



Study of the vaginal and rectal microflora in pregnant women, with emphasis on Group B streptococci

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Dedication

This thesis is dedicated to my lovely parents, Abdullah and Hanifa El Aila who taught me the value of education and have taken great pain to see me prosper in life. I am deeply indebted to them for their continued support and unwavering faith in me.

It is also dedicated to my wife Aziza and my daughters: Danya, Dimah and Lana for their constant moral support, encouragement and invaluable help at every stage of my research.

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List of abbreviations

AFLP	Amplification Fragment Length Polymorphism analysis
ARDRA	Amplified rDNA Restriction Analysis
BV	Bacterial Vaginosis
CAMP	Christie, Atkins and Munch-Petersen
CA	ChromID™ <i>Streptococcus</i> B agar
CDC	Centers for Disease Control
CFU	Colony Forming Unit
CNA	Colistin Nalidixic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	DeoxyriboNucleic Acid
FISH	Fluorescent <i>In Situ</i> Hybridization
FRET	Fluorescence <i>Resonance Energy Transfer</i>
GBS	Group B <i>Streptococcus</i> (<i>Streptococcus agalactiae</i>)
GBSDA	Group B <i>Streptococcus</i> differential agar
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
PB	Preterm Birth
PCR	Polymerase Chain Reaction
PID	Pelvic Inflammatory Disease
PFGE	Pulsed Field Gel Electrophoresis
qPCR	Quantitative Polymerase Chain Reaction (real-time PCR)
RAPD	Randomly Amplified Polymorphic DNA analysis
rDNA	rRNA gene
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
<i>Taq</i>	<i>Thermus aquaticus</i>
tDNA-PCR	tRNA intergenic spacer length polymorphism analysis
tRFLP	Terminal Restriction Fragment Length Polymorphism analysis
tRNA	transfer RNA
VMF	Vaginal microflora

List of bacterial species

Abbreviation

A. baumannii
A. meyeri
A. neuii
A. radingae
A. urogenitalis
A. christensenii
A. viridans
A. radiobacter
A. tetradius
A. vaginalis
A. vaginae
B. uniformis
B. breve
B. biavati
B. bifidum
B. infantis
B. longum
C. albicans
C. accolens
E. avium
E. faecalis
E. faecium
E. coli
F. magna
F. gonidioformans
G. vaginalis
K. planticola
L. acidophilus
L. amylolyticus
L. amylovorus
L. brevis
L. casei
L. casei paracasei
L. cellobiosus fermentum
L. coleohominis
L. fermentum
L. gallinarum
L. gasseri

Full name

Acinetobacter baumannii
Actinomyces meyeri
Actinomyces neuii
Actinomyces radingae
Actinomyces urogenitalis
Aerococcus christensenii
Aerococcus viridans
Agrobacterium radiobacter
Anaerococcus tetradius
Anaerococcus vaginalis
Atopobium vaginae
Bacteroides uniformis
Bifidobacterium breve
Bifidobacterium biavati
Bifidobacterium bifidum
Bifidobacterium infantis
Bifidobacterium longum
Candida albicans
Corynebacterium accolens
Enterococcus avium
Enterococcus faecalis
Enterococcus faecium
Escherichia coli
Fingoldia magna
Fusobacterium gonidioformans
Gardnerella vaginalis
Klebsiella planticola
Lactobacillus acidophilus
Lactobacillus amylolyticus
Lactobacillus amylovorus
Lactobacillus brevis
Lactobacillus casei
Lactobacillus casei paracasei
Lactobacillus cellobiosus fermentum
Lactobacillus coleohominis
Lactobacillus fermentum
Lactobacillus gallinarum
Lactobacillus gasseri

<i>L. helveticus</i>	<i>Lactobacillus helveticus</i>
<i>L. iners</i>	<i>Lactobacillus iners</i>
<i>L. johnsonii</i>	<i>Lactobacillus johnsonii</i>
<i>L. kitasatonis</i>	<i>Lactobacillus kitasatonis</i>
<i>L. mucosae</i>	<i>Lactobacillus mucosae</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
<i>L. rhamnosus</i>	<i>Lactobacillus rhamnosus</i>
<i>L. salivarius</i>	<i>Lactobacillus salivarius</i>
<i>L. vaginalis</i>	<i>Lactobacillus vaginalis</i>
<i>M. hominis</i>	<i>Mycoplasma hominis</i>
<i>N. gonorrhoeae</i>	<i>Neisseria gonorrhoeae</i>
<i>P. acidilactici</i>	<i>Pediococcus acidilactici</i>
<i>P. indolicus</i>	<i>Peptoniphilus indolicus</i>
<i>P. anaerobius</i>	<i>Peptostreptococcus anaerobius</i>
<i>P. bivia</i>	<i>Prevotella bivia</i>
<i>P. stutzeri</i>	<i>Pseudomonas stutzeri</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. capitis</i>	<i>Staphylococcus capitis</i>
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
<i>S. haemolyticus</i>	<i>Staphylococcus haemolyticus</i>
<i>S. hominis</i>	<i>Staphylococcus hominis</i>
<i>S. warneri</i>	<i>Staphylococcus warneri</i>
<i>S. agalactiae</i>	<i>Streptococcus agalactiae</i>
<i>S. anginosus</i>	<i>Streptococcus anginosus</i>
<i>S. bovis</i>	<i>Streptococcus bovis</i>
<i>S. intermedius</i>	<i>Streptococcus intermedius</i>
<i>S. mitis</i>	<i>Streptococcus mitis</i>
<i>S. mutans</i>	<i>Streptococcus mutans</i>
<i>S. parasanguinis</i>	<i>Streptococcus parasanguinis</i>
<i>S. porcinus</i>	<i>Streptococcus porcinus</i>
<i>S. pyogenes</i>	<i>Streptococcus pyogenes</i>
<i>S. salivarius</i>	<i>Streptococcus salivarius</i>
<i>T. vaginalis</i>	<i>Trichomonas vaginalis</i>
<i>V. cambriensis</i>	<i>Varibaculum cambriensis</i>
<i>W. paramesenteroides</i>	<i>Weissella paramesenteroides</i>

Summary

In a first part of the PhD research we established the degree of correspondence between the vaginal and the rectal microflora. The composition of the human vaginal microflora is affected by several host factors, including, among others, age, menarche, hormonal changes, sexual activity, pregnancy and the use of contraceptives or spermicides, as well as individual habits such as douching (405). Studies of vaginal lactobacilli have demonstrated that *Lactobacillus crispatus*, *L. jensenii*, *L. gasseri* and *L. vaginalis* are the most commonly recovered species of H₂O₂-producing lactobacilli (5, 172, 287, 380) and the absence of H₂O₂-producing lactobacilli from the vagina has been associated with an increased risk for bacterial vaginosis (BV) (161, 241). BV is a condition whereby the lactobacilli are overgrown by anaerobic bacteria, such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus* spp., *Mycoplasma* spp., *Peptostreptococcus* spp. and *Prevotella* spp. BV has been linked to increased shedding of HIV in the female genital tract (63), increased acquisition of HIV (161) and herpes simplex virus type 2 (63, 161) and with preterm birth (174). Several bacterial species are known to colonize both the gastrointestinal and the reproductive tract, and the rectum may play an important role as a source or reservoir for organisms that colonize the vagina (6, 238). The question remains whether these vaginal organisms are endogeneous or originate from the gastrointestinal tract.

To establish whether the rectum can serve as a possible bacterial reservoir for colonisation of the vaginal econiche, we cultured vaginal and rectal specimen from pregnant women at 35-37 weeks of gestation as part of the group B streptococci (GBS) screening program to prevent GBS neonatal disease, we identified the isolates to the species level with tRNA intergenic length polymorphism analysis (tDNA-PCR) and genotyped the isolates for those subjects from which the same species was isolated simultaneously vaginally and rectally, by RAPD-analysis (100). Also we compared the genotype of GBS isolates between the vagina and rectum (99). To further establish the potential role of the rectum as a reservoir for the vaginal microflora, we quantified the bacterial loads of six of the vaginally most important species by quantitative real-time

PCR (qPCR), for those women where these species were present simultaneously in both niches (96).

In summary, the species composition and the genotype similarity for those species simultaneously present in both niches, were unexpectedly high, indicating that indeed the rectum is probably the most important source of the vaginal microflora, or that strong dynamic exchange between both niches exist. This was further confirmed by the finding that for five of the six species tested (except for *G. vaginalis*), the vaginal and rectal bacterial load were in strong correspondence.

A second part of this PhD research regarded improving the prepartum detection of group B streptococci. Group B streptococci (GBS) are an important cause of neonatal sepsis and meningitis, and maternal infection. Early neonatal GBS infection can be prevented by identifying high-risk pregnancies and administering intrapartum antibiotics. Two different strategies, the screening-based and the risk-based approach, are used today in order to decrease the incidence of early-onset neonatal infection. To detect GBS colonization in pregnant women, the CDC recommends isolation of the bacterium from vaginal and anorectal swab samples by growth in a selective enrichment medium, such as Lim Broth (Todd-Hewitt broth supplemented with selective antibiotics), followed by subculture on sheep blood agar (57). However, this procedure may require 48 h to complete and its sensitivity might be improved.

We compared different sampling and culture techniques for the detection of group B streptococci (98) and found that rectovaginal sampling was the most sensitive method for sampling and that Granada agar and Chromagar were the most sensitive culture techniques, only slightly improved by Lim broth enrichment. Also we compared two different targets (*sip* and *cfb* genes), using two different real-time PCR (qPCR) approaches, *i.e.* the hydrolysis probe (Taqman, Roche) and hybridisation probe (Hybprobe, Roche) techniques, directly on the clinical sample and after Lim broth enrichment, with the previously optimized culture approach (97), *i.e.* Lim broth enrichment, followed by subculture on Chromagar (97).

We could establish that the sensitivity of these molecular techniques is significantly higher than that of the CDC recommended culture approach and also than that of our

improved culture approach. Rather surprisingly, the sensitivity of these molecular techniques is further significantly improved by Lim broth enrichment compared to direct application on the clinical sample. The consequences of this observation for the application of the rapid combined DNA extraction/qPCR techniques, as they are now available for intrapartum screening, are still unclear, and will be the basis for future research in the rapidly evolving GBS diagnostics.

Samenvatting

In het eerste deel van dit doctoraatsonderzoek, bepaalden we de overeenstemming tussen de vaginale en de rectale microflora. De samenstelling van de menselijke vaginale microflora wordt door verschillende gastheerfactoren bepaald, o.a. door leeftijd, menarche, hormonale veranderingen tijdens de menstruele cyclus, seksuele activiteit, zwangerschap en het gebruik van contraceptiva, maar ook door individuele gedragsverschillen (405). Studies van vaginale lactobacillen hebben aangetoond dat *Lactobacillus crispatus*, *L. jensenii*, *L. gasseri* en *L. vaginalis* de meest algemeen aanwezige H₂O₂-producerende lactobacillen zijn (5, 172, 287, 380) en de afwezigheid van H₂O₂-producerende lactobacillen in de vagina is geassocieerd geworden met een toegenomen risico op bacteriële vaginose (BV) (161, 241). BV is een toestand waarbij de lactobacillen zijn overgroeid door anaërobe bacteriën, zoals *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus* spp., *Mycoplasma* spp., *Peptostreptococcus* spp. en *Prevotella* spp. BV is daarenboven in verband gebracht met toegenomen excretie van HIV in de vrouwelijke genitale tractus (63), toegenomen besmettingsrisico door HIV (161) en herpes simplex virus type 2 (63, 161) en met vroegtijdige geboorte (174). De vraag blijft of deze bacteriën van endogene oorsprong zijn, dan wel bvb. vanuit de gastro-intestinale tractus opstijgen.

Verschillende bacteriële species zijn gekend om zowel de gastro-intestinale als de urogenitale tractus te koloniseren, en het rectum kan daarom een belangrijke rol spelen als reservoir voor de genitale microflora (6, 238). Om vast te stellen of het rectum inderdaad dergelijke rol kan spelen, kweekten we vaginale en rectale monsters bij vrouwen die 35-37 weken zwanger waren. Deze monsternamen gebeurtenis als onderdeel van de routine screening naar group B streptococci (GBS). We identificeerden de gekweekte isolaten met tRNA intergenische lengte polymorfisme analyse (tDNA-PCR) en genotypeerden ook de isolaten voor die vrouwen waarbij we hetzelfde species zowel rectaal als vaginaal aantreffen, door middel van RAPD-analyse (100). We vergeleken ook de GBS-isolaten voor beide niches (99). Om de potentiële rol van het rectum als reservoir voor vaginale microflora verder te documenteren, bepaalden we ook

kwantitatief het bacteriële inoculum van zes van de belangrijkste vaginale soorten, rectaal en vaginaal, d.m.v. real-time PCR (qPCR), voor die vrouwen waarbij deze soorten in beide niches waren aangetroffen (96).

Samengevat, konden we aantonen dat zowel de soortsaanstelling als de genotypische identiteit van de isolaten van die soorten die terzelfdertijd in beide niches werden aangetroffen, onverwachts hoog waren. Dit is een sterke aanduiding voor de rol van het rectum als reservoir voor de vaginale microflora, of voor een sterke uitwisseling tussen beide niches. Dit werd verder bevestigd door de vaststelling dat voor vijf van de zes gesteste soorten (uitgezonderd voor *G. vaginalis*) het vaginale en het rectale bacteriële inoculum sterk correspondeerden.

Een tweede deel van dit doctoraatsonderzoek betrof het verbeteren van de methodologie voor prepartum screening naar kolonisatie met GBS. GBS zijn een belangrijke oorzaak van neonatale sepsis en meningitis, en van maternale infectie. Vroege neonatale GBS-infectie kan voorkomen worden door hoog risico zwangerschappen op te sporen, zodat gepaste intrapartum antibiotica-therapie kan gestart worden. Twee verschillende strategieën, met name de screening-gebaseerde en de risico-gebaseerde benadering, worden momenteel toegepast om de incidentie van 'early-onset' GBS-infectie te doen afnemen. Om GBS-kolonisatie van zwangere vrouwen te detecteren, beveelt het CDC aan de bacterie uit vaginale en anorectale monsters te isoleren door middel van aanrijkingscultuur, bvb. met Lim Broth (Todd-Hewitt broth met selectieve antibiotica), gevolgd door subcultuur op schapenbloedagar (57). Het nadeel is dat deze procedure in totaal 48 uur in beslag neemt en de gevoeligheid ervan voor verbetering vatbaar is.

Wij vergeleken verschillende monsternamen- en kweektechnieken voor de detectie van GBS (98) en vonden dat rectovaginale staalname, zoals aanbevolen door het CDC, de meest gevoelige monsternamenmethode was, en dat Granada agar en Chromagar de meest gevoelige kweektechnieken waren, slechts weinig verbeterd door voorafgaande Lim broth aanrijking.

Ook vergeleken we twee verschillende genetische targets (de *sip* en *cfb* genen), gebruik makend van twee verschillende real-time PCR (qPCR) technieken, met name de

hydrolyse-probe (Taqman, Roche) en de hybridizatie-probe (Hybprobe, Roche) technieken, onmiddellijk op rectovaginaal monster en na Lim broth-aanrijking (97), met de eerder ge-optimaliseerde kweekmethode (93) (aanrijking op Lim broth, gevolgd door subcultuur op Chromagar). We konden aantonen dat de gevoeligheid van deze moleculaire technieken significant hoger lag dan die van de door het CDC aanbevolen kweek en ook dan die van de door ons ge-optimaliseerde kweek. Een verrassende bevinding was dat de gevoeligheid van deze moleculaire technieken nogmaals beduidend hoger lag na Lim broth aanrijking, in vergelijking met toepassing direct op het monster. De gevolgen van deze bevinding voor de toepasbaarheid van de snelle DNA-extractie/qPCR technieken, zoals ze sinds kort beschikbaar zijn voor intrapartum screening van GBS, zijn onduidelijk en zullen de basis vormen voor verder onderzoek in het snel evoluerende domein van GBS diagnostiek.

Research objectives

The main objectives of this doctoral thesis were to study vaginal and rectal colonization and to obtain a more complete knowledge of the composition of the vaginal microflora in pregnant women and to compare conventional culture methods and advanced molecular techniques in the prenatal screening programme of group B streptococci.

Specific objectives:

1. To characterize the vaginal microflora using culture-dependent and culture-independent analysis.
2. To establish the potential role of the rectum as a reservoir for the vaginal microflora, using tDNA-PCR, RAPD analysis and qPCR.
3. To improve prenatal screening of group B streptococci by comparing different selective media and different DNA detection chemistries.

Chapter I: General introduction

I.1. Female genital tract

The female genital tract is composed of a sequence of cavities. The external genital tract (vulva) leads into the vagina that connects in succession to the endocervix, the uterus and then to the Fallopian (or uterine) tubes (Figure 1). The exposure of the female genital tract to the external environment carries with it the risk, caused by infections, of potentially compromising reproductive functions (406). Among the defense mechanisms that are operational in preventing infections in this area, undoubtedly one of the most important is the composition of the microflora that colonizes the vagina (406).

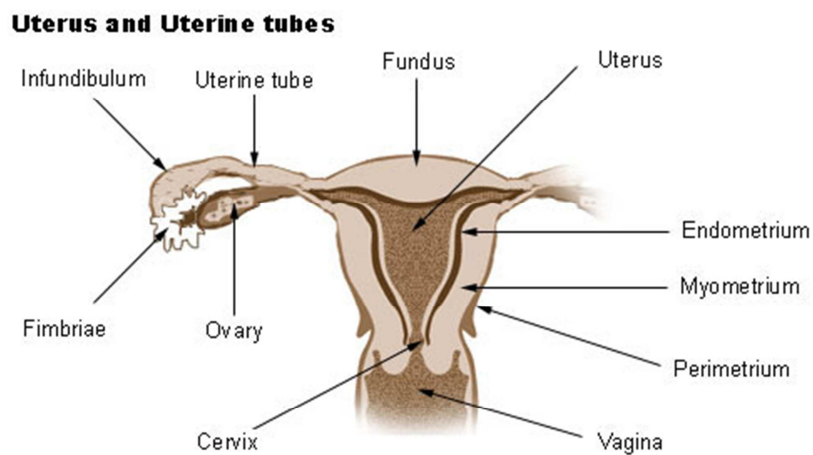


Figure 1: The female genital tract.

(from http://www.daviddarling.info/encyclopedia/F/female_genital_tract.html)

At birth, the vagina of the female infant is sterile. Because of the elevated glycogen content of the neonatal vaginal epithelial cells, with the glycogen derived from the mother at birth, the infant's vagina is colonized by lactobacilli migrating from the mother. This is in line with the generally recognized fact that the normal bacterial flora in humans originates from the mother (125). The vaginal microflora of the infant girl

becomes interspersed with contributions of coagulase negative staphylococci, streptococci, *Escherichia coli* and other intestinal bacteria. Small quantities of lactobacilli remain, however, and the oestrogen produced at menarche will cause a thickening of the vaginal mucosa (Figure 2), increased glycogen production (402). and possibly estrogenization and the expression of new receptors (Verstraelen, pers. comm.), a prerequisite for the propagation of lactobacilli, the dominant vaginal microflora of the adult female (291). The bacteria isolated from the vaginal secretions of women of childbearing age number around 10^7 or 10^8 CFU/g fluid (291). This microflora composition continues until the menopause, when it is replaced by a mixed flora not unlike that of the infant female, but with a considerable portion of *Mycoplasma* species and small quantities of anaerobic bacteria (including *G. vaginalis*). Hormone replacement therapy, when used, will cause lactobacilli to continue as the dominant microflora (125).

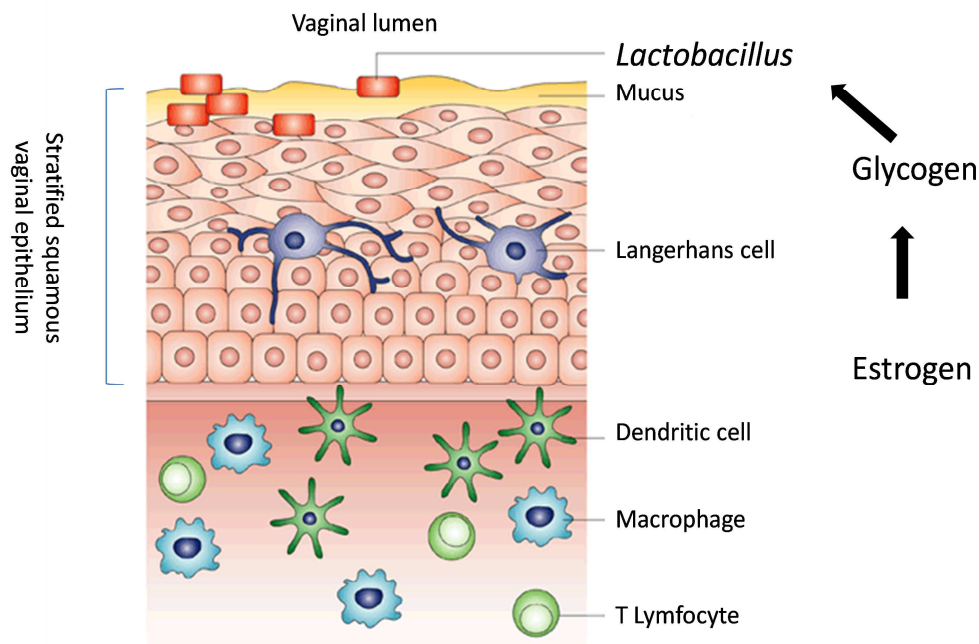


Figure 2: The vaginal epithelium (Adapted from (355)).

