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COLLAGENASE ACTIVITY IN *PREVOTELLA BIVIUS* ISOLATED FROM PATIENTS WITH PREMATURE RUPTURE OF MEMBRANES

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ABSTRACT

Bacterial vaginosis (BV) has been considered to be the most prevalent infection found in sexually active women. BV is thought to play an important role in the premature rupture of membranes (PROM) and preterm birth. Preterm delivery accounts for a substantial percentage of low birth weight infants and perinatal mortality and morbidity. *Bacteroides* and *Prevotella* species have been isolated from the amniochorion of women with preterm birth and PROM. Women with $>10^4$/mL *Prevotella bivius* (formerly *Bacteroides bivius*) have a 60-100% higher rate of preterm delivery. The purpose of this study was to determine wether some strains of *Prevotella* species isolated from PROM and BV patients produce proteases especially collagenase enzymes which faciliate the rupture of membranes leading to preterm birth.

Vaginal specimens have been obtained from 120 women with BV and premature rupture of membrane in 30-44 weeks gestational age. Twenty anaerobic coccobacilli consisting of *Bacteroides fragilis*, black pigmented *Bacteroides* and *Prevotella bivius* were isolated and identified. The isolates were examined for protease activity, using porcine skine gelatin and casein as substrates by Martleys method.

Elastase and collagenase activity were detected using elastin, guinea pig skin collagen, bovine achilles tendon collagen, FALGPAand GP,VPK as substrates by Levenson method.

Collagenase and elastase activity was detected in 90 and 100% of isolates. Gelatinase and caseinase activity was detected in 40 and 50 % of isolates. Collagenase produced by *Prevotella bivia* isolates was purified by ammonium sulphate precipitation, gel filtration and ion exchange chromatography. The collagenase was cleaved from the synthetic collagen substrate FALGPA, and GP, VPK substrates. The activity of the enzyme was inhibited by EDTA, Antipain and PMSF.

This study suggests that proteases produced by *Prevotella bivia* may be involved in the pathogenesis of premature rupture of membrane. PROM before 37 weeks has been reported to be significantly higher among patients with *Bacteroides* and *Prevotella* colonisation of the genital tract. The amniochorion consists of collagen and elastin which convey physical integrity to the placenta. Collagenase and elastase

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released into the genital tract may promote connective tissue destruction in the cervix and chorioamnion membranes.

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INTRODUCTION

Bacterial vaginosis (BV) has been reported as the most frequent vaginal infection within sexually active women. BV is a syndrome marked by increase in vaginal, milky creamy discharge, and an amine or fishy odour.[1] Microbiologically, it is characterised by a shift in the vaginal flora from dominant flora of *Lactobacillus spp.* to mixed vaginal flora that includes *Gardenella vaginalis*, *Bacteroides spp*, *Mobiluncus spp* and *Mycoplasma hominis*.[2] BV is a relatively benign disease, although if left untreated it can have extremely serious sequelae. A large body of evidence suggests that BV may play an etiologic role in preterm rupture of membranes (PROM) and labour. Preterm delivery accounts for a substantial percentage of perinatal mortality and morbidity, it has been defined on the basis of a birth weight under 2500 g.[3,4] Such births, increased risk of neurologic sequelae and the cost of society is overwhelming from a financial as well as a health perspective. The major role of *Bacteroides spp* in the etiology of PROM and preterm labour has been frequently noted.[5] Khorn et al.,[6] found that, among women with $>10^4$/mL *Prevotella bivius* in vaginal fluid, the rate of preterm delivery was 60-100 % higher than the rate in women with a lower number of these organisms. Premature rupture of membranes has been significantly reported among patients with *Bacteroides spp.*, and these patients are more likely to deliver their infants before 37 weeks with weight less than 2500 g.[5] The pathogenesis of PROM and preterm labour are multifactorial and remain largely unexplained. Elastin, collagen, and other structural proteins are important for physical integrity and function of the reproductive tract. Rupture occurs because of reduction of membrane strength. Collagenase, elastase and other proteases play dynamic roles in disease of other body sites. Bacterial collagenase has been implicated as a virulence factor in the reduction of the chorioamniotic strength.[7,8] Many cervical / vaginal microorganisms are recognised to produce collagenase as well as other proteases.[9] Production of protease has been reported as a virulence determinant in *Prevotella bivius*, *Bacteroides fragilis* and other anaerobic bacteria.[10] Infection following PROM is a constant threat for mother and child. It is also necessary to take into consideration the neonatal risks after PROM. Prevention of these complications will require better understanding and investigation of its causes. Study of possible virulence factors arising in the genital tract microflora may allow for increased understanding of pathogenesis, possible prevention and treatment.

