pH						
	2	3	4	5	6	7	8
E. coli	4	6	9	6	5	5	4
S. aureus	4	5	9	6	6	5	5
P. aeruginosa	-	-	-	-	-	-	-
B. subtilis	4	5	9.5	6	4	6	6
S. hemolyticus	4	4	8	5	6	5	5

### 3.5 Identification, Purification and Characterization of Antifertility Compounds with Microbicidal Activities (*Funded by CONRAD*)

Principal Investigator: **K.V.R. Reddy**

Project Associates: R.D. Yedery, Clara Aranha and Smita Mahale

Duration: 2001-2006

Naturally occurring antimicrobial proteins/peptides are abundant in the environment. They are present in plants and animals and are also produced by microorganisms. The widespread occurrence of these antimicrobial peptides suggests that they play a crucial role in immunity against pathogens. Crabs are amazingly resistant to microbial infections. Upon detection of bacteria, a complex genetic cascade is activated, which ultimately results in the synthesis of antimicrobial peptides and their release into the hemolymph.

The objective of the study is the identification of newer dual functional vaginal compounds from natural sources which exhibit antifertility and anti-STI/HIV activities. Earlier attempts to identify antimicrobial peptides/proteins led to the isolation of two cationic proteins (12 and 19kDa) (Annual Report 2002-03, p 121). One of these proteins (12kDa) named as *Scylla serrata* protein (SSP12) showed sequence homology with cystatin (a known antimicrobial compound). During the year, a 19 amino acid peptide that was present in the SSP12 has been synthesized using solid phase F-moc chemistry. Amino acid analysis and MALDI-TOF of the synthesized peptide was carried out to validate the amino acid composition and mass of the peptide (observed values vs. expected values) (Table 9). Using ELISA based chromogenic assay system with BAPNA (N $\alpha$ -Benzoyl-DL-Arginine P-Nitroanilide hydrochloride) as a substrate, it was further confirmed that 12kDa protein is a cystatin like molecule.

In an attempt to obtain the native protein, affinity purification was planned using antibodies as ligand. For the development of anti-peptide polyclonal antibodies, the peptide was conjugated with diphtheria toxoid using glutaraldehyde as a coupling agent. The antibodies developed showed a good titre (1:8000). ELISA results demonstrated the presence of SSP12 in the crab hemolymph and hemocytes (Fig. 89). N-terminal aminoacid sequencing and anti-STI/HIV studies are currently under progress.

Table 9: Amino acid analysis and determination of molecular weight of synthesized peptide

Amino acid	Expected value	Observed value
Asp	1	1.37
Thr	2	2.26
Glu	3	2.88
Gly	1	1.34
Ala	2	2.27
Val	3	2.43
Ile	1	1.12
Leu	1	0.93
Tyr	3	2.42
Lys	2	1.3

Expected mass	Observed mass
2187.4	2186

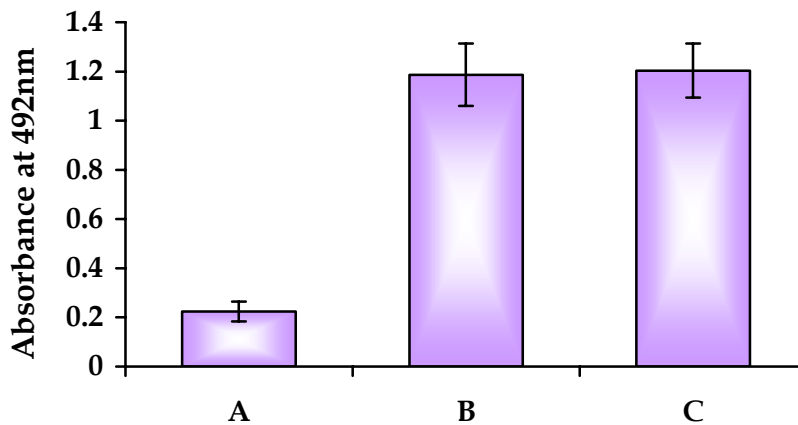


Fig. 89: Detection of peptide SSP12 in the hemolymph (B) and hemocytes (C) of crab, *Scylla serrata* by ELISA (A=PBS control).

### **3.6 Development of National Guidelines for Management of Reproductive Tract Infections including Sexually Transmitted Infections**

Principal Investigator: **S.L. Chauhan**

Project Associates: Beena Joshi, Ragini Kulkarni,  
Rajeshree Manjrekar, Kamal Hazari

Duration: 2005-2006

Reproductive tract infections (RTIs) including sexually transmitted infections (STIs) and HIV/AIDS are being increasingly recognized as a serious public health problem. The presence of RTI facilitates the acquisition and transmission of HIV infection. The risk when associated with diseases such as gonococcal, chlamydial infection and trichomoniasis, increases up to 4-fold in women. Therefore, achieving millennium development goal of reversing the trend of HIV spread by 2015 to the extent of 25 percent among young population, much will depend on effective strategies for prevention and treatment of RTI/STIs in the community.

In the Phase-1 of the National Reproductive and Child Health (RCH) program, RTI services could not be operationalised below the district levels and therefore management of RTIs is the most needed inclusion, particularly in the rural areas and urban slums of India in the upcoming Phase-2 of the RCH programme. This need has been reflected in the National Population Policy (2000) "to include STI/RTI and HIV/AIDS prevention, screening and management in maternal and child health services". Therefore, the Government of India is keenly considering introducing service for prevention and case management of RTI/STIs in RCH-2 programme.

As reported in the previous two annual reports, the institute has carried out a study to assess the capacity of primary health care system in a district in Maharashtra State to deal with RTIs. The study has provided pilot information on the primary health care system's capacity to provide quality services for RTIs. Subsequently, at the behest of the Ministry, Government of India, the institute has also initiated a WHO supported project for formulating "National Guidelines for Management of RTIs/STIs" that is envisaged to be incorporated in the RCH-2. The specific objectives are to (i) conduct a rapid review of management practices (operational, clinical, laboratory) on RTI/STIs at different levels of health system in India; (ii) share assessment findings with a group of experts and evolve operational as well as quality case management guidelines for RTI/STI services; and (iii) review the existing international guidelines for RTI/STIs and prepare operational and quality case management guidelines suitable for India's

health facilities. The inputs required for framing the guidelines for RTIs/STIs will be drawn from countrywide Rapid Assessment Survey in six zones of the country to assess their management practices (operational, clinical, laboratory) on RTIs/STIs at different levels (District, CHC, PHC and Sub-centre) of the health system. Thereafter, the working groups will take up the responsibility of formulating broad management guidelines on each of the discipline specific components (clinical, operational and laboratory). The developed draft guidelines will be presented for review in a broad-based national consultation meeting. Work on the project has been initiated.