I.2. Vaginal ecosystem

A healthy vaginal ecosystem is characterized by an intact vaginal epithelium and a microflora dominated by lactobacilli. *Lactobacillus* spp. account for more than 95% of all bacteria present (105, 347). Also, these organisms are believed to provide defense against infection by maintaining an acidic pH in the vagina (31). The lactobacilli of healthy women tend to contain more hydrogen peroxide-producing species, which damage organisms lacking free radical scavengers (105, 107). Some lactic acid bacteria produce hydrogen peroxide (oxidizing agent) and bacteriocins, which are antimicrobial substances. Bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria (122, 201). It may contribute to the colonisation resistance of the host, i.e. its protection against pathogens (35, 294).

The composition of the vaginal ecosystem is not static but changes over time and in response to endogenous and exogenous influences (109, 110, 284, 331). Variables include stage of the menstrual cycle, pregnancy, use of contraceptive agents, frequency of sexual intercourse, specific sexual partners, vaginal douching, use of panty liners or vaginal deodorants, and utilization of antibiotics or other medications with immune or endocrine activities. Exposure to an altered milieu will cause a fluctuation in the local environment and heighten or diminish the selective advantage of specific vaginal microbes. For example, the loss of lactobacilli from the vagina has been associated with sexual intercourse or with the use of antibiotics for non-vaginal illnesses (110).

I.3. The normal vaginal microflora

The vaginal microflora of healthy asymptomatic women consists of a wide variety of anaerobic and aerobic bacterial genera and species dominated by the facultative, microaerophilic, anaerobic genus *Lactobacillus*.

The first extensive study of the human vaginal microflora was published in 1892 by Döderlein (84). Döderlein and his contemporaries considered the vaginal flora to be homogeneous, consisting only of gram-positive bacilli. Döderlein's bacilli are now

known to be members of the genus *Lactobacillus*. A wide variety of microorganisms, including coliforms, diphtheroids, aerobic gram-positive cocci are recognized as a normal flora of the vagina. Anaerobic bacteria were recognized in the normal vagina as early as 1928 (159). In 1938, Weinstein reported that anaerobic organisms were present in the vaginal secretions of more than 90% of pregnant and nonpregnant women (391). Similarly, in 1947, Hite *et al.* reported the frequent observation of anaerobic organisms in the normal vagina (175).

I.3.1. Vaginal lactobacilli

Lactobacilli are Gram-positive, non-spore-forming microorganisms. Considering cellular shape, they can occur as rods or as coccobacilli. They are strictly fermentative, aerotolerant or anaerobic, requiring rich media to grow. They are catalase negative, even though pseudocatalase activity can be present in some species (116, 157). Since the first description of lactobacilli by Döderlein (84), it has been widely assumed that lactobacilli normally present in the vagina protect against the overgrowth of potentially pathogenic indigenous microflora and exogenous pathogens. The pathogenesis of **bacterial vaginosis** is thought to include the elimination or reduction of antibacterial activity expressed by indigenous vaginal lactobacilli (291, 337). Lactobacilli inhibit the *in vitro* growth of bacterial vaginosis-associated organisms, including species of *Gardnerella*, *Mobiluncus*, *Peptostreptococcus*, and *Bacteroides* (291, 337). Similarly, *Candida* vaginitis following systemic antibiotic therapy has been attributed to loss of the protective vaginal population of lactobacilli. *Lactobacillus* deficient conditions are associated with the development of numerous infections and promote the transmission of sexually transmitted diseases such as gonorrhoeae, chlamydiosis, syphilis, trichomoniasis, HIV, and HPV, whereby the latter may lead to cervical cancer (86). There are many different strains of lactobacilli present in the vagina, the most frequent being *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. iners* and, and there is a wide variation in species and relative numbers of species according to the population studied (275).

1.3.2. Taxonomy of vaginal lactobacilli

The genus *Lactobacillus* belongs to the lactic acid bacteria (LAB), to which also belong the genera *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Sporolactobacillus* and *Streptococcus*. LAB are representative of a group of bacteria, which are functionally related through their ability to produce lactic acid, either as a single product (homofermentative metabolism) or together with other products such as organic acids and ethanols (heterofermentative metabolism) (207). In 1928, Thomas identified the dominant vaginal species as being *L. acidophilus*. Determination of the species to which Döderleins bacillus belongs has been a long and complicated process (366). In early studies by Rogosa and Sharpe (301) and by Wylie and Henderson (412), the predominant species isolated from the vagina were identified as *L. acidophilus* and *L. fermentum*, with *L. brevis*, *L. casei*, *L. delbrueckii*, *L. jensenii*, *L. plantarum*, and *L. salivarius* isolated less frequently.

Considering DNA base composition of the genome, lactobacilli usually show a GC content ranging from 33 to 55 mol% (352). In 1975, Carlsson reported with the help of phenotypic methods, that species in the *L. acidophilus* group were the dominant residents of the ecological niche constituted by the healthy human vagina (48), an assumption later confirmed through DNA homology studies. In 1987, using genotypic studies of isolates from 27 women, Giorgi *et al.* (142) suggested that the identification of *L. acidophilus* was erroneous and that *L. crispatus*, *L. jensenii*, *L. fermentum*, *L. gasseri* and an unidentified species were the predominant vaginal species. In 1999, two DNA-based studies of 97 vaginal specimens from Japan and 215 from the United States gave similar results (5, 345). Antonio *et al.* (5) indicated *L. crispatus*, *L. gasseri*, 1086V (later identified as being *L. iners*) and *L. jensenii*, as the four most common species encountered in the vagina, applying whole-chromosomal DNA probes. *L. iners* was only recognized in 1999 (113) and was the last important vaginal *Lactobacillus* species thus far described.

The name *L. acidophilus sensu lato* is still in use to refer the name of a group of *Lactobacillus* species containing *L. acidophilus sensu stricto* (158), *L. amylolyticus* (27, 134), *L. amylovorus* (262), *L. crispatus* (50), *L. gasseri* (222), *L. gallinarum* (133), *L. helveticus* (189), *L. iners* (113, 134), *L. johnsonii* (133) and *L. kitasatonis* (260). *L. jensenii* is part of the *L. delbrueckii* group.

I.3.3. Lactobacilli and vaginal colonization resistance

I.3.2.1. Acid production

Lactobacilli are acidotolerant, and the most frequently described mechanism for the control of growth of other populations by lactobacilli has been via the production of lactic acid. Because human metabolism can only produce L-lactate (39) whereas bacteria can produce both D- and L-lactate (28, 244, 341) the presence of D-lactate in serum has been found to be a marker for bacterial infection (339, 340), and the presence of D-lactate can be similarly used as a marker for bacterial metabolism in other body fluids. The results reported by Boskey et al. (30) support the hypothesis that vaginal bacteria, not epithelial cells, are the primary source of lactic acid in the vagina.

With the onset of estrogen production during puberty, the amount of glycogen in the uterine wall and the vaginal epithelium increases. While it has been postulated that the breakdown of glycogen first to glucose, then to lactic acid by lactobacilli maintains the low pH characteristic of the healthy vaginal environment, the measured levels of lactic acid alone cannot account for the acid environment. Indeed, also the host releases many acidic secretions (249, 291) and therefore it is plausible that the predominance of lactobacilli reflects conditions provided by the host more than conditions created by the lactobacilli. The extent to which the microflora is determined by the environmental conditions provided by the host and the influence of microbial metabolites in determining the host environment remain to be established. *In vitro* studies showed that the concentrations of anaerobic bacteria increase with increasing pH, and that both *G. vaginalis* and *Prevotella* spp. are susceptible to low pH (31). McClean and McGroarty found that lactic acid and low pH had a greater inhibitory effect on *G. vaginalis* than

hydrogen peroxide. The *in vitro* experiments by Klebanoff *et al.* (205) showed that the hydrogen peroxide producing lactobacilli/myeloperoxidase/chloride system had maximum toxicity at a pH of between 5 and 6 (Figure 3).

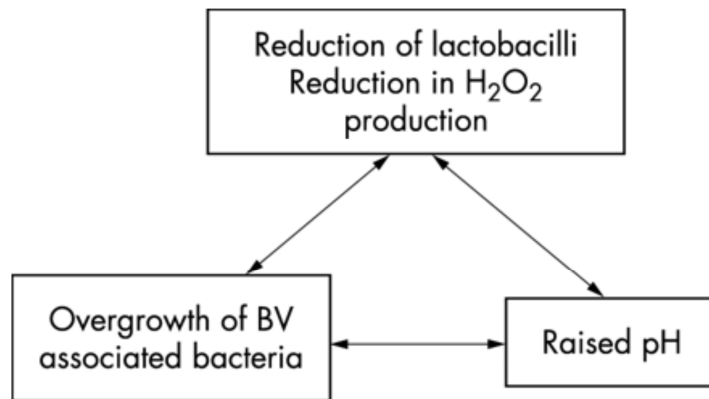


Figure 3: The inter-relationship between lactobacilli, vaginal pH, and bacterial overgrowth in the etiology of bacterial vaginosis.

I.3.2.2. Hydrogen peroxide production

Hydrogen peroxide is postulated to have a crucial role in protecting against the overgrowth of the pathogens in the vagina, since it can be inhibitory to bacteria, fungi, viruses and mammalian cells (29, 107). Hydrogen peroxide alone or in combination with halide and peroxidase, also present in vaginal secretions (205), has potent toxic properties. *In vitro* studies have documented that hydrogen peroxide producing lactobacilli are cidal to HIV 1 (204), *G. vaginalis*, *Bacteroides* sp. (205), *Neisseria gonorrhoeae* (350) and *Candida albicans* (121). The inhibition of the growth of one bacterial species by H₂O₂ generated by another species is a well-recognized mechanism of bacterial antagonism (71, 396). Lactobacilli, as well as other lactic-acid-producing bacteria, lack heme and thus do not utilize the cytochrome system (which reduces oxygen to water) for terminal oxidation. Lactobacilli utilize flavoproteins, which generally convert oxygen to H₂O₂. This mechanism, together with the absence of the heme protein catalase, generally results in the formation of H₂O₂ in amounts that are more than the capacity of the organism to degrade it. The H₂O₂ formed may inhibit or kill

other members of the microflora (71, 396), particularly those which lack or have low levels of H₂O₂-scavenging enzymes, such as catalase peroxidase.

The microbicidal activity of H₂O₂ is considerably increased by the enzyme peroxidase in the presence of a halide ion (202). Among the peroxidases, which can function in this way are those of milk and saliva (lactoperoxidase), neutrophils and monocytes (myeloperoxidase), eosinophils (eosinophil peroxidase) (191), and genital tract secretions (rat uterine fluid and human cervical mucus) (26, 206). The peroxidase and the halide ion greatly enhance the H₂O₂-dependent microbial antagonism between two bacterial species (203), between bacteria and viruses (204), between bacteria and spermatozoa, and between bacteria and mammalian cells (66). Hydrogen peroxide production of bacterial strains is easily measured on a culture medium called TMB (plus) agar (107, 249, 287), containing 3,3',5,5'- tetramethylbenzidine dihydrochloride, a chromogen that colors blue in the presence of H₂O₂ and peroxidase, when exposed to (oxygen rich) air (Figure 4).

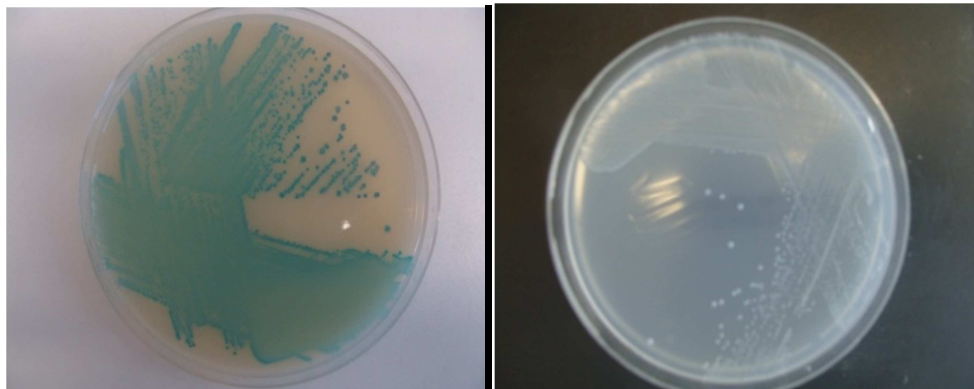


Figure 4: Culture of a vaginal swab on TMBplus agar.

Left: Bacteria positive for hydrogen peroxide production (blue colonies); Right: Bacteria negative for hydrogen peroxide production (white colonies).

I.4. Bacterial vaginosis

I.4.1. History and microbiology

Bacterial vaginosis was first recognized in the late nineteenth century. In 1914, Curtis (70) reported an association between abnormal anaerobic vaginal microflora and vaginal discharge. In 1955, Gardner & Duke reported the clinical and bacteriological

aspects of a syndrome they termed *Haemophilus vaginalis*-vaginitis (nonspecific vaginitis), now renamed bacterial vaginosis (BV). Without using anaerobic culture techniques, the microaerophilic microorganism *Haemophilus* (later termed *Corynebacterium*, now renamed *Gardnerella*) *vaginalis* was said to be the sole etiological agent of BV, a claim that was almost immediately called into question (135). With today's improved culture methods, it is generally recognized that anaerobic bacteria dominate the BV ecosystem, that *G. vaginalis* resides in small or moderate amounts in the vaginal ecosystem of every healthy woman, and that the bacteria tend to increase in number in a BV flora. But *G. vaginalis* has proved to be inadequate for accurate diagnosis of BV, even when semiquantitative culturing methods are used (179, 214). Because much of the vaginosis-associated microflora is anaerobic, the term 'anaerobic vaginosis' has been suggested (25). Several changes in nomenclature occurred over the years. In 1984, the term bacterial vaginosis was proposed (237) and became widely accepted. The term 'bacterial vaginosis' indicates that bacteria rather than fungi or parasites apparently cause this syndrome.

I.4.2. Definition

Bacterial vaginosis is a polymicrobial syndrome in which the normal vaginal lactobacilli, particularly those producing hydrogen peroxide, have been replaced by a variety of anaerobic bacteria and mycoplasmas. Common bacterial species of bacterial vaginosis include *G. vaginalis* (49, 135, 149), *Mobiluncus* species (169), *Bacteroides* species (333), and *Mycoplasma hominis* (214) (Figure 5).

Up to 50% of women with BV remain asymptomatic (410). Symptomatic BV on the other hand is most typically accompanied by foul-smelling, profuse vaginal discharge in the absence of any appreciable signs of inflammation(381). With bacterial vaginosis, concentrations of aerobes and anaerobes are generally elevated 100 to 1000-fold over levels in overtly healthy subjects (347). In addition to the microbiological alterations, biochemical changes, such as an increase in vaginal pH (4), production of volatile amines (62), bacterial sialidase (51), endotoxins (336) and cytokines (420), also occur in BV.

Immunoglobulins A (IgAs) against the hemolytic *G. vaginalis* toxin (Gvh) and sialidase and prolidase activities have been measured in the vaginal fluid of BV-positive women (52).

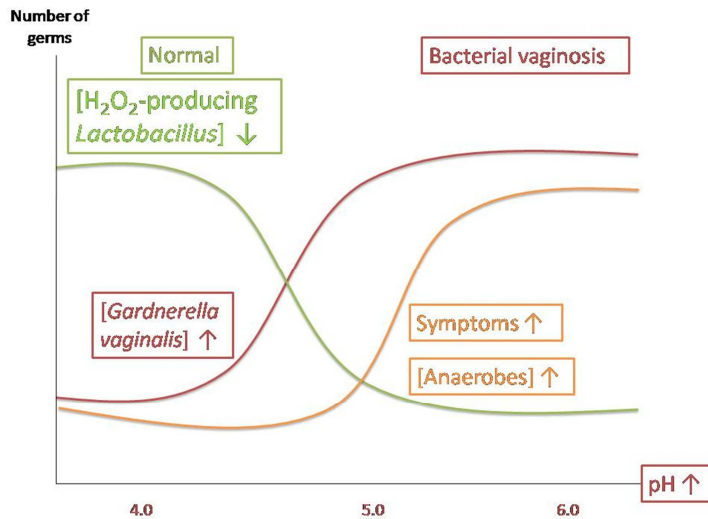


Figure 5: Schematic overview of the transition from a healthy vaginal microflora to bacterial vaginosis.

Despite the substantial microbial overgrowth, BV does not seem to be associated with signs of local inflammation. Polymorphonuclear leukocytes are usually absent or present in low numbers in the vaginal fluid of women with BV, and for this reason the term vaginosis has been used rather than vaginitis.

I.4.3. Prevalence and epidemiology

Bacterial vaginosis (BV) is a very common condition. Studies have revealed that the prevalence varies widely depending on the patient population. In the United States, the prevalence of BV was 29.2%, corresponding to 21 million women with BV and whereby only 15.7% of the women with BV reported vaginal symptoms. Prevalence was 51.4% among non-Hispanic blacks, 31.9% among Mexican Americans, and 23.2% among non-Hispanic whites (212). BV is diagnosed in 9-38% of women attending gynecology clinics (73, 218, 418), in 61% of women attending sexually transmitted disease clinics (4, 103, 108, 347), in 24 to 51% of lesbian women (178) and in up to 85% of female sexual

workers studied in Africa (106). Studies of pregnant women have documented similar prevalence rates to those seen in nonpregnant populations, ranging from 6% to 32% (171, 216, 252, 272, 280). Several risk factors for the acquisition of BV have been identified. Black race (145), smoking (192), sexual activity (42, 384), contraceptive practice and the use of vaginal douching (161) have all been found to be associated with increased prevalence.

I.4.4. Pathogenesis

The massive overgrowth of vaginal anaerobes is associated with increased production of proteolytic carboxylase enzymes, which act to break down vaginal peptides to a variety of amines, which, at high pH, become volatile and malodorous, especially trimethylamine (16). The amines are associated with increased vaginal transudation and squamous epithelial cell exfoliation, creating the typical discharge (342). In conditions of elevated pH, *G. vaginalis* more efficiently adheres to the exfoliating epithelial cells, creating clue cells (i.e., vaginal epithelial cells studded with coccobacillary organisms) (135, 235, 342) (Figure 6). The presence of clue cells is the single most reliable predictor of BV (108). Amines further provide a suitable substrate for *M. hominis* growth. What remains unknown is whether the loss of lactobacilli precedes or follows this massive upheaval in anaerobic microflora (342). Since the original transmissible nature of BV was demonstrated by Gardner & Dukes in 1955 (135), several studies have confirmed that *G. vaginalis* and other BV-associated organisms may be transferred sexually (102, 180, 279, 358). However, transmission alone is not sufficient to cause disease because most of the microorganisms are normally found in low numbers in the healthy vagina.

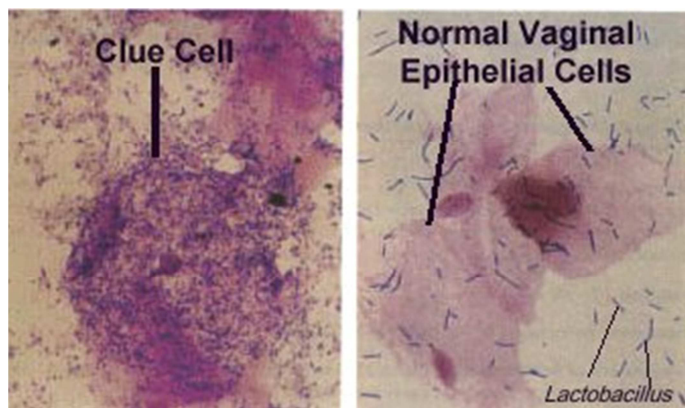


Figure 6: A clue cell (left) versus normal vaginal epithelial cell (right).

I.4.5. Complications of bacterial vaginosis

In pregnant women, BV has been associated with an approximately twofold risk for premature rupture of membranes, and for giving birth to babies with low birth weight (171). BV is associated with chorioamnionitis and there is strong evidence of a causal relationship between ascending intrauterine infection and spontaneous preterm labor and delivery (144, 171, 173, 248, 380, 384), and with serious sequelae related to the first trimester miscarriage in women undergoing *in vitro* fertilization (289), amniotic fluid infections (334), postpartum and postabortal endometritis (390), and postabortal pelvic inflammatory disease (PID) (221).

The aforementioned observations were the basis for recommending screening of all pregnant women for BV as a strategy to reduce the rate of preterm delivery (211). However, routine screening is not the present standard of care because of the highly contradictory results of prospective studies of treating pregnant women with BV (124). In non-pregnant women (357), bacterial vaginosis increases the risk of posthysterectomy infections (220, 346) and PID (171, 399), and increases the risk of transmission of sexually transmitted infections (161, 241, 400).