In this investigation we examined *Prevotella bivius* which was isolated from the genital tract of pregnant women with bacterial vaginosis and rupture of membranes, for collagenase, elastase and non-specific protease activity for the major role of protease enzymes as virulence factors in the pathogenesis of rupture of membranes and premature term and delivery.

MATERIAL AND METHODS

Isolation and identification of *Bacteroides* and *Prevotella spp*

Vaginal specimens from women with BV and premature rupture of membranes in 30- 44 weeks gestational age have been selected. A total of 120 specimens were evaluated with gram stain.

Vaginal specimens were plated into fastidious anaerobic agar (FAA) supplemented with 5% horse blood, incubated for 48 hr in an anaerobic chamber at 37°C. The gram negative coccobaccili colonies were selected and confirmed with black pigmentation, kanamycin activity, growth in 20 % bile salts, Indole, fumarate, motility, urease and xylose, sucrose fermentation tests. The pure *Prevotella* and *Bacteroides* isolates were stored in supplemented brain heart infusion (BHI) broth containing 20 % glycerol in –70°C.

Protease assay

Prevotella bivius was grown in BHI supplemented with hemin, yeast extract, and vitamin K at 37°C for 2 days in an anaerobic chamber (85 % N_2, 10% H_2, 5% CO_2). Cell free culture supernatant was obtained from 2 days old-culture by centrifugation at 12000 g for 30 min. Ammonium sulphate was added to achieve 80 % saturation. The precipitate was collected and dialyzed against 0.05 M Tris- HCl buffer, pH 8.00. The clear supernatant was applied to a DEAE-Sepharose CL-6B column equilibrated with 0.05M Tris-HCl, pH 8.00, after washing the column with 2 volumes of starting buffer, the elution was accomplished by a linear gradient of 0-500 mM NaCl in 0.05 mM Tris- HCl buffer pH.8.00. Fractions of 5 mL were collected and enzyme activity was detected by hydrolysis of chromogenic substrate (BZ-VAL-GLY-pNA) and collagen slide gel assay.[11]

Caseinase assay

Martley's caseine agar was modified for proteolytic enzyme assay within culture supernatant and whole cells of *Prevotella bivius*. At pH[6] small wells were cut in the media and were filled with the culture supernatant.[12] Trypsin (1mg/mL) as control positive was used. Caseine breakdown was

visualised in the media as a white zone of percipitation around colonies and supernatant filled wells (Fig.1).

Collagenase assay

A quantitative microassay for detection of bacterial and tissue collagenase using the method of Levenson.[11] Nobel

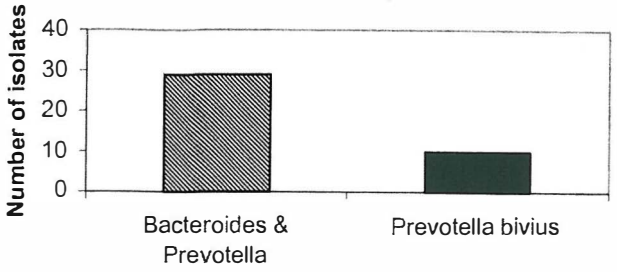

Fig. 1. Percentage of *Prevotella bivius* isolated from PROM patients.