I.4.6. Diagnosis of bacterial vaginosis

I.4.6.1. Clinical features by Amsel criteria

Bacterial vaginosis is a syndrome that can be diagnosed both clinically and microbiologically. In 1983, Amsel and colleagues (4) published a paper outlining clinical diagnostic criteria, and these are still in use today. The clinical diagnosis of bacterial vaginosis is made if three of the four following signs are present:

- 1) An adherent and homogenous vaginal discharge**
- 2) Vaginal pH greater than 4.5**
- 3) Presence of clue cells**
- 4) positive whiff test (An amine odor after the addition of potassium hydroxide (10% KOH))**

Unfortunately, although these criteria are extremely useful in diagnosing symptomatic and asymptomatic BV, this method is unreliable because of the lack of microscopy-related skills and availability of pH paper in the offices of most general practitioners, and therefore, thousands of women who complain of a malodorous discharge are invariably empirically diagnosed with BV without confirmation. A rapid, inexpensive point-of-care diagnostic test is, therefore, urgently required.

I.4.6.2. Commercial point-of-care tests for the diagnosis of BV

In clinical practice, different commercial point-of-care tests for diagnosing BV are available, based on biochemical reactions or on nucleic acid technology.

I.4.6.2.1. pH measurements:

Two point-of-care tests for assessment of vaginal pH have been commercialized:

1- A self-test pH glove: A glove with an integrated pH indicator paper, developed in Germany in the early 1990s, by which women can monitor their vaginal pH by inserting one finger into the vagina. The glove has been broadly applied in population-based screening programs in Germany for the prevention of preterm birth, by which women were instructed to consult their physician if the pH was 4.7 or more (181).

2- Presence of trimethylamine in combination with vaginal pH assessment: This point-of-care test, the FemExam® (CooperSurgical, Inc, CT, USA) test card, is based on determining of pH and trimethylamine levels in vaginal fluid for the diagnosis of BV; however the test did not compare favorably with Amsel's criteria nor Nugent criteria in published studies (283, 293, 395).

I.4.6.2.2. Presence of trimethylamine (electronic sensor array or electronic nose)

The volatile organic amino acids responsible for the characteristic odor in BV have been used as a target in BV diagnosis. Osmetech Microbial Analyser™ (OMA™) is an electronic nose system in which vaginal swabs are placed in a sample vial and analysed directly without the need for complex extraction procedures. The sample head space is passed over an application specific array of conducting polymer sensors, each of which has specific interactions to different volatile organic species based upon their size, shape and functional group (61). In a large diagnostic study, Hay *et al.* (162) obtained a sensitivity of 81.5% and specificity of 67.1% with the electronic nose compared with Amsel's criteria, and a sensitivity and specificity 82.9 and 77.3% compared with Gram stain diagnosis.

I.4.6.2.3. Testing for sialidase activity

The BVBlue® Test (Gryphus Diagnostics, AL, USA) is a chromogenic diagnostic test based on the presence of elevated sialidase enzyme in vaginal fluid samples. The sialidase is produced by bacterial pathogens associated with BV, including *Gardnerella* and *Mobiluncus* (261).

I.4.6.2.4. Testing for proline aminopeptidase activity

Proline aminopeptidase activity (Pip Activity Test card™, Quidel Corp., CA and CooperSurgical, CT) is a rapid and reliable laboratory test for diagnosis of bacterial vaginosis and would be helpful in the clinical diagnosis of this disease. Elevated proline aminopeptidase activity has been shown to predict accurately women with a clinical diagnosis of bacterial vaginosis (319). However, this assay has significant practical disadvantages, the most notable of which is the production of a carcinogenic end product, alpha-naphthylamine (319).

I.4.6.2.5. DNA probe for *G. vaginalis* rRNA

The Affirm™ VP III Microbial Identification Test (BD Diagnostic Systems, NJ, USA) DNA hybridization assay is used for the detection and identification of *Candida* species, *G. vaginalis* and *Trichomonas vaginalis* from vaginal fluid specimens in both symptomatic and asymptomatic patients (41). It is based on the principles of nucleic acid hybridization and uses two distinct single-stranded probes for each organism, a capture probe and a color development probe. After completion of the test, the results of the assay can be visually observed (41). The rapid turnaround time (between 30 and 45 min) enables the availability of results to clinicians within 24 hours.

I.4.6.3. Gram stain

To perform a Gram stain, vaginal discharge is collected on a glass slide, allowed to air-dry, Gram-stained in the laboratory, and examined under oil immersion at a 100 times magnification for the presence of bacteria. This diagnostic method has several advantages, including a permanent record, a high frequency of interpretable results, low cost, and ease of transport and storage (214). Over the past two decades, there have been several Gram stain diagnostic schemes used for the evaluation of BV.

In 1892, Döderlein (84) described three grades of "vaginal cleanliness" based on microscopic examination of unstained vaginal fluid. Grade I indicates a "clean vagina" whereby lactobacilli predominate. Grade III is Döderlein's "pathological flora" where the discharge is described as "profuse and purulent or rather scanty and watery," and therefore appears to describe patients with bacterial vaginosis (BV) or trichomoniasis. Grade II is intermediate between Grades I and III and is associated with a mixed microflora including fewer lactobacilli than seen in Grade I.

Numerous methods for microscopic diagnosis of BV have been described since that time. Dunkelberg (92) used the Gram stain to diagnose BV by the detection of clue cells. In 1983, Spiegel and colleagues (348) published a paper describing an objective way to diagnose bacterial vaginosis by Gram's stain. An inverse relationship between the quantity of the *Lactobacillus* cell morphology type, *i.e.* large gram-positive rods and of the *Gardnerella* cell morphology type, *i.e.* small gram-variable rods, was noted on Gram

stain ($P < 0.001$)(348). When Gram stain showed a predominance (3 to 4+) of the *Lactobacillus* cell type with or without the *Gardnerella* cell type, it was interpreted as normal. When Gram stain showed mixed flora consisting of gram-positive, gram negative, or gram-variable bacteria and the *Lactobacillus* cell type was decreased or absent (0 to 2+), the Gram stain was interpreted as consistent with BV (348). Spiegel and coworkers (348) demonstrated high intraobserver reliability for 20 specimens examined by three separate microbiologists. Although used for many years, the Spiegel system was criticized because there was no account for the spectrum of severity (266).

In 1991, Nugent and colleagues (266) addressed this problem and developed a scoring system, presented in The scoring criteria summed the weighted quantitation (0, 1 to 4+) of the following morphotypes to yield a score of 0 to 10 for each person: large gram-positive rods (lactobacillus morphotypes) (weighted such that absence yielded the highest score), small gram-negative to -variable rods (*G. vaginalis* and *Bacteroides* spp. morphotypes), and curved gram-negative rods (*Mobiluncus* spp. morphotypes) (Table 1).

Table 1. Laboratory examination of vaginal smears and the determination of the Nugent Score. Cell types are scored as the average number seen per field by light microscopy of Gram stained smears at x1000 magnification with oil immersion. Total score= lactobacilli + *G. vaginalis* and *Bacteroides* spp. + curved rods.

Score	<i>Lactobacillus</i> cell types	Number of <i>Gardnerella</i> and <i>Bacteroides</i> spp. cell types	Number of Curved bacteria cell type (<i>Mobilincus</i>)
0	> 30	0	0
1	5-30	< 1	1-5
2	1-4	1-4	> 5
3	< 1	5-30	
4	0	> 30	

The criterion for bacterial vaginosis is a score of 7 or higher. A score of 4 to 6 is considered intermediate, and a score of 0 to 3 is considered normal. Nugent scoring for Gram stained vaginal smears has shown high intercenter and intracenter reliability as well as high intraobserver and interobserver reproducibility (187, 243), but the reading of the slides needs to be done by trained staff and is rather time-consuming. Gram stain of vaginal secretions is more reliable than wet mount, with a sensitivity of 93% and specificity of 70%, but it is rarely used by practitioners (266).

Another grading scheme for Gram stains was proposed in 2002 by Ison and Hay (185). The Ison and Hay criteria that were developed initially in a study of pregnant women just defined normal microflora, BV, and intermediate grades (185). It is based on the estimation the ratios of the observed cell types rather than on the exact number of the bacteria. This system was updated (185) to include two additional Gram stain categories (Table 2). Grade 0 was used to indicate smears with no bacteria and Grade IV those with only gram-positive cocci attached to epithelial cells. Both of these patterns may be most common in women who recently received antibiotic therapy. The scheme of Ison and Hay shows resemblances to the wet smear categorization (Reinheitsgrade) proposed by Schröder in 1921 (324).

Table 2. Grading scheme according to Ison and Hay (163, 164, 185).

Grade	Categorization	Gram stain microscopy
0		Epithelial cells with no bacteria seen
I	Normal vaginal microflora	<i>Lactobacillus</i> cell type
II	Intermediate vaginal microflora	Reduced <i>Lactobacillus</i> cell type with mixed bacterial cell types
III	Bacterial vaginosis	Mixed bacterial cell types with few or absent <i>Lactobacillus</i> cell types
IV	Gram positive cocci	Epithelial cells covered with Gram positive cocci only

In 2005, Verhelst *et al.* (380) published a modification based on these Gram stain criteria. Grade I was divided into grade Ia, Ib and Iab and an additional category was created, called grade I-like. A grade Ia slide contains particularly thick (plump) and short *Lactobacillus* cell types whereas grade Ib consists mainly of long, small and less stained Gram positive rods. Grade Iab is a mixture of both types. In the additional category, grade I-like, especially diphtheroid cell types can be seen (Figure 7), and culture revealed *Bifidobacterium* species in 19 out of 36 grade I-like samples.

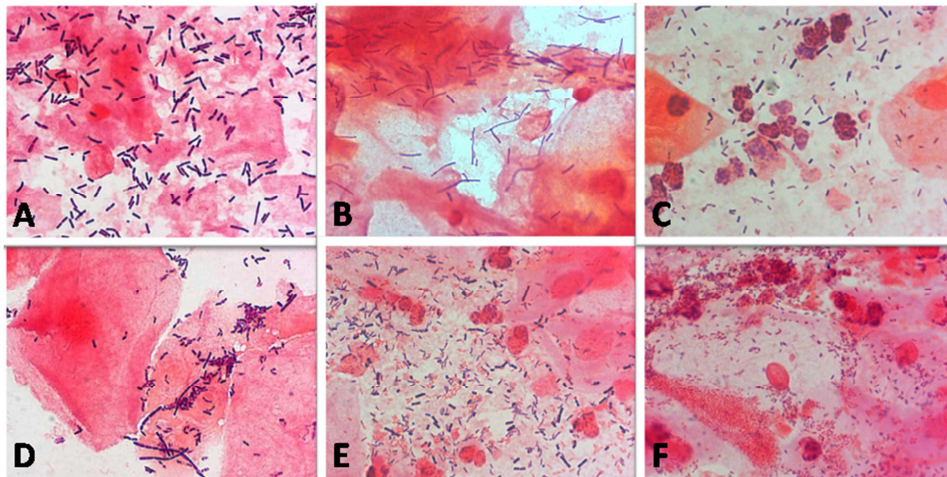


Figure 7: Gram stain of vaginal swabs with grades according to (380).
A: grade Ia; B: grade Ib; C: grade Iab; D: grade I-like; E: grade II; F: grade III.

I.4.6.4. Fresh wet-mount microscopy

Wet mounting has the advantage that it can be performed immediately and therefore allows immediate preventive, diagnostic, or therapeutic action where indicated. Using saline without additive colouring or fixation, Albert Döderlein (84) was able to show lactobacilli in vaginal secretions of healthy women and a *Lactobacillus*-deficient microflora in women with postpartum endometritis. His successor, Schröder, started to use this information in a broader clinical context, and the first VMF grading scheme was initiated (324).

Schmidt *et al.* evaluated a scoring system (Table 3), weighting small bacterial morphotypes versus lactobacillary morphotypes. The wet mount criteria of Schmidt and Hansen (314-316) resembles Nugent scoring (266) in that it ranks the quantities of

lactobacilli and cocci in the same way, although the demarcations of the intervals differ (105). Moreover, the Schmidt system does not incorporate the presence of *Mobiluncus*. The Schmidt method has been validated for diagnosis of BV in primary care populations (105). For wet mount, the numbers of lactobacilli and of *G. vaginalis* are evaluated, whereas for Nugent, next to these cell types (Gram positive rods: lactobacillary cell types, small Gram negative to variable rods: *Gardnerella* and *Bacteroides* spp. cell types) also curved Gram variable rods are taken into account but with a lower weight than the others.

An international workshop on vaginal smear-based diagnosis of bacterial vaginosis showed that the interobserver reproducibility of interpretations of Nugent scores, Ison and Hay scores and wet smear scores for the diagnosis of bacterial vaginosis was high. (126). The scheme of Ison and Hay also showed resemblances to the wet mount categorization (Reinheitsgrade) proposed by Schröder in 1921 (Grade 1: Döderleins bacilli only, Grade II: Döderleins bacilli and other organisms, Grade III: organisms other than Döderleins bacilli) (324).

Based on wet mount, Donders *et al.* (87) distinguishes lactobacillary grades I, IIa, IIb and III. They also compared the accuracy of lactobacillary grading screened by wet mount with that using Gram stained specimens in a small study and found a higher false positive rate in Gram stained specimens than in wet mounts (88). In addition, they use terms as aerobic vaginitis, desquamative vaginitis and BV and evaluate the presence of leukocytes and parabasal cells (86, 87).

Table 3. Scoring system for evaluation of bacterial cell types in wet mount preparation at x400 magnification with phase-contrast microscopy

Score	Number of <i>Lactobacillus</i> Cell morphology types per field*	Number of small bacterial cell morphology types per field*
0	> 30	0
1	16-30	1-5
2	6-15	6-15
3	1-5	16-30
4	0	> 30

Bacterial cell morphology type ('morphotype') score (BMS) = lactobacillary 'morphotype' score + small bacterial 'morphotype' score. A bacterial 'morphotype' score of 7-8 is indicative of bacterial vaginosis.

* Number of 'morphotypes' is the average number observed

I.4.6.5. Treatment

The Centers for Disease Control and Prevention (CDC 2006) recommends testing all symptomatic women and treating those who are positive for BV. The recommended treatment for BV is metronidazole 500 mg orally twice daily for 7 days, or metronidazole gel 0.75% intravaginally daily for 5 days, or clindamycin cream 2% intravaginally once per day for 7 days (411). Alternative treatments include clindamycin, 300 mg orally twice a day for 7 days or clindamycin ovules 100 mg intravaginally once at bedtime for 3 days (411). The oral metronidazole can cause some minor but unpleasant side effects, but is believed to be the most effective treatment. The gels do not typically cause side effects, although yeast vaginitis can occur as a side effect of the medication. Tinidazole (Tindamax) is an antibiotic that appears to have fewer side effects than metronidazole and is also effective in treating bacterial vaginosis. In addition to being labeled for the treatment of trichomoniasis and bacterial vaginosis, it is also indicated for the treatment of giardiasis and amebiasis. Several studies (17, 231, 257) have indicated at least equivalent therapeutic efficacy compared with metronidazole, with some evidence of possible superior activity. However, additional studies will be required to support this claim. Secnidazole (267, 309) and ornidazole (104, 308, 309) have been

investigated *in vivo*, and seem to be as effective as metronidazole. Secnidazole has been also shown to be equally effective as metronidazole *in vitro* (74). An advantage of secnidazole over metronidazole is its longer half life time, such that only one dose needs to be taken, avoiding patient compliance related problems.

I.5. Culture-dependent methods to study VMF

I.5.1. Culture media

Different media, selective, semi-selective and non-selective, have been used for culturing vaginal /rectal swabs. Non-selective media are generally used to estimate total numbers of both aerobic and anaerobic organisms. Media used for the recovery of facultative anaerobes are 5% sheep blood in tryptic soy agar, Columbia agar, mannitol salt agar, and MacConkey agar. Chocolate agar is used for the recovery of *G. vaginalis*, whereas the culture media for recovering anaerobes are prereduced *Brucella*-base agar with 5% sheep blood, enriched with hemin and vitamin K1 and prereduced *Brucella*-base agar with 5% laked sheep blood, 100 mg/mL kanamycin, and 7.5 mg/mL vancomycin, supplemented with hemin and vitamin K1 (81). Several selective/ semi selective media have been developed for cultivation of lactobacilli, *i.e.* Rogosa agar (300), de Man, Rogosa and Sharpe medium (MRS) (80), LBS (*Lactobacillus* selective agar (258)) and LAMVAB (*Lactobacillus* anaerobic MRS agar with vancomycin and bromocresol green (160)).

HBT Bilayer Medium was described in 1982 by Totten *et al.* (367) for the selective isolation and detection of *G. vaginalis* from clinical specimens. It also supports the growth of the more fastidious *Lactobacillus* species, such as *L. iners*. Colonies of *G. vaginalis* may be differentiated by a diffuse, beta-hemolytic reaction, which they produce in the presence of human blood. Still, the fact that *L. iners* can grow on HBT, produces small colonies and stains Gram-negative (75) , is probably the main reason why several *L. iners* isolates have been misidentified as *G. vaginalis* (307). *Gardnerella* selective agar with 5% human blood is a partially selective and differential medium for the isolation of *G. vaginalis* from clinical specimens. It is based on Columbia CNA agar, which provides nutrients and the antimicrobials colistin and nalidixic acid, which inhibit Gram negative

but not Gram positive bacteria, and amphotericin B, which inhibits vaginal yeasts. The medium is supplemented with Proteose Peptone to improve the growth of *Gardnerella*. Human blood is added as a nutrient and to detect the characteristic diffuse beta hemolysis of the organism (373). A7 medium (Northeast Laboratory, Waterville, Maine) is used for the isolation of *Mycoplasma* and *Ureaplasma* species and *Trichomonas vaginalis* organisms can be detected with Diamond's medium (PML Microbiologicals, Warwick, R.I.)

The medium TMB-Plus consists of *Brucella* agar base, 3,3,5,5-tetramethylbenzidine, horseradish peroxidase, starch, vitamin K, hemin, magnesium sulfate, manganese sulfate, and horse serum. It is used for detecting H₂O₂ production by lactobacilli (287) (Figure 4). The disadvantage of selective media is their not absolute selectivity and their toxicity against certain isolates of the species, yielding some false positive and false negative results.

I.5.2. Phenotypic identification methods

Biochemical identification methods for vaginal bacterial species are cumbersome and have very limited discriminatory power. For example, there are no good biochemical reactivity identification schemes to discriminate between the different *Lactobacillus* species. Also, based on a limited number of characteristics, *L. iners* has been misidentified as *G. vaginalis* (see also I.4.6.5.1).

Boyd *et al* (36), evaluated the API 50 CH identification system for the identification of 97 strains of commensal lactobacilli. They observed a high level of intraspecific phenotypic variability and a limited number of useful differences between species. This, in combination with a limited database for these species, limits the usefulness of phenotypic identification methods.

I.5.3. Molecular identification and typing of cultured bacteria

I.5.3.1. PCR-independent methods

Using a DNA-hybridization method, Giorgi *et al.* (142) identified *L. crispatus*, *L. jensenii* and *L. gasseri* but not *L. acidophilus*, as the predominant vaginal *Lactobacillus* species colonizing the vagina of women with normal vaginal microflora. Song *et al.* (345)

identified *Lactobacillus* isolates from stool specimens of mothers and infants and from vaginal swabs of women to study the ecology of intestinal and vaginal lactobacilli (5).

Also, Antonio *et al.* (5) used whole chromosomal DNA hybridization probes for known *Lactobacillus* species to identify isolates cultured from sexually active women. He identified *L. iners* as a member of the vaginal microflora and reported the species specificity of H₂O₂-production by lactobacilli.

The most commonly employed target for molecular identification of bacteria is the small ribosomal subunit or 16S rRNA gene. Kullen *et al.* (215) and Tarnberg *et al.* (361) used **sequencing** of the V1-V3 region of the 16S rRNA gene for accurate identification of bacteria in the *L. acidophilus* group.

I.5.3.2. PCR-dependent methods

The combination of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (**DGGE**) has been used to characterize the microflora isolated from different parts of the human body (43, 46, 246). Primers designed to amplify DNA have been largely based on universal bacterial 16S ribosomal subunit gene sequences (114, 166, 297, 311, 423). DGGE and temporal temperature gradient gel electrophoresis (**TTGE**) have been successfully used for examination of the vaginal microflora (43, 46).

Gancheva *et al.* (134) used molecular techniques such as Randomly Amplified Polymorphic DNA analysis (RAPD) and Amplification Fragment Length Polymorphism analysis (**AFLP**) for the identification on species level of members of the *L. acidophilus* group although these techniques are more commonly used for the typing of strains (intraspecies variability) than for identification.

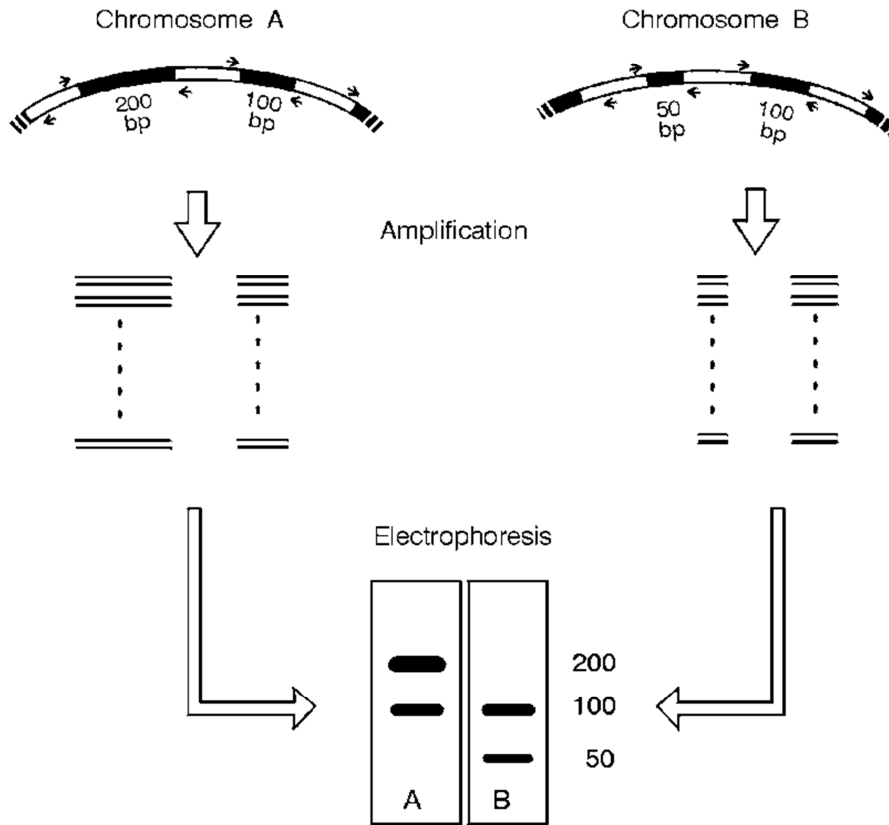
In our laboratory we use a technique that not only allows to unambiguously identify *Lactobacillus* species but also most cultivable species present in the vaginal microflora. tRNA-intergenic spacer length polymorphism PCR (**tDNA-PCR**), first described in 1991 (393) allows rapid, discriminative and low cost identification of most cultivable bacteria.

I.5.3.2. Principle of tRNA intergenic polymorphism length analysis (tDNA-PCR).

Baele et al.(10) applied tDNA-PCR using universal primers, followed by separation of the PCR products with capillary electrophoresis, in order to evaluate the discriminatory power of this technique within the genus *Lactobacillus* and were able to discriminate 21 of 37 tested species. It consists of the amplification of the spacer regions in between tRNA genes by use of consensus primers, which are complementary to the highly conserved edges of the flanking tRNA genes and are directed outwardly (Figure 8). The resulting PCR fragments can be separated by capillary electrophoresis. tDNA-PCR makes use of primers complementary to regions.

It has been applied (combined or not with capillary electrophoresis) for *e.g.* *Acinetobacter*, (94, 398), *Enterococcus* (9), *Lactobacillus* (10), *Legionella* (76), *Listeria* species (375), *Staphylococcus* (77, 232) and *Streptococcus* (77). Verhelst *et al.* (379) combined culture with tDNA-PCR (9, 10, 393) and sequencing of the 16S rRNA-gene to characterize the vaginal microflora (5, 10, 45, 46, 377). Interestingly, tDNA-PCR is one of the few approaches that enables differentiation of most *Lactobacillus* species, including *Lactobacillus crispatus*, *L. gasseri* and *L. iners*, which were previously all lumped together into the *L. acidophilus* complex, moreover in a rapid and relatively cheap manner. We applied it for this doctoral research for the identification of rectal and vaginal microflora (100).

INTERREPEAT SPACER LENGTH
POLYMORPHISM ANALYSIS



- Consensus primers, outwardsly directed
- ▭ Repeat element
- Interrepeat spacer

Figure 8: The principle of tRNA intergenic polymorphism length analysis (tDNA-PCR).

Several molecular techniques have been applied to study the genetic diversity of lactobacilli, such as restriction fragment length polymorphism analysis (RFLP) (143), ribotyping (299), pulsed-field gel electrophoresis (PFGE) (378) and multilocus sequence typing (MLST) (47). Nigatu *et al.* (264) reported that RAPD can be used as a rapid, efficient and reproducible genetic tool for the classification and identification of *Lactobacillus* species.

I.5.3.3. Principle of RAPD (Random Amplification of Polymorphic DNA)

RAPD (pronounced "rapid") stands for Random Amplification of Polymorphic DNA, whereby the fragments of DNA that are amplified are random. In this reaction, a single primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocyclic amplification (Figure 9). The presence of each amplification product identifies complete or partial nucleotide sequence homology, between the genomic DNA and the oligonucleotide primer, at each end of the amplified product. On average, each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphism between individuals. (392, 404). Because the RAPD technique is an amplification-based assay, only nanogram quantities of DNA are required, and automation is feasible. Because it relies on a large, intact DNA template, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. Despite producing simple patterns, RAPD has been used extensively to fingerprint bacteria (1, 115) and fungi (69, 147), to study phylogenetic and taxonomical relationships in plants (182), and to establish molecular relatedness and genetic variation in animals (271).

I.6. DNA-based culture-independent methods to study VMF

I.6.1. PCR-independent methods

An approach complementing broad range PCR is characterization of the vaginal bacterial community by using nucleic acid probes, oligonucleotides complementary to rRNA gene targets. Probes are designed using sequences generated from broad range PCR and sequencing experiments which can have a wide range of phylogenetic specificities ranging from domain to strain levels.

FISH (fluorescence in situ hybridization) is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on the genome of the

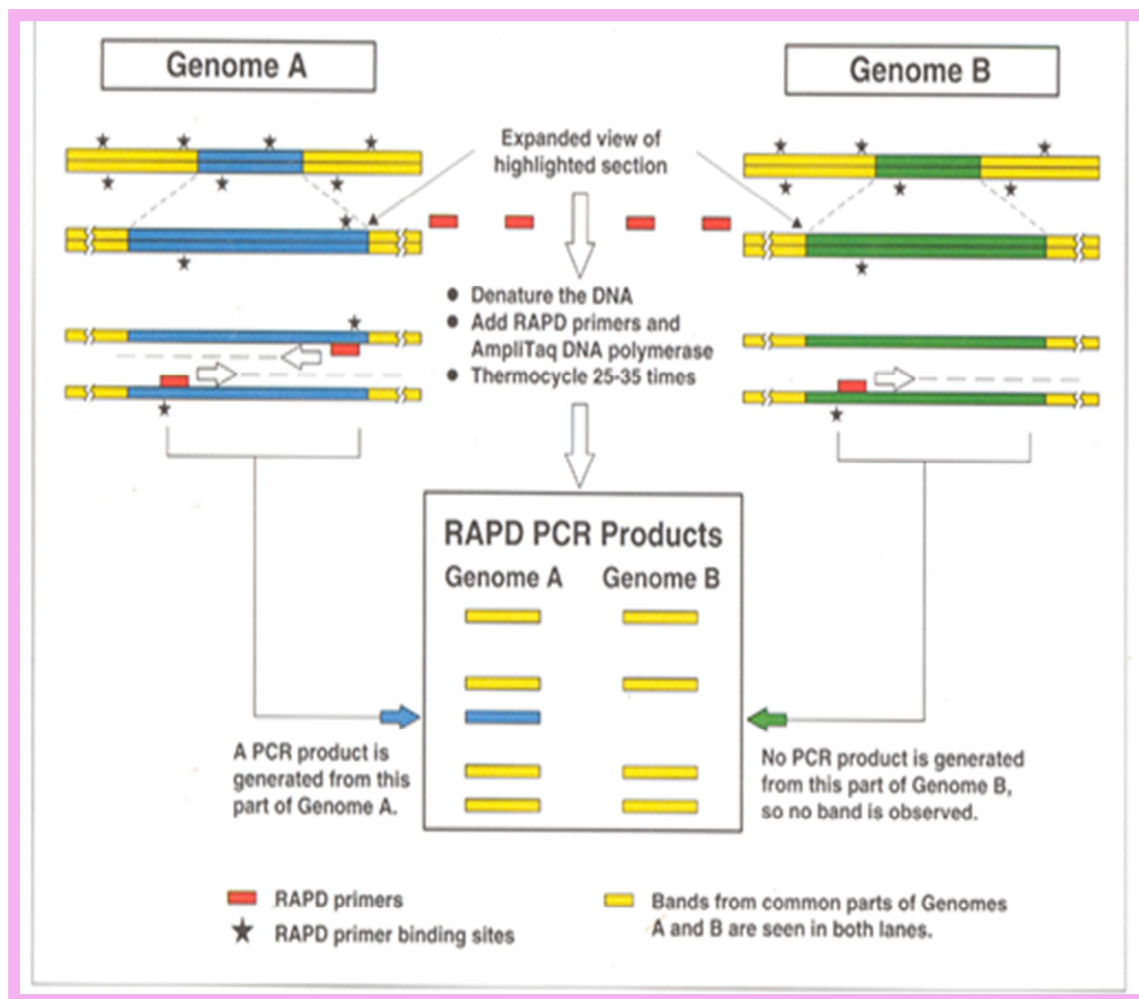


Figure 9: The principle of RAPD PCR analysis.

bacteria in the sample. FISH uses fluorescent probes (often complementary to a sequence in the 16S or 23S rRNA gene) that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out where the fluorescent probe bound to the bacteria. FISH evaluation of vaginal biopsy specimens demonstrates that the presence of an adherent vaginal biofilm that predominately hybridizes with a *G vaginalis* probe is sensitive and specific for detecting bacterial vaginosis (359). The main disadvantage of these probe-based methods is their high workload, preventing fast analysis. In addition, these techniques can detect only species for which DNA probes have been prepared. An

advantage is that hybridization occurs without amplification, overcoming biases in quantification imposed by PCR amplification procedures and thus providing quantitative data.

I.6.2. PCR-dependent methods

Studies on many habitats have demonstrated the limitations of cultivation-dependent methods to assess microbial community composition. In most instances, this is because readily cultivated populations represent a small fraction of the extant community (245). In recent years, culture-independent methods based on the analysis of 16S and 18S rRNA gene sequences have been used to overcome many of these limitations (389). The **16S rRNA gene** which encodes a constituent part in bacterial ribosomes has a structure and size that makes it an excellent molecule to manage and manipulate, and has proven to be very useful in the study of vaginal lactobacilli. 16S rRNA gene has regions of sequence conservation that can be targeted with broad range PCR primers and areas of sequence heterogeneity that can be used to identify bacteria or infer phylogenetic relationships (13, 273, 317, 394).

These methods have been successfully used in numerous studies to explore the microbial diversity in various habitats (46, 422, 424). Also they can reveal the existence of novel taxa and result in a perception of diversity that is quite different from that provided by cultivation-dependent methods (290). These studies have often included construction and analysis of 16S rRNA gene clone libraries to provide precise information as to the phylogeny of the constituent populations. In addition to being widely used for studies on the ecology of terrestrial and aquatic habitats (91, 95, 245), they are increasingly being used to study human and animal flora, including that of the colon and subgingival crevice (46, 177, 195, 213, 274, 356).

Several manuscripts about **cloning** of the vaginal microflora have been published recently, using the 16S rRNA-gene (128, 183, 268, 379) or the chaperonin-60 gene (170). The gene encoding chaperonin-60 (cpn60) offers several advantages over the widely used 16S recombinant RNA (rRNA) gene as a target for microbial species

identification and phylogenetics. The *cpn60* universal target region generally provides more discriminating and phylogenetically informative data than the 16S rRNA target, particularly between closely related species (40). Verhelst *et al.* (379) published in 2004 the results of a 16S rRNA-cloning experiment of the vaginal microflora of eight women and discovered the association between the previously not recognized bacteria *A. vaginae*, with *G. vaginalis* and BV. The association was confirmed by means of Terminal Restriction Fragment Length Polymorphism-analysis (**tRFLP**) (364, 383) and with DGGE (117). T-RFLP is a molecular technique for profiling of microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. The method is based on digesting a mixture of PCR amplified variants of a single gene using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer. The result is a graph image where the X axis represents the sizes of the fragment and the Y axis represents their fluorescence intensity. Verhelst *et al.*, (380) reported that the agreement between Gram stain categorization as grade Iab and t-RFLP-analysis positive for *L. crispatus* was 92.9% whereas *L. crispatus* was detected by tRFLP only in 27.3%, 20.0%, 22.5% and 0% for grades Ib, I-like, II and III, respectively. These results indicate the possibility to recognize *L. crispatus* bacteria upon cell morphology, a finding that is of importance since this species is clearly associated with healthy microflora, and possibly better ensures stable healthy microflora than other lactobacilli (371). Verstraelen *et al.* (382) showed with tRFLP that the presence of *L. crispatus*, even when accompanied by the other *Lactobacillus* species, *L. jensenii*, *L. gasseri* and/or *L. iners*, is a prominent stabilizing factor to the vaginal microflora.

Screening clones in a library by amplified ribosomal DNA restriction analysis (**ARDRA**) in order to limit the number of clones to be sequenced is also commonly used (376, 379). ARDRA is based on the restriction digestion of 16S rRNA gene clones or amplified DNA and electrophoretic separation on high percent agarose or polyacrylamide gels (128).

The recently available high-throughput 454 **pyrosequencing** now allows for in-depth sequencing and analysis of microbial community composition, and also for a dramatic

increase in throughput via parallel in-depth analysis of a large number of samples with limited sample processing and lower costs (156, 255, 385). This technique has been successfully used in various ecosystems including skin (120), chronic wounds (90), and oral microbiota (198). Ling *et al.* (229) confirmed that the barcoded pyrosequencing approach can be a powerful tool for characterizing the microbiota in vaginal ecosystems compared with classical molecular ecological approaches, such as PCR-DGGE. They observed that at a high taxonomic level, the phylum of Bacteroidetes, Actinobacteria and Fusobacteria were significantly associated with BV. Also their data indicated that the vaginal communities including *Gardnerella*, *Atopobium*, *Megasphaera*, *Eggerthella*, *Aerococcus*, *Leptotrichia/Sneathia*, *Prevotella* and *Papillibacter* were clearly associated with BV.

The broad-range bacterial PCR studies identified key organisms related to BV and opened the door for detection of these bacteria by either **species specific PCR** or **quantitative real time PCR**. Several assays for the detection of *G. vaginalis*, targeting respectively, the 16S-23S rRNA spacer region (269, 372, 421) or the 16S rRNA gene (263) were designed. In addition, species specific PCRs were developed for the fastidious anaerobic *Mobiluncus* spp. (269, 330) and *Mycoplasma* spp. (421), which is of special importance for the latter, as this species is not detected by Gram staining. Recently, several research groups applied species specific PCR for *A. vaginae* since this species was found to be more specific for BV than *G. vaginalis* (44, 118, 128, 379). Species specific PCR studies that applied combination of key organisms suggested that PCR-based amplification might potentially be used for molecular diagnosis of BV and resulted in the application of qPCR allowing also quantification of the bacteria.

I.6.3. Quantitative real time PCR

The first efforts to investigate the applicability of qPCR for the diagnosis of BV came from Zariffard *et al.* (421), who determined the feasibility of using this technique to detect and quantify *Lactobacillus* species, *G. vaginalis* and *Mycoplasma hominis* in the genital tract of 21 women using stored vaginal samples. The results show that samples from women that were diagnosed clinically as having BV had significantly higher

numbers of *G. vaginalis* but significantly lower numbers of lactobacilli. In another study, Sha *et al.* (332) compared Nugent score with Amsel criteria and quantitative bacterial PCR for diagnosing BV in 203 samples from women with Nugent scores of 7-10 (BV-group) and 203 samples from women with Nugent scores of 0-3 (no-BV group). Although there was significant overlap in the log₁₀ *Lactobacillus* counts between the two groups, their data demonstrate that quantitative bacterial PCR for *G. vaginalis*, *M. hominis* and lactobacilli significantly correlates with the Nugent Gram-stain method with regard to diagnosis of BV.

Several research groups also applied an *A. vaginae* qPCR to study the role of this species in BV (38, 119, 360, 368). Bradshaw *et al.* (38) and Ferris *et al.* (117) studied the presence of *A. vaginae* before and after treatment with metronidazole. Bradshaw *et al.* found higher recurrence rates in women for whom both *A. vaginae* and *G. vaginalis* had been detected pretreatment, and Ferris *et al.* found that high pretreatment *A. vaginae* concentrations are predictive of adverse treatment outcomes for BV patients. De Backer *et al.* (75) used qPCR and found that the presence of *A. vaginae* seems to be diagnostically a more valuable marker for BV than the presence of *G. vaginalis*.

Recently, a few research groups applied broader sets of qPCR for the diagnosis of BV.

Menard *et al.* (254) used series of species-specific primers for qPCR targeting *Lactobacillus* species, *G. vaginalis*, *Mobiluncus curtisii*, *M. mulieris*, *Ureaplasma urealyticum*, *A. vaginae*, *M. hominis*, and *Candida albicans*. They were able to document that the presence of *A. vaginae* at a level of 10⁸ copies/ml or more and of *G. vaginalis* at a level of 10⁹ copies/ml or more was highly predictive for BV (254).

Fredricks *et al.* (129) used eight qPCR assays targeting both easily cultivated vaginal bacteria (*G. vaginalis* and *L. crispatus*) and fastidious bacteria (BVAB1, BVAB2, BVAB3, *Leptotrichia/Sneathia*, *Atopobium* and *Megasphaera*-like species) to determine how concentrations of vaginal bacteria change in women with BV by comparing women who cured to women with persistent BV one month following vaginal metronidazole treatment. Successful antibiotic therapy resulted in 3- to 4-log reduction in the median

bacterial loads of BVAB1, BVAB2, BVAB3, a *Megasphaera*-like bacterium, *Atopobium* species, *Leptotrichia/Sneathia* species and *G. vaginalis*.

With the advent of qPCR, it has also become possible to quantify the bacterial loads of different species at different sites (*e.g.* vaginal vs rectal microflora)(96, 100) or at different time points (*e.g.* during different periods of the menstrual cycle (Lopes *et al.* in preparation)).

A huge range of qPCR formats has been described. Besides detection of the amplified DNA by means of the DNA binding dye SYBR Green (24, 75, 119), different probe-based formats have been described, using hybridization probes (8, 196, 208), a hydrolysis probe (22, 129, 194, 253)(Figure 10), molecular beacons (369, 370) and scorpions (302, 397)(Figure 11).

I.6.3.1. DNA binding dyes

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light (Figure 10A). Thus, as a PCR product accumulates, fluorescence increases (259). The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers (298) and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles. Accurate results demand a specific PCR, which can be confirmed via dissociation curve analysis, where the presence of different PCR products is reflected in the number of first-derivative melting peaks (298) or gel analysis (226).

I.6.3.2. Hybridization probes

In this technique (Figure 10C), one probe is labeled with a donor fluorochrome (*e.g.* FAM) at the 3' end and a second probe is labeled with an acceptor fluorochrome (*e.g.* LC Red640, LC red 705) at its 5' end. When the two fluorochromes are in close vicinity (*i.e.* within 1–5 nucleotides), the emitted light of the donor fluorochrome will excite the

acceptor fluorochrome, a process referred to as fluorescence resonance energy transfer. This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals (374).

I.6.3.3. Hydrolysis probe

In the hydrolysis probe assay (Figure 10B), exemplified by the TaqMan chemistry, also known as the 5' nuclease assay, fluorescence is generated upon probe hydrolysis to detect PCR product accumulation. TaqMan probes depend on the 5'-nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end (139), which allows the quencher to reduce the reporter fluorescence intensity by FRET when the probe is intact (67). These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'-nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage. Several different fluorophores (*e.g.* 6-carboxyfluorescein, acronym: FAM, or tetrachlorofluorescein, acronym: TET) and quenchers (*e.g.* tetramethylrhodamine, acronym: TAMRA, or dihydrocyclopyrroloindole tripeptide minor groove binder, acronym: MGB) are available (217).