agar (1%) was boiled and cooled to 50°C. A glass slide was dipped into the agar, the agar allowed to dry for 15 min at 37°C in low humidity. One drop of collagen is sandwiched between two agar-coated slides. The thin film of collagen (guinea pig skin collagen and type 1 from bovine achilles tendon from Sigma LTD) adhering to the agar was then dried and collagenase test solution and *Clostridium* collagenase (Sigma) as positive control were placed on the slide and then incubated at 37°C in humidity for a time range from 5min-3hr. The slide was then stained in Coomassie blue for 30 sec. The collagen film stains dark blue, complete digestion of the film by the collagenase results in clear areas of lysis (Fig. 2) where the sample was applied.

Elastase assay

Elastase assay was performed with collagen film microassay. Substrate for elastase was elastine from bovine neck ligament (Sigma).[11]

Gelatinase assay

Gelatine agar was modified for anaerobic bacteria. Porcine skin gelatine (Sigma) was added to media as substrate. Small wells were cut in the media and were filled with the culture supernatant of *Prevotella bivius*. After 3 days incubation, 1-5 mL of mercuric chloride was added to the plates. Gelatine liquefying was visualised with a clear zone around colonies.

RESULTS

A total of 20 *Bacteroides* and *Prevotella* spp. were isolated from women with BV and PROM in weeks 30-44 gestational age (Fig.3).

Bacteroides fragilis was recovered from 3 (15%) of 20, black pigmented *Bacteroides* accounted for 5 (25 %) of 20

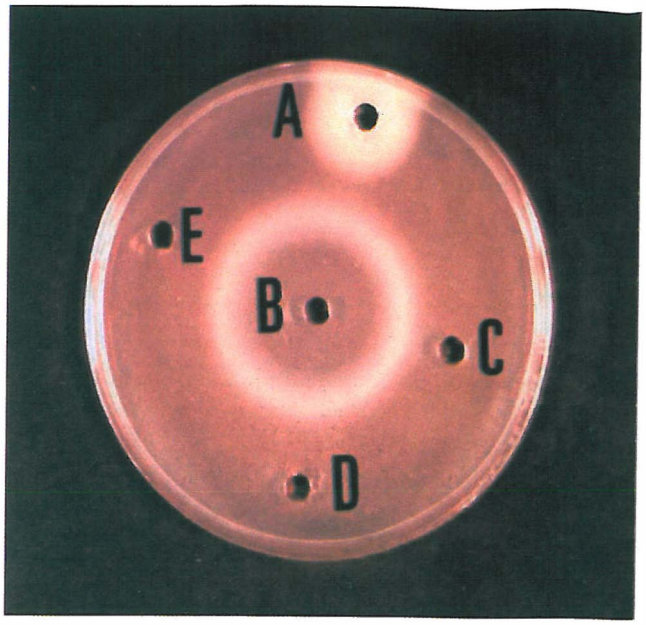

Fig. 2. Collagen film
bated with *Clostridium* collagenase as positive control. B; Collagen coated slide incubated with *Prevotella* collagenase at 37°C for 1hr and then stained with Coomassie. C; Collagen coated slide incubated with saline as negative control. Lysis occurs directly under sample.