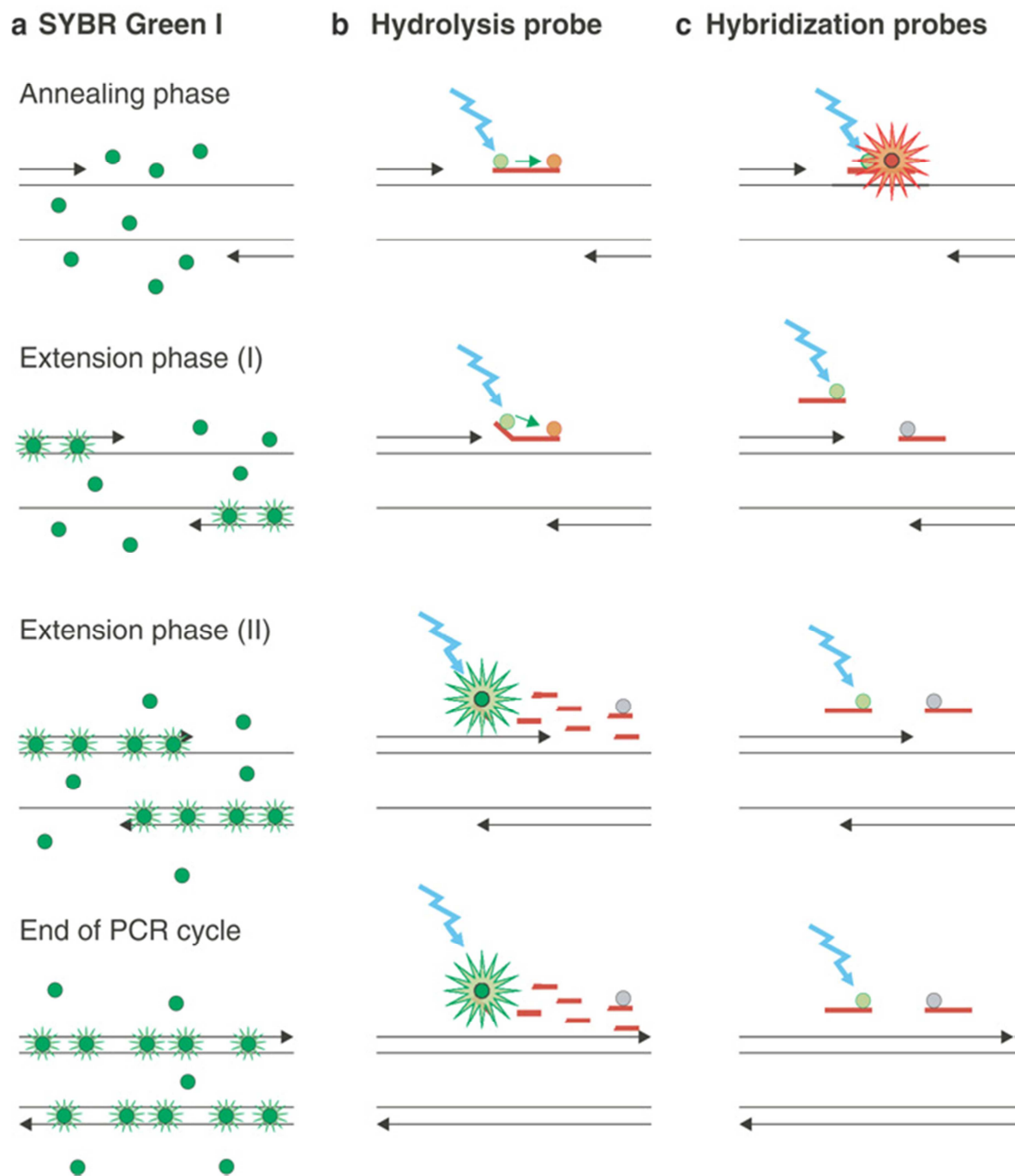


Figure 10: Real-time PCR detection chemistries (374).

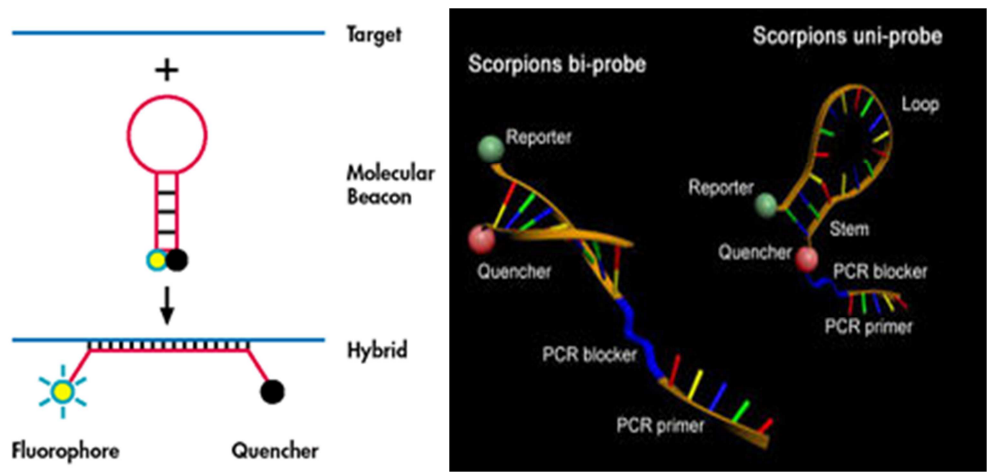


Figure 11: Molecular beacon (left) and scorpion technology (right).
 (Adapted from http://www.gibthai.com/services/technical_detail.php)

I.7. Prenatal screening of Group B streptococci

I.7.1. Group B streptococci

The Group B *Streptococcus* (GBS) (*Streptococcus agalactiae*) is an important cause of neonatal infection. In 1935, Rebecca Lancefield described asymptomatic vaginal carriage of GBS (219). Three years later, Fry reported three cases of fatal puerperal sepsis with GBS (132) and shortly thereafter, Hill and Butler described severe and fatal cases, following childbirth in Melbourne (168). In the 1970s, GBS emerged as the primary pathogen causing neonatal sepsis (127). This disease affects 0.6 per 1,000 births (20).

GBS is a Gram-positive diplococcus (Figure 12), which grows under both aerobic and anaerobic conditions (14). The organism has a polysaccharide capsule that defines the individual serotypes of GBS. Several serotypes of GBS have been identified and they are numbered Ia, Ib, II-VIII. The most common serotypes causing neonatal infections are Ia, III and V.

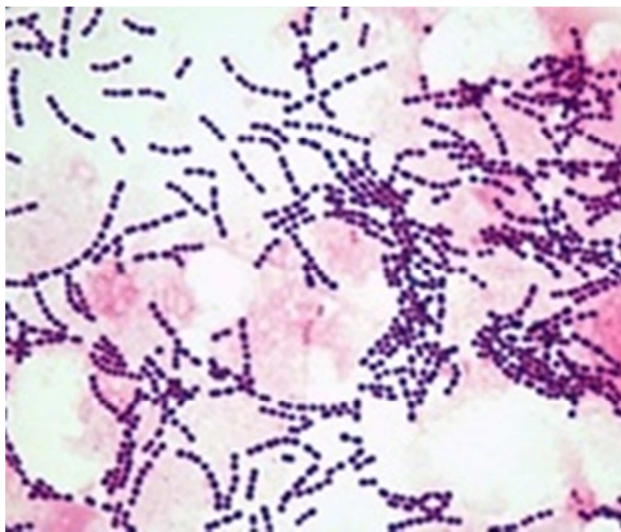


Figure 12: Gram stain of *Streptococcus agalactiae*.

I.7.2. The *Streptococcus agalactiae* pan-genome model

A bacterial species can be described by its pan-genome, which is composed of a core genome containing genes present in all strains, and a dispensable genome containing genes present in two or more strains and genes unique to single strains (250). *S.*

agalactiae species can be described by a pan-genome consisting of a core genome shared by all isolates, accounting for 80% of any single genome, plus a dispensable genome consisting of partially shared and strain-specific genes. Mathematical extrapolation of the data suggests that the gene reservoir available for inclusion in the *S. agalactiae* pan-genome is vast and that unique genes will continue to be identified even after sequencing hundreds of genomes. (363). Presently, the pan-genome of Group B *Streptococcus* (GBS), contains 2713 genes, of which 1806 belong to the core genome, and 907 belong to the dispensable genome. The GBS pan-genome is predicted to grow by an average of 33 new genes every time a new strain is sequenced (250). The dispensable genome contributes to the species' diversity and probably provides functions that are not essential to its basic lifestyle but confer selective advantages including niche adaptation, antibiotic resistance, and the ability to colonize new hosts (363). The genomes of multiple, independent isolates are required to understand the global complexity of bacterial species. The concept of the pan-genome is not just a theoretical exercise; it also has fundamental practical applications in vaccine research. Analysis of multiple GBS genomes was found to be instrumental for the development of vaccines (234) and for the functional characterization of important genetic determinants (223). Recently, it was shown that the design of a universal protein-based vaccine against GBS was only possible using dispensable genes (234). In addition, the sequencing of multiple genomes was instrumental in discovering the presence of pili in GBS, an essential virulence factor that had been missed by all conventional technologies (251).

I.7.3. Epidemiology

Both men and women can be colonized with GBS in the lower gastrointestinal and genital tract and the bacteria can be transmitted sexually. An estimated 20-30% of all pregnant women are GBS carriers and the colonization can be permanent but also intermittent or transient and 50–70% of infants born to these women will themselves become colonized with the bacterium. Selective culture methods are used to optimize the isolation of GBS from a complex microflora in the vagina and rectum (137, 224, 285).

Because colonization can be intermittent or transient, culture status can vary between pregnancies, and therefore screening during each subsequent pregnancy is advised. The predictive value of prenatal screening improves with shorter intervals between culture and delivery (37, 417), so that prenatal screening at 35–37 weeks of gestation is currently recommended. Chemoprophylaxis with penicillin at delivery should be based upon the 35–37 week culture even if earlier cultures were obtained (137).

Cross-sectional studies in the United States have found GBS colonization rates to be higher in African-American women compared with Caucasians or Asians (292) and international reports confirm racial or ethnic differences are likely evident after accounting for methodological differences (323, 354, 365). Nearly 80% of all invasive GBS infections occur in the first few days of life (325). The CDC reported in 1997 that most of the cases were caused by bacteremia. Ten percent of those infected with GBS had meningitis, but not all cases of meningitis were associated with bacteremia. Gender does not appear to play a role in the development of either early- or late-onset GBS infection (14).

The incidence of GBS infection varies geographically as well as from hospital to hospital (55, 148). This might be related to the presence or absence of formal policies about the selective use of intrapartum chemoprophylaxis(55).

GBS infection has been known to recur. Estimates place the recurrence rate at approximately 1 % for newborns who were symptomatic and treated appropriately. Baker *et al.* (15) and Schuchat *et al.* (14, 325) noted that 50% of newborns with recurrent GBS infection have a new site of involvement with the second episode.

I.7.4. Pathogenesis

The gastrointestinal tract is the usual reservoir in females, whereas GBS can be isolated in 45% to 63% of male urethras (55, 56, 59). The bacteria are also found in the genital tract, urinary tract, throat, and respiratory tract. Genital tract colonization poses the most important threat to the newborn, because of exposure during the birth process, and to the mother, because of ascending infection after the membranes rupture.

Colonization is found in the rectum more often than the vagina, in the vagina more than the urethra, and in the urethra more than the cervix (Figure 13).

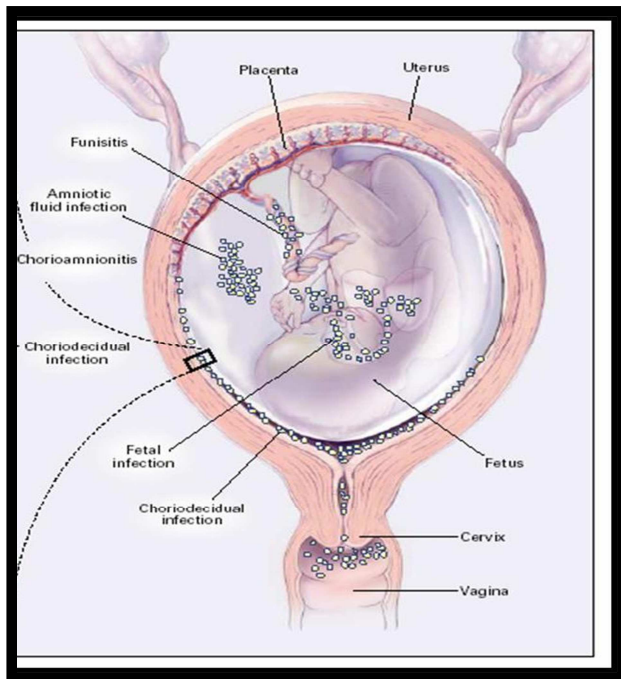


Figure 13: GBS colonization in pregnant women.

Vertical transmission with colonization of the newborn is reported to occur in 5-40% of all pregnancies (148, 197, 276, 312) with 38% of GBS-colonization in women with membranes ruptured less than 12 hours and 73% in women with a longer duration of membrane rupture (167, 296). Increased duration of internal monitoring and increased number of vaginal examinations also augment the risks for the laboring woman and her newborn (326, 415). Intrapartum fever is seen more frequently in women of affected newborns (327, 344). One to two percent of newborns exposed to GBS during the birth process subsequently develop GBS disease (82, 153, 344). The neonatal mortality rate with GBS sepsis is 5% to 6%, with major neurological sequelae in surviving newborns (247). Most mothers of neonates with early-onset disease did not receive intrapartum antibiotics (233).

I.7.5. Maternal complications and complications during pregnancy

GBS colonization in women is rarely life threatening but can cause infections and complications during the antepartum, intrapartum, and postpartum. Approximately 50,000 colonized women are affected annually with GBS-related illness in the United States (54, 55). Bacteremia is the primary symptom for 64% of pregnant women with GBS disease (323). Failure to recognize and treat bacteremia, which can be asymptomatic in about 6% of pregnant women, can lead to further urinary tract complications such as pyelonephritis (138, 419). Pregnancy-related infections are sepsis, amnionitis, urinary tract infection, and stillbirth.

I.7.6. Clinical features

In neonates two syndromes exist: early-onset (< 7 days old) and late-onset (7-90 days old). Both include sepsis, pneumonia and meningitis.

I.7.6.1. Early-onset GBS

Early-onset GBS infection is caused by vertical transmission whereby the organism ascends into the chorioamniotic space prior to birth. Between 75% and 80% of newborns with early-onset GBS infection are born at term. However, premature newborns are more likely to develop early-onset GBS infection than full-term newborns (14, 312). Information from the National Institute of Child Health and Human Development (NICHD) Neonatal Research Network (353) found that GBS is the cause of approximately 31 % of all infections in low-birthweight newborns.

I.7.6.1.1. Manifestation of early-onset GBS

The most common sign of newborns with early-onset GBS infection is respiratory distress. Their chest x-rays are often indistinguishable from those of newborns with respiratory distress syndrome. Only about one third of newborns with congenital pneumonia will have infiltrates apparent on chest x-rays; Pleural effusions also may be present (14). In addition, infected newborns may have other nonspecific symptoms such as lethargy, poor feeding, temperature instability, and glucose instability. More ominous

signs of severe invasive infection include hypotension, which may occur in up to 25% of all cases; fetal asphyxia, related to being infected *in utero*; rapidly worsening respiratory distress; and persistent pulmonary hypertension. Newborns with meningitis may not have signs different from those of newborns with GBS septicemia or pneumonia, although seizures can occur in the first 24 hours of life (14).

I.7.6.1.2. Risk factors for early-onset GBS

Risk factors for early-onset GBS disease have been well-characterized and include maternal GBS colonization, prolonged rupture of membranes (*i.e.* more than 18 hours), frequent vaginal examination during labor, preterm delivery, GBS bacteriuria during pregnancy, delivery of a previous infant with invasive GBS disease, maternal chorioamnionitis as evidenced by intrapartum fever, young maternal age, African or Hispanic ethnicity and low levels of antibody to type-specific capsular polysaccharide antigens (37, 328, 329, 416). It is possible for newborns with no known maternal predisposition for GBS to develop invasive disease. In addition, nosocomial early-onset GBS infection has been known to occur (14).

I.7.6.2. Late-onset GBS infection

Newborns who present with late-onset GBS do not have signs of infection in the first week of life. The symptoms also may be somewhat indistinct, such as lethargy, poor feeding, or irritability. Most of the isolates of late-onset GBS infection are serotype III, and approximately 30% to 40% of the newborns with late-onset GBS will have meningitis (14, 329). Newborns with meningitis will generally have fever, and 20% to 30% will have had symptoms of an upper respiratory tract infection. Otitis media may be present. Permanent neurologic sequelae in varying degrees are evident in a large number of newborns with GBS meningitis (14).

I.7.6.2.1. Manifestations of late-onset GBS

Late-onset GBS infection also can have a focal presentation. Facial cellulitis is being seen with increasing frequency, more often in males than females. Septic arthritis and osteoarthritis also are seen more often in late-onset infection. Just as with early-onset GBS infection, there have been isolated reports of the infection occurring in almost every

organ (14). Late-onset GBS infection has a mortality rate of 2% to 6%, and the incidence has not changed following the advent of intrapartum chemoprophylaxis (55, 323). However, as with early-onset disease, the initial presentation may vary markedly. Newborns with late-onset infection who present with symptoms such as apnea, seizures, leukopenia, or neutropenia are more likely to have a fatal outcome.

I.7.6.2.2. Risk factors for late-onset GBS

Risk factors associated with late-onset GBS infection have not been well defined. Vertical transmission and nosocomial acquisition are the most likely sources (14, 325, 329). Prematurity and African ethnicity are recognized also as a risk factors for late onset GBS (228). Baker and Edwards (14) reported that although GBS can be acquired nosocomially, it does not appear to cause outbreaks in nurseries. Newborns who are colonized with GBS do not need to be isolated. Colonization has been reported to last for several months after birth, even in newborns treated for GBS infection (3). Newborns who present with late-onset GBS do not have signs of infection in the first week of life.

I.7.7. Treatment

The most recent treatment recommendations for newborns are ampicillin with the addition of an aminoglycoside (3). This broad-spectrum coverage allows the newborn to be treated for other causes of sepsis, including *Escherichia coli*, *Listeria monocytogenes* and *Enterococcus fecalis* (176, 310). Johnson *et al.* (188) suggested that ampicillin alone might be adequate empiric coverage for asymptomatic full-term newborns. Their review of more than 5,000 newborns found that only one benefited from the addition of gentamicin, whereas all were exposed to the ototoxic side effects of the drug. The American Academy of Pediatrics (3) recommends that antibiotics be discontinued after 48 hours of treatment if laboratory results are negative and the clinical course is not consistent with invasive disease. Baker & Edwards (14) recommended not to continue antibiotic treatment for more than 72 hours, unless blood or cerebral spinal fluid cultures are positive or the clinical course is consistent with septicemia or pneumonia.

I.7.8. Prevention

Schuchat *et al.* (325) suggested that GBS infection is now a public health concern and the focus should be primarily on prevention, not treatment. The US Centers for Disease Control and Prevention (53) suggests that prevention of GBS infection might be compared with other perinatal prevention strategies, such as the use of folic acid to prevent neural tube defects and vaccination to prevent transmission of hepatitis B infections. The dramatic decrease in the incidence of early-onset GBS infection since the adoption of guidelines for selective intrapartum chemoprophylaxis supports this view (321).

It is important to note that, the decrease in incidence has occurred only in early-onset cases. Further prevention strategies that address late-onset GBS infection, the incidence of which has remained stable, are necessary. It has been suggested that intrapartum chemoprophylaxis interrupts the vertical transmission of GBS from mother to newborn but has no effect on infection by GBS to which the newborn may subsequently be exposed (55). The concept of a vaccine for GBS is based on the finding that the absence of antibodies to the GBS capsule is correlated with the development of invasive infection (13). Because there are multiple capsular serotypes of GBS, vaccine development will most likely need to be specific to the serotypes causing the (late-onset) infections.

I.8. Laboratory diagnosis of Group B streptococci

I.8.1. Culture

In 2002, the Centers for Disease Control and Prevention (CDC) recommended that all pregnant women be screened for carriage of GBS at between 35 and 37 weeks of gestation (57, 58). The CDC currently recommends screening cultures using selective broth medium for the recovery of GBS from vaginorectal specimens. Because the sensitivity of selective culture methods for detection of GBS colonisation depends on the timing and method of collection and on the processing of the specimens, the protocols

for sample collection, as recommended by the CDC, should be strictly followed (327). A good laboratory algorithm includes inoculation - whereby the inoculum size has not been specified by the CDC, into a selective broth medium, *i.e.* Todd-Hewitt broth supplemented either with 15 µg colistin and 10 µg nalidixic acid (Lim broth)(227) or with 8 µg gentamicin and 15 µg nalidixic acid (12), and incubation for 18–24 h, followed by inoculation onto sheep-blood agar and incubation for an additional 18–24 h. If no GBS are identified, the plate should be re-incubated for an additional 24 h (59). Culturing GBS to sufficient numbers to carry out subsequent identification can therefore take 2–3 days. However, this method has a slow turnaround time requiring 36–72 h before results can be issued. Also, this method requires an experienced technician to identify suspect colonies, which are not always β-hemolytic.

In the recent guidelines (2010) of the Centers for Disease Control and Prevention (CDC)(58), the following key changes were made from the 2002 guidelines: GBS identification options were expanded to include a positive identification from chromogenic media and identification directly from enriched broth (Figure 14). Nucleic acid amplification tests (NAAT), such as commercially available PCR assays, can also be used after enrichment, if laboratories have validated NAAT performance and included appropriate quality controls. Also the direct plating option can be included in addition to enriched culture, although it has lower sensitivity than enriched culture and should not be used as sole means to detect GBS.

Group B streptococci can be confirmed using biochemical tests like a positive catalase or hippurate hydrolysis reaction, the CAMP test (Figure 15), a positive bile esculin test (111) and serological tests. The CAMP test (named after Christie, Atkins and Munch-Petersen) is used for the presumptive identification of GBS, yielding positive results for up to 98% of GBS isolates (209).