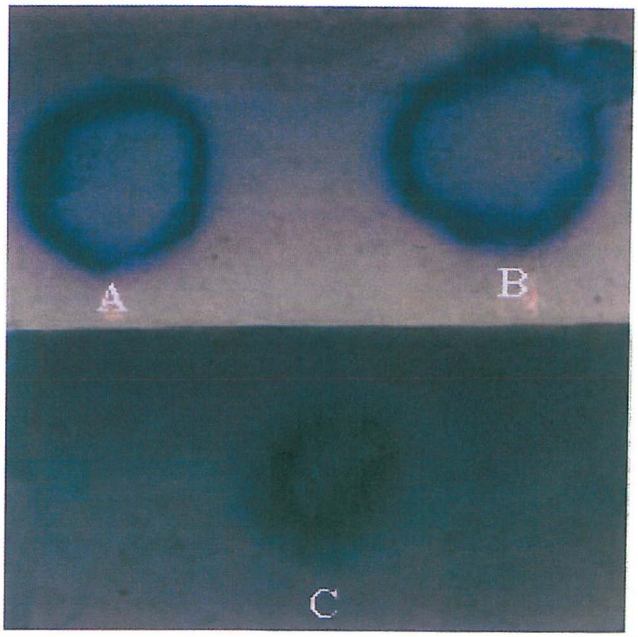

Fig. 3. Proteolytic response of culture supernatant of *Prevotella bivius* on modified
control. B; supernatant of *Prevotella bivius*. E,C, D; BHI without *Prevotella bivius* as negative control.

and *Prevotella bivius* was isolated from 10 patients (50%) with PROM and BV in 30-44 weeks of gestation (Table I).

Table I. Percentage of *Bacteroides* and *Prevotella* species isolates from patients with BV and PROM in 30 - 44 weeks gestation.

	No	%
Bacteroides fragilis	3	15
Black pigmented *Bacteroides*	5	25
Provotella bivius	10	50
Others	2	10
Total	20	100

So recovery of *Bacteroides fragilis* and black pigmented *Bacteroides* and *Prevotella bivius* were associated to preterm delivery and BV.

We confirmed *in vitro* production of a variety of proteases by *Prevotella bivia* isolated from the lower genital tract in PROM patients. *Prevotella bivius* may constitute normal lower genital tract microflora. Collagenase production was examined by whole cell, culture supernatant and partially purified collagenase, using different collagen substrates and clostridium collagenase as positive control using collagenase film microassay. Incubation time for digestion was 3-5hr, but complete digestion depends on collagenase concentration and time. A collagen film microassay was performed for elastase assay. Substrate for elastine was elastine from bovine neck ligment but elastine was insoluble.

We have demonstrated collagenase production by *Prevotella bivia* which are of possible significance in the pathogenesis of reproductive tract disease.

To assess protease production by *Prevotella bivius*, we determined collagenase, elastase, caseinase and gelatinase production among the 10 isolates of *Prevotella bivius*. Elastase activity was detected in 100% (10/10), collagenase activity in 90% (9/10), caseinase activity in 60% (6/10) and gelatinase activity in 40% (4/10) of *Prevotella* isolates (Table II).

DISCUSSION

Premature rupture of membranes (PROM) occurs in 3% of pregnancies and is responsible for approximately one-third of all preterm births.[14] Choriodecidual infection or inflammation appears to play an important role in the etiology of PROM.[4,13,14]

We have isolated *Bacteroides* and *Prevotella* from the genital tract in BV and PROM patients and confirm protease production in *Prevotella bivius* isolated from genital tract infection. *Prevotella bivia* accounted for 50% of isolates. Recent sudies indicated that microorganisms associated with BV can be isolated more frequently from amniotic fluid or chorioamnion of women who deliver prematurely than those who deliver at term.[14,15] Very little is known of the pathophysiologic mechanisms by which bacterial vaginosis may cause preterm labour or premature rupture of membranes. In 1995, Hillier et al. undertook a large study of the association between bacterial vaginosis and the preterm delivery of low birth weight infants.[16] In 2000, Goldenberg et al[15] found that up to 80% of women who deliver before 37 weeks gestation have evidence of bacterial infection in the amniotic fluid compared with only 30% at >37 weeks gestation. Minkoff et al. studied 233 women with PROM, they found women colonized with *Bacteroides* spp had a significantly smaller infant, more frequent preterm rupture of membrane and preterm delivery.[5] *Bacteroides bivius* was associated with a two-fold increased risk of delivery at >37 weeks gestation.[6] *Prevotella bivius* constitute normal lower genital tract microflora and are frequently recovered from the lower genital tract. Amniotic fluid contamination with *Prevotella bivius* have also been significantly associated with delivery of infants weighing < 2500 gm.[16] *Bacteroides fragilis* has been reported in the cervix and amniotic fluid and placenta of patients with premature rupture of membrane.[17]