It is based on the fact that GBS produce an extracellular protein called the CAMP factor. This protein acts synergistically with *Staphylococcus aureus* β-toxin to hydrolyse red blood cells. However, a small percentage of group A streptococci and some strains of *Listeria monocytogenes* are also CAMP-positive (209). Hippurate hydrolysis, for which

approximately 96% of GBS isolates are positive (112) is another physiological characteristic used for the presumptive identification of GBS (306).

More recently, a selective and differential agar, known as Granada agar, has also been used for the presumptive identification of GBS (78, 140). In principle only GBS produce a red-orange carotenoid pigment when grown anaerobically on this starch containing medium (79, 304). Pigment is produced in 93.0–98.5% of GBS human isolates (140, 303). The production of the carotenoid pigment is linked to the GBS β -hemolysin/cytolysin (β H/C) which is encoded by *cylE* (230). Several in-vitro and animal model studies have demonstrated the important role of β H/C in the virulence of GBS (230) and showed that non-hemolytic strains are less virulent. Non hemolytic GBS and non pigmented GBS occur in 1-2% of pregnant women. Several studies reported GBS infection with non hemolytic strains of GBS(65, 98, 151, 270, 386).

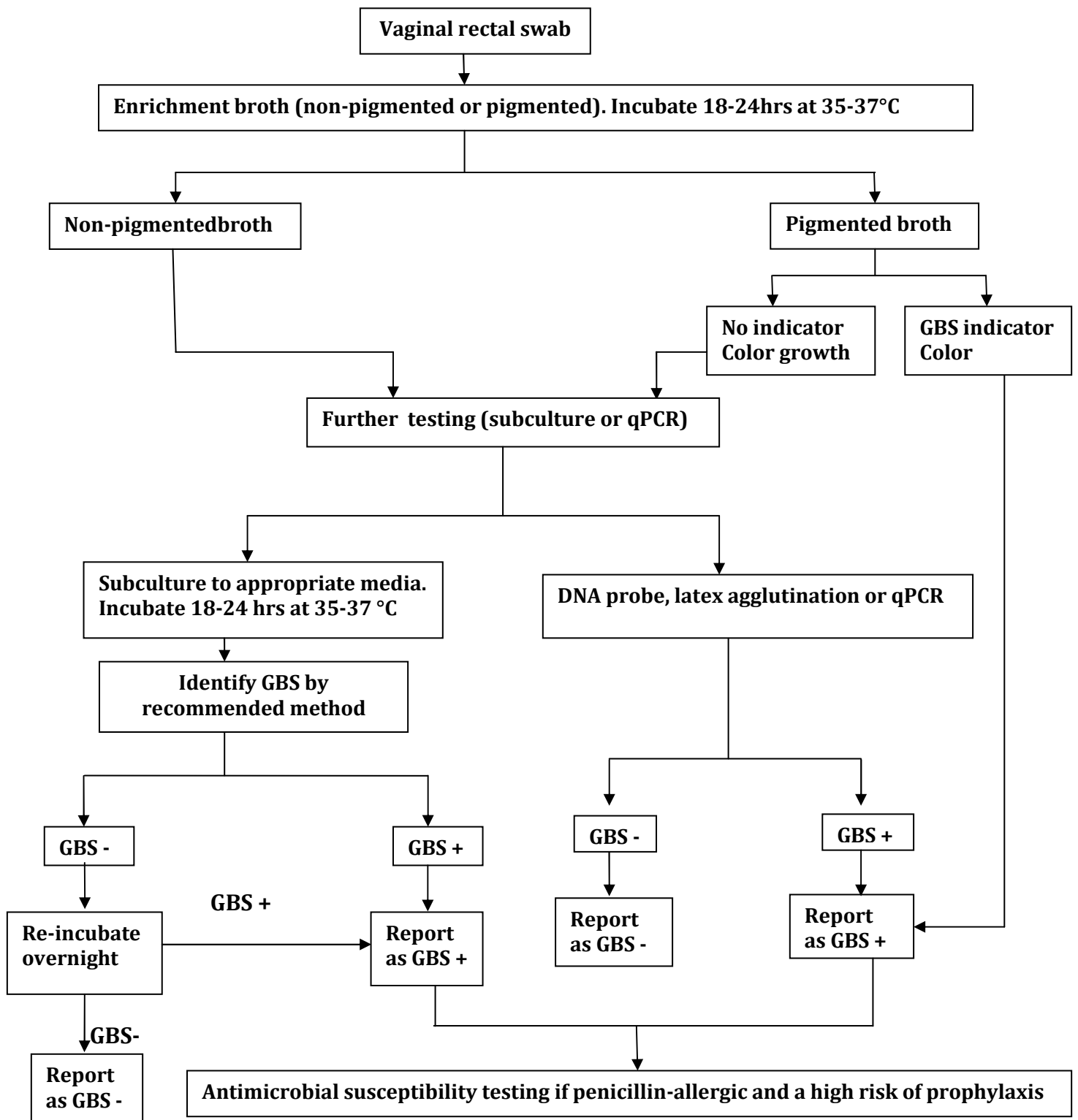


Figure 14: Algorithm for recommended prenatal group B streptococcal laboratory testing(CDC 2010).



Figure 15: CAMP test for *S. agalactiae* (positive, upper left streak) and *Enterococcus faecalis* (negative, lower right streak), with vertical streak of *Staphylococcus aureus* on blood agar.

A major limitation of Granada agar is the requirement for anaerobic culture conditions, although the requirement for anaerobic incubation can be circumvented by the application of a cover slide on the inoculum (303). Another selective and chromogenic medium, known as ChromID™ Strepto B agar (ChromAgar) developed by bioMérieux, it is based on the detection of enzymatic activity, not specified by the company, using chromogenic substrates. It consists of a nutritive base combining different peptones, 3 chromogenic substrates and antibiotics. These components enable the screening, under aerobic conditions, of *S. agalactiae* by the spontaneous appearance of pink to red, round and pearly colonies. Most other bacterial species are either inhibited or the colonies produced are of a different color (e.g. violet, blue, colorless etc.). During this doctoral research we compared applicability, sensitivity and specificity of both media (98).

I.8.2. Immunological methods

GBS isolates are identified reliably by the production of group B Lancefield antigen (306). Consequently, many latex agglutination tests and immunoassays that detect this

antigen for GBS identification have been developed (11, 149, 387, 413). The ability to identify GBS by these methods depends on the amount of the bacteria inoculated in the assay. Their sensitivities for detection of GBS directly from clinical specimens varied from 19% to 82% when selective broth media were used to recover GBS from clinical samples (413). In general, these immunological tests are not sufficiently sensitive for the direct detection of GBS from clinical samples, and only women with heavy colonisation can be readily identified by these methods (11, 210).

I.8.3. DNA hybridisation-based methods

The most widely used hybridisation-based test to date is the Accuprobe Group B *Streptococcus* Identification Test (Gen-Probe, San Diego, CA). This assay, which targets specifically the GBS ribosomal RNA, is suitable to identify GBS from 18 h to 24 h cultures in selective enrichment broth (33, 200, 403). Compared with the standard culture method, this probe-based assay had a sensitivity of 94.7–100% and a specificity of 96.9–99.5% for screening for GBS colonization in pregnant women (33, 200, 403). When shorter pre-incubation periods were used, the sensitivity of this test was 44% for a 2.5 h incubation (94% specificity) and 71% for a 3.5 h incubation (90% specificity) (33, 414). Bourbeau *et al.* (34) demonstrated significant potential labour savings utilizing this product, compared with the traditional culture methods.

Another test based on DNA hybridisation is the Affirm GBS Microbial Identification System (MicroProbe, Bothell, WA, USA) (34). However, even after a 16–24 h pre-incubation, the sensitivity of the test was only 81%. Such a test does not offer any advantages over culture methods (305). Thus, available probe hybridization methods are suitable for GBS identification from overnight cultures in selective enrichment broth but are poorly sensitive for direct detection and identification of GBS from vaginorectal swabs obtained from pregnant women.

I.8.4. Conventional PCR-based methods

Polymerase chain reaction (PCR)-based assays offer promising tools for sensitive, specific and rapid detection of GBS directly from clinical specimens. A number of PCR assays targeting different genes for the specific detection of GBS have been developed. These include genes encoding C protein (7, 242, 388), the 16S rRNA , and the 16S–23S spacer region (154). Ke *et al.* (196) developed a GBS-specific PCR assay that targets the *cfb* gene, which encodes the CAMP factor (21, 196) that is present in virtually every strain of GBS (401). It allows the detection of as little as one genomic copy of GBS. This PCR assay achieves almost the same sensitivity as standard culture methods when vaginal and/or rectal specimens from pregnant women are tested for GBS colonization (21). Although 2% of GBS isolates were phenotypically negative for the CAMP test (209), molecular characterisation of the *cfb* gene showed that this gene was present in virtually every isolate (281).

I.8.5. Real-time PCR-based methods

Several PCR-based assays for the detection of GBS have been developed, but these require procedures not applicable readily to clinical laboratories (7). However, the development of real-time PCR (qPCR) has provided new platforms for bacterial detection and identification. qPCR technology offers several advantages over conventional PCR methods, such as a lower risk of PCR contamination, shorter turnaround time, less labour, less biosafety risk (no pouring of gels and staining with EtBr) and quantitative results (165). Some of the most popular commercially available systems coupling PCR technology with real-time detection of PCR products include the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), the LightCycler™ (Idaho Technologies, Idaho Fall, ID; and Roche Diagnostics, Indianapolis, IN), and the Smart Cycler® (Cepheid, Sunnyvale, CA). GBS-specific PCR assays have been developed using the latter two platforms.

I.8.5.1. GBS-specific qPCR with the LightCycler

The LightCycler is a real-time amplification-detection apparatus with air thermal cycling and fluorescently monitored product analysis in a closed-tube assay format (184, 407, 408). An important advantage of the LightCycler is its rapid temperature transition rate of up to 20 °C per second, allowing completion of 45 cycles of amplification in 20–30 min (409). Bergeron *et al.* (21) developed a qPCR assay that targets the *cfb* gene and that is able to identify pregnant women colonized by GBS in 30–45 min, with a sensitivity of 97% and a specificity of 100% as compared with the standard culture method. In another study, Bergh *et al.* (22) developed a qPCR assay that targets the *sip* gene, which encodes the *sip* surface immunogenic protein. It is universally present across all serotypes of GBS (22). Bergseng *et al.* (23) reported that the qPCR assay targeting the *sip* gene is fast, highly sensitive and specific for detecting GBS colonization in pregnant women at delivery, and has the potential for intrapartum detection of GBS colonization. During this doctoral research, we compared two different targets (*sip* and *cfb* genes), using two different real-time PCR (qPCR) approaches, i.e. the hydrolysis probe assay (Taqman, Roche) and the hybridisation probes assay (Hybprobe, Roche) with standard culture to detect GBS colonization in pregnant women (Figure 16).

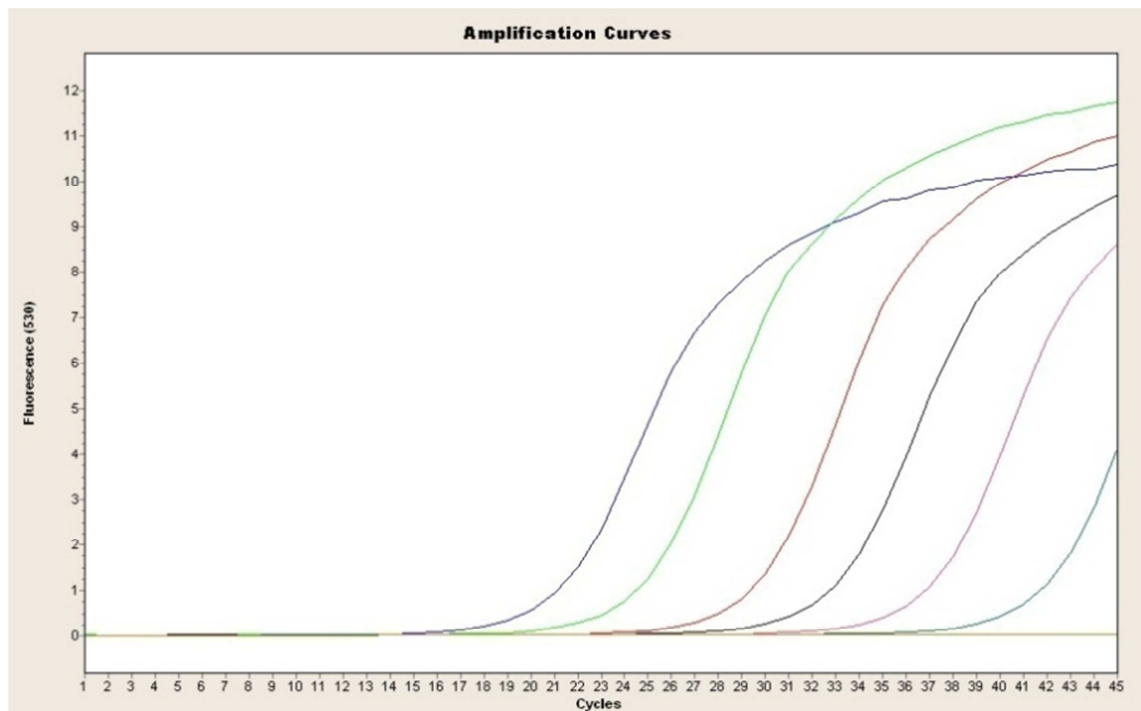


Figure 16: Real time PCR analysis of GBS (serial dilution) using the LightCycler.

I.8.5.2. GBS-specific qPCR with the Smart Cycler

The Smart Cycler is a miniature analytical thermal cycling instrument designed for qPCR. It was first described by Northrup *et al.* (265) and is manufactured by Cepheid. The system is designed to allow low-power operation, high-temperature transition speed and thermal uniformity (64). The BD GeneOhm StrepB test (BD GeneOhm Sciences, San Diego, CA; formerly IDI-StrepB), the Cepheid GeneXpert GBS and Smart GBS (Cepheid, Sunnyvale, CA) offer detection of GBS directly from rectovaginal swabs for antepartum or intrapartum detection of GBS colonization.

I.8.5.2.1. BD GeneOhm™ StrepB assay

The BD GeneOhm™ StrepB Assay (previously marketed as IDI Strep B) is a qualitative *in vitro* diagnostic test for the rapid detection of Group B *Streptococcus* (GBS) in pregnant women at the time of delivery or at any other stage of their pregnancy. The BD GeneOhm™ StrepB test results can be available in less than one hour (338), enabling new capabilities for earlier and more definitive treatment of mother and newborn. The

test can be performed on the SmartCycler automated analyzer. The PCR master mixture contained all reagents necessary for amplification of the GBS *cfb* gene, if present, and of the internal control template. The GBS amplicon is a 154-bp DNA fragment. The internal control is a 180-bp DNA fragment, consisting of a 134-bp sequence not found in GBS flanked by the complementary sequence of each of the 2 GBS-specific primers. Amplified DNA is detected with hybridization probes labeled with quenched fluorophores (molecular beacons). The fluorophores attached at the 5' end of the beacons are 5'-carboxyfluorescein and tetrachlorofluorescein, for the detection of GBS amplicons and internal control amplicons, respectively. The beacon-target hybrids fluoresce at wave lengths characteristic of the fluorophore used in each of the particular molecular beacons used. The concomitant amplification of the internal control allows for verification of the efficiency of the PCR and alerts the user to the presence of significant PCR inhibition within the test sample.

Comparison of the LightCycler assay and the GeneOhm StrepB Test, starting from Lim broth cultures showed sensitivities of 100% and 92.5%, respectively, and specificities of 95.9% and 92.5%, respectively, with culture as the gold standard (146). In a multicenter study, the sensitivity and specificity of the IDI-StrepB assay, at the time of labor, was 94% and 95.9% respectively, compared to culture (72). When compared to either the sensitivity of antenatal cultures (54%) or of risk factor analysis (42%), the sensitivity of the IDI-StrepB assay was superior (72).

I.8.5.2.2. Cepheid GeneXpert GBS assay

The Cepheid Xpert GBS™ assay, performed on the GeneXpert System, is another qualitative *in vitro* diagnostic test designed to detect GBS DNA in vaginal/rectal specimens using qPCR technology. This new assay is intended for use as a method for rapid detection of GBS colonization in antepartum and intrapartum women with results available in just over an hour. Its automated system allows for sample DNA purification, nucleic acid amplification, and detection of the target sequence in the biological samples using qPCR (60). Single-use GeneXpert cartridges hold the sample and PCR reagents and host the PCR process. The GBS assay is based on the principle that GBS can be distinguished from other streptococci by the cell wall associated group B carbohydrate.

The assay reagents concurrently amplify and detects a target within a 3' DNA segment adjacent to the *cfb* gene of *S. agalactiae*. Each cartridge includes internal controls to validate sample processing and PCR performance. The sample swab and the 2 reagent solutions are placed into the appropriate chambers of the GeneXpert GBS cartridge, which is then loaded into the machine, a 2-minute process. In a completely automated fashion, the GeneXpert system elutes the bacteria from the swab, mixes the sample reagent with a sample-processing control (*Bacillus globigii*) and the treatment reagent, and captures and lyses the bacterial cells with subsequent elution of the DNA. The DNA solution is mixed with dry PCR reagents and transferred into the reaction tube for qPCR. The process is completed in less than 75 minutes while the computer collects cycle data to detect the presence or absence of the GBS target. As a point-of-care intrapartum screening test, the Xpert GBS had a sensitivity of 95.8% and a specificity of 64.5%, whereas the antenatal culture was 83.3% sensitive and 80.6% specific when intrapartum culture was used as the gold standard (136).

I.8.5.2.3. Cepheid Smart GBS

The Cepheid Smart GBS test, performed on the SmartCycler, is a qualitative in vitro diagnostic test, designed to detect Group B *Streptococcus* (GBS) DNA in vaginal/rectal specimens using proven real-time PCR technology. The test can be performed directly from antepartum and intrapartum vaginal-rectal swabs and from Lim broth cultures. The test uses PCR primers specific to a unique sequence region of *S. agalactiae*, followed by fluorogenic target-specific hybridization to detect the amplified DNA. Specimens are collected using the Cepheid GBS collection device, and verification and validation of performance characteristics of specimen transport and storage conditions using such is an important component of test implementation. The manufacturers reported assay sensitivity from a multisite study of antepartum and intrapartum women to be 81.6% for direct vaginorectal swabs compared to results obtained using the CDC culture method (57). Jordan *et al.* (193) reported that the Smart GBS assay had excellent performance characteristics compared to broth-based culture and was found to be substantially equivalent or better than the BD Genome StrepB direct assay based on sensitivity, specificity and accuracy.

Chapter II: Experimental work

II.1. Publication 1

El Aila NA, Tency I, Claeys G, Verstraelen H, Saerens B, Santiago GL, De Backer E, Cools P, Temmerman M, Verhelst R, Vaneechoutte M.

Identification and genotyping of bacteria from paired vaginal and rectal samples from pregnant women indicates similarity between vaginal and rectal microflora.

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Research article

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Identification and genotyping of bacteria from paired vaginal and rectal samples from pregnant women indicates similarity between vaginal and rectal microflora

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Abstract

Background: The vaginal microflora is important for maintaining vaginal health and preventing infections of the reproductive tract. The rectum has been suggested as the major source for the colonisation of the vaginal econiche.

Methods: To establish whether the rectum can serve as a possible bacterial reservoir for colonisation of the vaginal econiche, we cultured vaginal and rectal specimens from pregnant women at 35-37 weeks of gestation, identified the isolates to the species level with tRNA intergenic length polymorphism analysis (tDNA-PCR) and genotyped the isolates for those subjects from which the same species was isolated simultaneously vaginally and rectally, by RAPD-analysis.

One vaginal and one rectal swab were collected from a total of each of 132 pregnant women at 35-37 weeks of gestation. Swabs were cultured on Columbia CNA agar and MRS agar. For each subject 4 colonies were selected for each of both sites, i.e. 8 colonies in total.

Results: Among the 844 isolates that could be identified by tDNA-PCR, a total of 63 bacterial species were present, 9 (14%) only vaginally, 26 (41%) only rectally, and 28 (44%) in both vagina and rectum. A total of 121 (91.6%) of 132 vaginal samples and 51 (38.6%) of 132 rectal samples were positive for lactobacilli. *L. crispatus* was the most frequently isolated *Lactobacillus* species from the vagina (40% of the subjects were positive), followed by *L. jensenii* (32%), *L. gasseri* (30%) and *L. iners* (11%). *L. gasseri* was the most frequently isolated *Lactobacillus* species from the rectum (15%), followed by *L. jensenii* (12%), *L. crispatus* (11%) and *L. iners* (2%).

A total of 47 pregnant women carried the same species vaginally and rectally. This resulted in 50 vaginal/rectal pairs of the same species, for a total of eight different species. For 34 of the 50 species

pairs (68%), isolates with the same genotype were present vaginally and rectally and a high level of genotypic diversity within species per subject was also established.

Conclusion: It can be concluded that there is a certain degree of correspondence between the vaginal and rectal microflora, not only with regard to species composition but also with regard to strain identity between vaginal and rectal isolates.

These results support the hypothesis that the rectal microflora serves as a reservoir for colonisation of the vaginal niche.

Background

The composition of the human vaginal microflora is affected by several host factors, including, among others, age, menarche, sexual activity, pregnancy and the use of contraceptives or spermicides, as well as individual habits such as douching [1]. Several bacterial species are known to colonize both the gastrointestinal and the reproductive tract, and the rectum has been suggested to play an important role as a source or reservoir for organisms that colonize the vagina [2,3]. It is important to establish to which degree this is also the case for lactobacilli, the predominant group of microorganisms of the normal vaginal microflora, because these bacteria are generally known to produce endogenous microbicides such as lactic acid, which acidifies the vagina, and hydrogen peroxide (H₂O₂), toxic to other bacteria and viruses, including HIV [4]. Studies of vaginal lactobacilli have demonstrated that *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. vaginalis* are the most commonly recovered species of H₂O₂-producing lactobacilli [5-9] and the absence of H₂O₂-producing lactobacilli in the vagina has been associated with an increased risk for bacterial vaginosis (BV) [10,11]. BV has been linked to increased shedding of HIV in the female genital tract [12], increased acquisition of HIV [10] and herpes simplex virus type 2 [10,12] and with preterm birth [13].

In order to document in more detail a possible rectal origin of the vaginal microflora, this study was set up not only to compare the bacterial species present in vagina and rectum, but in addition, to compare the genotypes of those strains belonging to species that were present simultaneously at both sampling sites of the same subject.

Methods

Patients

The study was approved by the research ethics committee (IRB protocol nr 2007/096) of Ghent University Hospital, Belgium. All women attending the clinic were included and participating women gave a written informed consent. Between April and December 2007, 132 paired vaginal and rectal swabs were collected from pregnant women at 35 - 37 weeks of gestation.

Sampling procedures

All specimens were collected using nylon flocked swabs that were submerged into 1 ml of liquid Amies transport medium (eSwab, Copan Diagnostics, Brescia, It.). For rectal specimens, a swab was carefully inserted approximately 1.5 - 2 cm beyond the anal sphincter and then gently rotated to touch anal crypts.

Vaginal samples were collected by inserting a swab into the vagina. The swab was rolled round through 360 degrees against the vaginal wall at the midportion of the vault. At Ghent University Hospital, the routine screening for group B streptococci of pregnant women is always performed during the prenatal consultation at 35-37 weeks' gestation according to the CDC guidelines for the prevention of perinatal Group B streptococcal disease [14]. All study samples were collected by midwives and transported to the Laboratory for Bacteriology Research of the University of Gent within 4 hours.

Culture and Gram staining

A total of 70 µl from the Amies liquid transport medium of each of the vaginal and rectal swabs was inoculated onto Columbia CNA agar with 5% sheep blood (Columbia CNA agar, Becton Dickinson, Erembodegem, Belgium) respectively De Mann Rogosa Sharp Agar (MRS, Oxoid, Hampshire, UK) and then incubated at 37 °C in an anaerobic chamber (10% H₂, 10% CO₂, 80% N₂) (Bug-Box, LedTechno, Heusden-Zolder, B.) for 72 h. Another 50 µl of the vaginal swab suspension was taken for smear preparation for the Gram stain.

Gram stain based grading was carried out according to modified Ison & Hay criteria [15], as described by Verhelst *et al.* [16].

Grade Ia specimens contained mainly *Lactobacillus crispatus* cell types, i.e. plump, quite homogeneous lactobacilli, grade Ib contained non-*L. crispatus* cell types, i.e. long or short, thin lactobacilli, grade Iab contained mixtures of *L. crispatus* and non-*L. crispatus* cell types, grade I-like contained irregular-shaped Gram positive rods, grade II contained a mixture of *Lactobacillus* cell types and bacterial

vaginosis-associated bacteria (*Gardnerella*, *Bacteroides-Prevotella* and *Mobiluncus* cell types), whereas samples devoid of *Lactobacillus* cell types with the presence of only *Gardnerella*, *Bacteroides-Prevotella* or *Mobiluncus* cell types were classified as grade III. Finally, samples were classified as grade IV when Gram positive cocci were predominantly present and as grade 0 when no bacterial cells were present [16].

DNA-extraction from isolates

DNA was extracted from cultured isolates by alkaline lysis as follows: One bacterial colony was suspended in 20 µl of lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and heated at 95 °C for 15 min. The cell lysate was diluted by adding 180 µl of distilled water. The cell debris was spun down by centrifugation at 16,000 g for 5 min. Supernatants were used for PCR or frozen at -20 °C until further use.

Identification of isolates

From all 132 women, 8 colonies per subject, i.e., one colony of each of the two most abundant colony types from both Columbia CNA and MRS agar plates and for both rectal and vaginal swabs were picked, i.e. a total of 1056 isolates. Isolates were identified by tRNA intergenic length polymorphism analysis (tDNA-PCR) as described before [8,17-19]. Briefly, the tRNA-intergenic spacer regions were amplified by PCR using consensus primers, applicable to most bacterial species, and the resulting fingerprints, obtained by separation of the amplified spacers by capillary electrophoresis on an ABI310, were compared with those of a large library of reference strains of the different species, shown in previous studies to be part of the vaginal microflora. Isolates with fingerprints that did not match fingerprints already present in the library were considered as not identifiable.

Genotyping of isolates

Isolates of species present in both vagina and rectum of the same subject were genotyped using RAPD-analysis with RAPD Ready-to-Go beads (GE Healthcare, Buckinghamshire, UK) as described previously [20] with primer OPM1 (5' GTT GGT GGC T) at a final concentration of 2 µM, including 0.2 µM of fluorescent TET-labeled OPM1 primer. After 5 min at 94 °C, 5 min at 35 °C and 5 min at 72 °C, reaction mixtures were cycled 30 times in a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, Ca.), with the following conditions: 30 s at 94 °C, 1 min at 35 °C, and 1 min at 72 °C, with a final extension period of 5 min at 72 °C. Reaction vials were then cooled to 10 °C until electrophoresis.

Capillary electrophoresis

A volume of 11.9 µl of deionized formamide (ACE formamide, Lucron, De Pinte) was mixed with 0.6 µl of an inter-

nal size standard mixture containing 0.3 µl of the ROX-400 high-density size standard (Applied Biosystems, Foster City, Ca.) and 0.3 µl of Map marker 1000 size standard (BioVentures, Murfreesboro, Tn.). One µl of RAPD-PCR product was added. The mixtures were denatured by heating at 95 °C for 3 min and placed directly on ice for at least 10 min. Capillary electrophoresis was carried out using an ABI-Prism 310 genetic analyzer (Applied Biosystems) at 60 °C, at a constant voltage of 1.5 kV, and at a more or less constant current of approximately 10 mA. Capillaries with a length of 47 cm and diameter of 50 µm were filled with performance-optimized polymer 4. Electropherograms were normalized using Genescan Analysis software, version 2.1 (Applied Biosystems).

Data analysis

tDNA-PCR and RAPD fingerprints were obtained as table files from the GeneScan Analysis software (Applied Biosystems) and analyzed with BaseHopper, an in house software program [17]. The obtained tDNA fingerprints were compared with those of a library of tDNA fingerprints obtained from reference strains, representing most vaginal species isolated in previous studies and previously identified by 16S rRNA gene sequencing [8].

Similarity between RAPD fingerprints was calculated using the Dice algorithm. Clustering analysis was done with the Neighbor module of the Phylip software <http://evolution.genetics.washington.edu/phylip.html>, using the Neighbour joining algorithm. Isolates of which the RAPD fingerprints were clustered together, were inspected visually to confirm similarity.

Results

Categorization of vaginal microflora

Samples were categorized as grade Ia for 55 subjects (41.6%), grade Ib for 37 (28.0%), grade Iab for 13 (9.8%), grade I-like for 5 (3.8%), grade II for 14 (10.6%), grade III for 6 (4.5%) and grade 0 for 2 (1.5%).

The most common genus recovered from grade Ia, Ib and Iab specimens was *Lactobacillus*. Grade Ia samples contained predominantly *L. crispatus* (75.0%) and *L. jensenii* (43.6%), whereas *L. gasseri* (40.5%) and *L. iners* (27%) were the most frequently present species in grade Ib specimens. The five grade I-like specimens were found to contain respectively *Bifidobacterium bifidum* and *Enterococcus faecalis*, *L. gasseri* and *E. faecalis*, *L. jensenii*, *L. gasseri* and *Gardnerella vaginalis* or *L. rhamnosus*.

The most characteristic cultured organisms in grade II and grade III specimens were *G. vaginalis* (28% and 33%, respectively), *Actinomyces neuii*, *Aerococcus christensenii*, *Atopobium vaginae* and *Fingoldia magna*. The lactobacilli cultured from the six grade III specimens were respectively

L. iners, *L. gasseri*, *L. jensenii*, *L. crispatus*, *L. rhamnosus* and a combination of *L. gasseri* and *L. vaginalis*.

Rectal and vaginal prevalence of different bacterial species

For a total of 132 women, 4 colonies each were picked from the vaginal and rectal sites, i.e. a total of 1056 colonies were picked and subjected to identification by tDNA-PCR. Of these, 844 could be identified.

A total of 103 isolates gave no amplification or tDNA-PCR patterns composed of only a few and short tRNA intergenic spacers. Most of the isolates for which no amplification or only a few fragments could be obtained, are probably corynebacteria, which yield poor tDNA-PCR fingerprints (unpublished data).

Finally, 109 isolates gave uninterpretable patterns, due to mixed cultures, as was confirmed by 16S rRNA gene sequencing for 20 of these, whereby the sequences could not be interpreted because of ambiguities, pointing to mixtures.

The frequency of vaginal and rectal colonization by lactobacilli and the other most prominent bacterial species is shown in Table 1. A total of 63 bacterial species were identified, 9 (14%) occurring only vaginally, 26 (41%) only rectally and 28 (44%) in both vagina and rectum, with 8 species that could be isolated simultaneously from rectum and vagina of 47 subjects.

Overall, 121 of 132 pregnant women (92%) carried vaginal lactobacilli and 52 (39%) carried rectal lactobacilli. Seventy two pregnant women (54.5%) carried lactobacilli only vaginally, three only rectally (2%) and 49 (37%) in both sites, i.e. only three women out of 52 from whom lactobacilli could be isolated rectally, did not carry lactobacilli vaginally. *L. crispatus* was the most frequently identified *Lactobacillus* species isolated from the vagina (40%, i.e., 53/132 subjects positive of which 10 also carried *L. crispatus* rectally), followed by *L. jensenii* (32%), *L. gasseri* (30%), *L. iners* (11%) and *L. vaginalis* (10%).

Besides these five *Lactobacillus* species, all other species were encountered in no more than five subjects, except for *G. vaginalis* (8% of subjects positive).

L. gasseri was the most frequently isolated *Lactobacillus* species from the rectum (20/132 subjects positive), followed by *L. jensenii* (16) and *L. crispatus* (14). *L. iners* was isolated rectally from only 2 subjects. *L. vaginalis* was isolated only from the vagina, whereas *L. fermentum*, *L. coleohominis* and *L. fermentum* were only isolated from the rectum, at a frequency of $\leq 1\%$. Rectally, the most abundant species that could be cultured, were *Streptococcus*

anginosus group (47/132 subjects positive), *Finegoldia magna* (40), *Peptoniphilus indolicus* (25) and *E. faecalis* (21).

Forty six pregnant women (35%) were colonized by at least 2 different *Lactobacillus* species, with 42 of them only vaginally, two only rectally and another two both in the vagina and rectum. Taking into account vaginal and rectal colonization by more than one *Lactobacillus* species, 18 women (13.6%) were colonized by both *L. crispatus* and *L. jensenii*, of which 17 vaginally and one rectally, five (4%) by *L. jensenii* and *L. gasseri* of which 3 vaginally and 2 rectally and another 18 (13.6%) with other combinations of *Lactobacillus* species. Seven subjects (5.3%) were colonized vaginally by more than two *Lactobacillus* species. In total, of the 121 women colonized vaginally by lactobacilli, 42 (32%) were colonized by two or more *Lactobacillus* species and of the 52 women colonized by lactobacilli rectally, two (1.5%) were colonized by two *Lactobacillus* species. A total of 47 (35%) of 132 pregnant women were colonized both vaginally and rectally with the same species and 3 of these 47 women carried two species both vaginally and rectally. The species found to be simultaneously present in the same subject both rectally and vaginally were *L. crispatus* (n = 10 subjects), *L. jensenii* (14), *L. gasseri* (16), *S. anginosus* (3), *S. agalactiae* (3), *S. salivarius* (1), *E. faecalis* (2) and *Bifidobacterium* species (1). In summary, 50 vaginal/rectal pairs of the same species were observed in 47 subjects, for a total of eight different species.

Genotyping of bacterial isolates from the vagina and the rectum

For 34 of the 50 vaginal/rectal species pairs, isolates with the same genotype were present vaginally and rectally.

Table 2 presents the genotyping results for each of the 50 vaginal/rectal species pairs. We found the same genotype for both rectal and vaginal *L. crispatus* isolates in 7/10 subjects, for *L. gasseri* in 14/16, for *L. jensenii* in 7/14, for *Bifidobacterium* species in 1/1, for *E. faecalis* in 1/2, for *S. agalactiae* in 2/3, and for *S. anginosus* in 2/3.

Figure 1 shows the genotyping results for four pregnant women for which rectal and vaginal isolates belonged to a single genotype, for *L. gasseri* (Figure 1), *L. crispatus* (Figure 2), *L. jensenii* (Figure 3) and *E. faecalis* (Figure 4).

Genotyping results: variability among multiple isolates per subject

For only 2 of the 9 vaginal samples of which more than one *L. crispatus* isolate was picked, all the isolates belonged to the same genotype. For the 7 other vaginal samples, 2 to 3 genotypes of *L. crispatus* were present. In 7 of the 10 cases for which rectal/vaginal pairs of *L. crispatus*

Table 1: Vaginal and rectal prevalence of 63 bacterial species among 132 pregnant women

	Species	Only vaginal ^a	Only rectal	Vaginal + rectal	Overall
1	<i>Acinetobacter baumannii</i> group	0	8	0	8
2	<i>Actinomyces meyeri</i>	0	2	0	2
3	<i>Actinomyces neuii</i>	2	0	0	2
4	<i>Actinomyces radingae</i>	0	1	0	1
5	<i>Actinomyces urogenitalis</i>	0	1	0	1
6	<i>Aerococcus christensenii</i>	3	2	0	5
7	<i>Aerococcus viridans</i>	0	1	0	1
8	<i>Agrobacterium radiobacter</i>	0	2	0	2
9	<i>Alloscardovia omnicolens</i>	0	1	0	1
10	<i>Anaerococcus tetradius</i>	0	1	0	1
11	<i>Anaerococcus vaginalis</i>	0	4	0	4
12	<i>Atopobium vaginae</i>	2	2	0	4
13	<i>Bacteroides uniformis</i>	1	1	0	2
14	<i>Bifidobacterium bifidum</i>	2	6	1	9
15	<i>Bifidobacterium longum</i> subsp. <i>longum</i>	2	5	0	7
16	<i>Bifidobacterium longum</i> subsp. <i>infantis</i>	0	2	0	2
17	<i>Bifidobacterium breve</i>	0	1	0	1
18	<i>Corynebacterium accolens</i>	0	1	0	1
19	<i>Dialister</i> sp.	5	0	0	5
20	<i>Enterococcus faecalis</i>	5	19	2	26
21	<i>Enterococcus faecium</i>	1	3	0	4
22	<i>Enterococcus avium</i>	0	1	0	1
23	<i>Escherichia coli</i>	1	7	0	8
24	<i>Finexdial magna</i>	2	40	0	42
25	<i>Fusobacterium gonidioformans</i>	0	3	0	3
26	<i>Gardnerella vaginalis</i>	10	2	0	12
27	<i>Klebsiella planticola</i>	0	1	0	1
28	<i>Lactobacillus casei</i>	2	1	0	3
29	<i>Lactobacillus fermentum</i>	1	0	0	1
30	<i>Lactobacillus coleohominis</i>	2	0	0	2
31	<i>Lactobacillus crispatus</i>	43	4	10	57
32	<i>Lactobacillus fermentum</i>	2	0	0	2
33	<i>Lactobacillus gasseri</i>	24	4	16	44
34	<i>Lactobacillus helveticus</i>	1	1	0	2
35	<i>Lactobacillus iners</i>	14	2	0	16
36	<i>Lactobacillus jensenii</i>	28	2	14	44
37	<i>Lactobacillus mucosae</i>	0	1	0	1
38	<i>Lactobacillus rhamnosus</i>	3	3	0	6
39	<i>Lactobacillus salivarius</i>	1	1	0	2
40	<i>Lactobacillus vaginalis</i>	13	0	0	13
41	<i>Lactobacillus coleohominis</i>	1	0	0	1
42	<i>Pediococcus acidilactici</i>	1	0	0	1
43	<i>Peptoniphilus indolicus</i>	1	25	0	26
44	<i>Peptostreptococcus anaerobius</i>	1	4	0	5
45	<i>Peptostreptococcus</i> sp.	0	8	0	8
46	<i>Prevotella bivia</i>	4	2	0	6
47	<i>Pseudomonas stutzeri</i>	0	1	0	1
48	<i>Staphylococcus aureus</i>	0	1	0	1
49	<i>Staphylococcus capitis</i>	1	4	0	5
50	<i>Staphylococcus epidermidis</i>	3	13	0	16
51	<i>Staphylococcus haemolyticus</i>	2	1	0	3
52	<i>Staphylococcus hominis</i>	1	4	0	5
53	<i>Staphylococcus warneri</i>	0	2	0	2
54	<i>Streptococcus agalactiae</i>	1	4	3	8
55	<i>Streptococcus anginosus</i>	3	44	3	50
56	<i>Streptococcus bovis</i>	0	8	0	8
57	<i>Streptococcus intermedius</i>	0	1	0	1
58	<i>Streptococcus mitis</i> group	0	7	0	7
59	<i>Streptococcus mutans</i>	1	0	0	1
60	<i>Streptococcus parasanguinis</i>	0	1	0	1
61	<i>Streptococcus salivarius</i>	3	2	1	6
62	<i>Varibaculum cambriense</i>	0	1	0	1
63	<i>Weissella paramesenteroides</i>	0	1	0	1
		193	270	50	513

a: Number of pregnant women carrying this species.

were observed, the one or two rectal *L. crispatus* isolates had the same genotype of at least one of the one to four vaginal isolates. For only two of the 11 women for which more than one vaginal *L. jensenii* isolate was present, the genotype of all isolates was identical. For another two of these 11 women, all four isolates were genotypically different. For half (7/14) of the subjects with *L. jensenii*, the presence of identical rectal and vaginal isolates could be established.

For the other species, a similar pattern of large heterogeneity among the vaginal strains of the same species was also observed and about half of the women were found to carry at least one vaginal isolate genotypically identical to at least one rectal isolate.

Discussion

During the last decade, the composition of the vaginal microflora has been well characterised, using culture based and culture independent methods [8,21-27]. However, the origin of lactobacilli and BV associated bacteria remains less well-understood and different opinions exist as to whether the vaginal bacteria are largely endogenous or whether there is continuous recolonization from the rectum. Few studies have addressed the species composition of both vagina and rectum in the same subjects [2,3,5] and only the study of Marrazzo *et al.* [3] genotyped paired rectal/vaginal isolates, from lesbian women. To our knowledge, this is the first study among pregnant women to address the relatedness of vaginal and rectal strains to the degree of clonal identity of the strains.

Rectal and vaginal occurrence of lactobacilli

Table 3 compares the findings of this study with those of two other groups with respect to rectal and vaginal colonization by lactobacilli. The four species predominant in the vagina, as established in this study, i.e. *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners* are in correspondence with previous studies [2,3,6,16,28-30].

Both Antonio *et al.* [2] and Marrazzo *et al.* [3] reported *L. crispatus* and *L. jensenii* twice as much vaginally compared to their rectal occurrence, whereas we report four, respectively three times higher abundance of these species vaginally. Both groups also found approximately equal abundance for *L. gasseri* in rectum and vagina, whereas we could isolate twice as much *L. gasseri* from the vagina. *L. iners* was found ten times more often vaginally than rectally in this study and 20 times more vaginally than rectally in that of Antonio *et al.* [2], while this species was virtually absent from the study of Marrazzo *et al.* [3]. *L. vaginalis* was not found by Marrazzo *et al.* [3] and virtually absent in the study of Antonio *et al.* [2], whereas we found a vaginal carriage rate of approximately 10%.

The differences in *Lactobacillus* vaginal microflora between studies may be attributed to several factors. One suggestion is that the intestinal lactobacilli differ geographically [5], and the same may be true for the vaginal lactobacilli [31]. Also the populations studied differ, e.g. pregnant women in our study vs. nonpregnant women in the study of Antonio *et al.* [2] and lesbian women in the study of Marrazzo *et al.* [3]. Strong differences may exist between women, e.g. on average only 30% of the women carry *L. crispatus*, differences between Caucasian and black women have been reported [32], and differences may be present for each woman because samples can be taken during different phases of the menstrual cycle. More technically related factors concern variations in the way that samples are taken, transported and treated, the fact that culture media and incubation methods may strongly influence the outcome, e.g. incubation in an anaerobic chamber yields more *L. vaginalis* than incubation in an anaerobic jar (see below) and the use of MRS agar precludes isolation of *L. iners*, and that identification has often been based on phenotypic methods [33,34].