Prevotella bivius have been isolated from 50 % of PROM patients in 30-44 weeks gestation. Multiple isolates of *Prevotella bivius* were examined for protease activity, using different substrates. *Prevotella bivius* produce a variety of protease which are of possible significance in the pathogenesis of reproductive tract disease. We have found collagenase activity associated with more than 90% of isolates. A large proportion of the isolates we have examined have the ability to digest collagen. Microbial proteases such as collagenase and elastase released into the genital tract milieu may act to structurally damage connecting tissue in the cervix and chorioamnion membranes. McGregor found increased concentration of vaginal wash protease >10 tripsin units was associated with increased risk of PROM.[9,10]

Collagen is a major component of the amniotic membrane. Collagen in amnion plays a large role in the stress tolerance of the fetal membrane and protects against the occurrence of PROM.[9,10,18,19] It is known that collagen content in preterm amnion with premature rupture of the membranes was significantly lower than that of preterm amnion without premature rupture of the membranes.[19,20,21] Kanayama demonstrated that collagen content in the am-

Table II. Percentage of proteolytic activity of *Prevotella bivius* using elastine, casein and gelatine as substrates. Proteolytic activity of *Prevotella bivius* was measured during exponential growth phase in the culture supernatant.

Substrate	Whole cell		Culture supernatant	
	no.	%	no.	%
Elastine	10	100	10	100
Collagen	9	90	9	90
Casein	6	60	6	60
Gelatin	4	40	4	40

nion with PROM is significantly lower than that of amnion without PROM.[22] Therefore the cause of PROM in the preterm gestation may be strongly related to types and contents of collagen, especially to the reduction of type III collagen.[22] Decreased membrane collagen content has been demonstrated in the setting of PROM[20] Increases in amniotic fluid matrix metaloprotease as well as decreases in tissue inhibitors of matrix metaloprotease have been identified among women with preterm PROM.[7,8] Extracellular protease production constitutes a potential factor that may alter or inactivate a variety of host proteins including surface antimicrobial factors and collagen structure.[10] We found that *Prevotella bivius* in lower genital tract sites of women with PROM produce collagenase, elastase and nonspecific protease.

Production of collagenase may correlate with pathogenicity of the genital tract of women with BV. We have found that collagenase activity was associated with more than 90% of isolates. The collagenase we have examined has the ability to cleave synthetic collagen substrates such as FALGPA, and GP, VPK substrates. The activity of the enzyme was inhibited by EDTA, antipain and PMSF. There is good evidence supporting the role of protease production by bacteria related to porphyromonas activity in the oral cavity. In the past decade several studies have been conducted to purify protease enzymes from cell extracts and culture media of *Porphyromonas gingivalis* (formerly *Bacteroides*) in the periodental disease. This organism is an important pathogen in the oral cavity. *Bacteroides spp* are known to produce a number of enzymes which are capable of degrading different tissue components associated with the pathogenesis of BV. Protease enzymes reduce the chorioamnion membrane strength *in vitro*.[9]

It is even possible that high concentrations of bacteria in the lower genital tract could produce enough protease to weaken the fetal membrane strength causing premature rupture of membranes. Certain complications of pregnancy such as PROM, preterm labour and abortion are frequently associated with histological tissue findings of inflammation and microbiologic recovery of normal vaginal microflora.[23] This association suggests that the presence of protease release by vaginal microorganisms or an inflammatory response to infection may lead to fetal membrane damage. Concentrations of protease, collagenase and phopholipase and other factors may originate from the cumulative presence of genital microorganisms and the host responses are suggested as possible virulence factors for preterm birth.[13] Proof of the relevance of these findings requires clinical correlation and further experiments.

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