In addition, Kim *et al.* [35], reported that the vaginal microflora is not homogeneous throughout the vaginal tract but differs significantly within an individual with regard to anatomical site and sampling method used.

Apparently, when based on culture, the vaginal carriage rate for *L. crispatus* ranges between 20 and 40% (Antonio *et al.* [6], Kiss *et al.* [28], this study), although Marrazzo *et al.* [3] report a carriage rate of approximately 65%. The high percentage reported by the latter group could be related to the study of a different population (lesbian women), to the use of culture methods better suited for *L. crispatus* and corresponds better with results obtained by non culture based methods, as reported previously [16,22,26,27,32,36-38].

Interestingly, in a previous study of our group [8], we isolated almost no *L. vaginalis*, using anaerobic jars and GasPak (Becton Dickinson), yielding an atmosphere of 15% CO₂, 80% N₂, and less than 1% O₂. Since we started using an anaerobic chamber, with an atmosphere of 10% H₂, 10% CO₂, and 80% N₂, the number of *L. vaginalis* isolations has increased significantly and this species is now among the five most abundant vaginal species. Possibly, the virtual absence of *L. vaginalis* in the studies of Antonio *et al.* [2] and Marrazzo *et al.* [3] might be explained by the use of anaerobic culture in jars.

Comparing the reported culture results for *L. iners* remains also problematic, because this species does not grow on MRS agar, specifically designed to culture lactobacilli, and the small colonies it forms on most media may be more easily overlooked.

Table 2: Genotyping results for the 50 cases in which the same species could be isolated from vagina and rectum of the same subject

Subjects, arranged per species	V1 ^a	V2	V3	V4	R1	R2	R3	R4	V = R ^c
<i>Lactobacillus crispatus</i>									
1 RVS003	B ^b	A		C			A		1
2 RVS011			A	A			A		2
3 RVS013		A	B				A		3
4 RVS020	A			B				C	
5 RVS023			A	B			C		
6 RVS028	A			B			C	D	
7 RVS043			A				A		4
8 RVS061	A	A	A	A				A	5
9 RVS069	B	A	C	A			A		6
10 RVS099	B		A					A	7
<i>Lactobacillus jensenii</i>									
11 RVS015	B	C	D	A		A			8
12 RVS019	B	A	C	A	D		E		
13 RVS022		A	A	B		A	A		9
14 RVS023		A						B	
15 RVS038		A		B	C				
16 RVS040		A	B				C		
17 RVS057	B	C	A	A			D		
18 RVS058	A	A		A	B				
19 RVS080	A		B	A			B		10
20 RVS092				A				A	11
21 RVS093	A	A		A				A	12
22 RVS097	C	A	B	A			A		13
23 RVS111				A			A	A	14
24 RVS113			A		B				
<i>Lactobacillus gasseri</i>									
25 RVS024			A		A		A	A	15
26 RVS025	B		A	A			C		
27 RVS031		A	A	B				C	
28 RVS035	A	A					A		16
29 RVS044			A				A		17
30 RVS051		A	A				A		18
31 RVS054			A					A	19
32 RVS060			A		A				20
33 RVS072				A			A		21
34 RVS084	B			A				A	22
35 RVS090			A	A			A	A	23
36 RVS091	A			A			A	B	24
37 RVS095		B	A	A				A	25
38 RVS109			A	B			A		26
39 RVS110	A	A	B	B			B	A	27
40 RVS136	A	A	A	A			A		28
<i>Streptococcus anginosus</i>									
41 RVS003			A		B				
42 RVS058			A			A	B		29
43 RVS068				A		A	A		30
<i>Streptococcus agalactiae</i>									
44 RVS027	B		A			A		A	31
45 RVS064	A	A	A	A			A		32
46 RVS076		A		B	C	D			
<i>Enterococcus faecalis</i>									
47 RVS086	A	A	A	A	A	A	A	A	33
48 RVS122				B	A	A			
<i>Streptococcus salivarius</i>									
49 RVS075				A				B	
<i>Bifidobacterium bifidum</i>									
50 RVS033	A			A			A		34

a: V: vaginal isolates 1-4, R: rectal isolates 1-4.

b: Different genotypes are designated A, B, C, D or E per subject. Genotypic similarity/difference only relates to the other isolates of the same subject, i.e. genotype A of subject 1 does not indicate similarity to genotype A of subject 2.

c: Women for which the genotype of at least one vaginal isolate was identical to that of at least one rectal isolate.

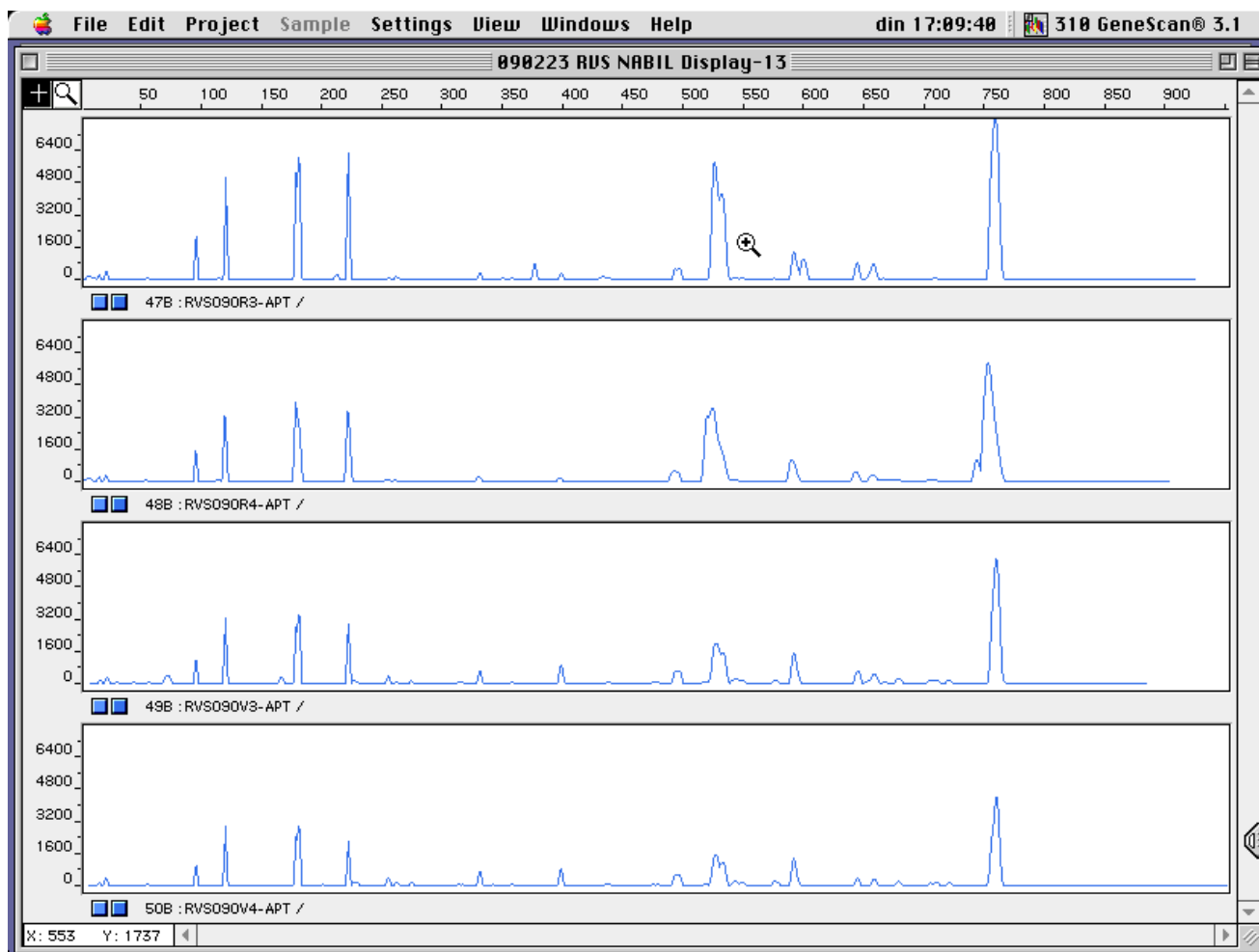


Figure 1
RAPD fingerprints of vaginal and rectal *L. gasseri* isolates of subject RVS090. Vaginal isolates: V3 and V4; rectal isolates: R3 and R4. x-axis: length of amplified DNA fragments expressed in bps. y-axis: peak height (fluorescence intensity of DNA-fragment as measured by ABI310 capillary electrophoresis).

The rectal occurrence of lactobacilli in culture-based studies may be underreported. Because of lactobacilli are the predominant species vaginally, they can be easily overgrown by the predominant rectal bacteria as they represent about 0,01% of the overall cultivable bacterial intestinal population [39,40]. However, high occurrence of intestinal lactobacilli has been reported [41,42]. The difference with our study might be explained by the fact that these studies used fecal samples, whereas we started from rectal swabbing.

The virtual absence of *L. iners* from the rectum in this culture-based study is in correspondence with the findings of the other culture-based studies [2,3]. However, preliminary data, obtained by analyzing the same samples using *L. iners* specific realtime PCR, indicate that for most women for which this species could be isolated by culture

from the vagina, also the rectal sample is *L. iners* PCR positive (data not reported).

In this population of pregnant women, we isolated lactobacilli more frequently from the vagina (121 subjects, 91.6%) than from the rectum (52 subjects, 39.3%), which is in correspondence with the findings of Antonio *et al.* [2], who reported vaginal recovery of lactobacilli in 74% and rectal recovery in 51% of a total of 531 nonpregnant females.

Although for many women from which lactobacilli could be isolated from the vagina, no lactobacilli were isolated rectally, most of the women that carried lactobacilli rectally, also had vaginal lactobacilli, i.e. few women carried lactobacilli only rectally.

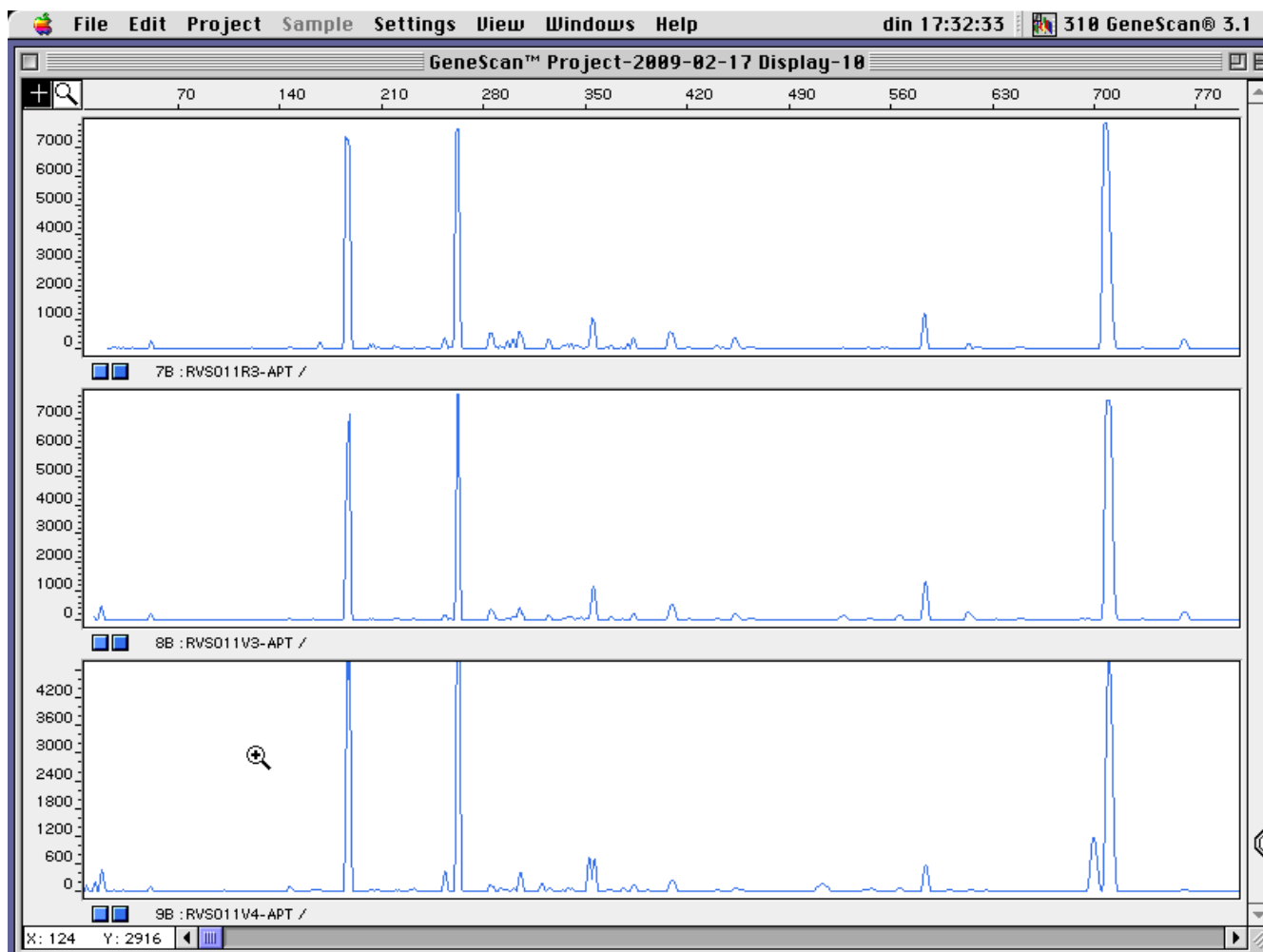


Figure 2
RAPD fingerprints of vaginal and rectal *L. crispatus* isolates of subject RVS011. Vaginal isolates: V3 and V4; rectal isolate: R3. x-axis: length of amplified DNA fragments expressed in bps. y-axis: peak height (fluorescence intensity of DNA-fragment as measured by ABI310 capillary electrophoresis).

Number of *Lactobacillus* species per individual

In our study, a total of 46 (35%) of 132 pregnant women carried two or more *Lactobacillus* species vaginally and/or rectally. Marrazzo *et al.* [3] reported 72% of 237 participants to be colonized overall by lactobacilli and 24% to be overall colonized with more than one *Lactobacillus* species. Antonio *et al.* [6] reported that 8% carried more than one *Lactobacillus* species in the vagina.

A total of 18 women in this study were colonized by both *L. crispatus* and *L. jensenii* (17 vaginally and one rectally). Vaginal colonization of women with *L. crispatus* and *L. jensenii* has been suggested to be advantageous in the maintenance of a normal microflora and the prevention of sexually transmitted diseases [2,6].

Genotyping results: clonal identity between vaginal and rectal isolates

RAPD was used in this study to detect genotypic similarity of vaginal and rectal strains of the same bacterial species. We could show that for 34 of the 50 pairs (68%) for which several isolates of the same species were present both in vagina and rectum, genotypic identity could be observed between at least one of the vaginal and at least one of the rectal isolates. In another study, on the same population of women, we compared the genotypes of rectal and vaginal *Streptococcus agalactiae* (group B streptococci: GBS) isolates and found clonal identity between isolates from both sites in 18 of the 19 subjects [43], confirming that also for GBS the rectally occurring strains are frequently identical to their vaginal counterparts. Because of the close proximity of the rectum to the vagina, the isolation of H₂O₂-producing vaginal *Lactobacillus* species from the

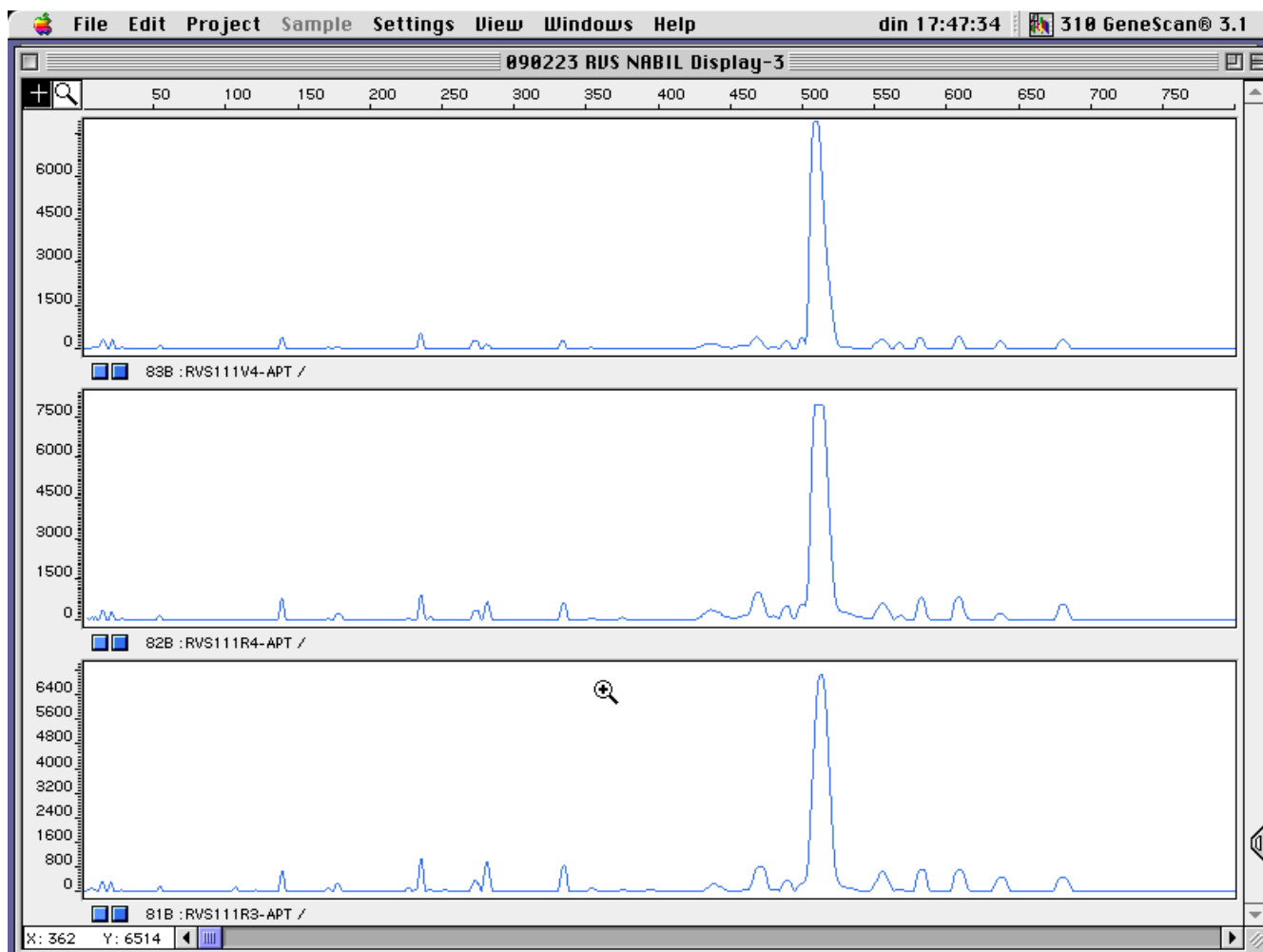


Figure 3
RAPD fingerprints of vaginal and rectal *L. jensenii* isolates of subject RVS111. Vaginal isolate: V4; rectal isolates: R3 and R4. x-axis: length of amplified DNA fragments expressed in bps. y-axis: peak height (fluorescence intensity of DNA-fragment as measured by ABI310 capillary electrophoresis).

rectum suggests that it may play a role as a reservoir for these microorganisms [2].

Vaginal colonization by *Lactobacillus* species was found to be transient in many females [7], and the rectum may be a source for vaginal recolonisation by lactobacilli after a disturbance of the ecology that follows douching, menses or sexual intercourse. Studies conducted between 1960 and 1980 indicated that while most *Lactobacillus* strains found in the human intestinal tract are allochthonous, *L. acidophilus*, *L. fermentum* (now *L. reuteri*) and *L. salivarius* can be isolated from individuals over longer periods [44-48]. Since on the basis of current taxonomy *L. crispatus* [49], *L. gasseri* [50] and *L. iners* [51] belong to the *L. acidophilus* complex the rectum may be a source for vaginal recolonization by these *Lactobacillus* species.

Genotyping results: variability among multiple isolates per subject

We found a surprising high genotypic heterogeneity within species. For the 50 species for which isolates were available from both vagina and rectum of a total of 47 pregnant women, on average of 2.2 vaginal respectively 1.3 rectal isolates were genotyped and on average 1.6 vaginal genotypes and 1.1 rectal genotypes were found for these species. It can be expected that more species pairs, more intraspecies genotypic diversity and more identical genotypes in rectum and vagina will be found, when more isolates would be picked. Although we did not sample the same subjects at different time intervals, this finding suggests the occurrence of changes in the composition of the vaginal microflora, whereby different strains of a limited number of species may replace each other, and are may be exchanged between vagina and rectum.

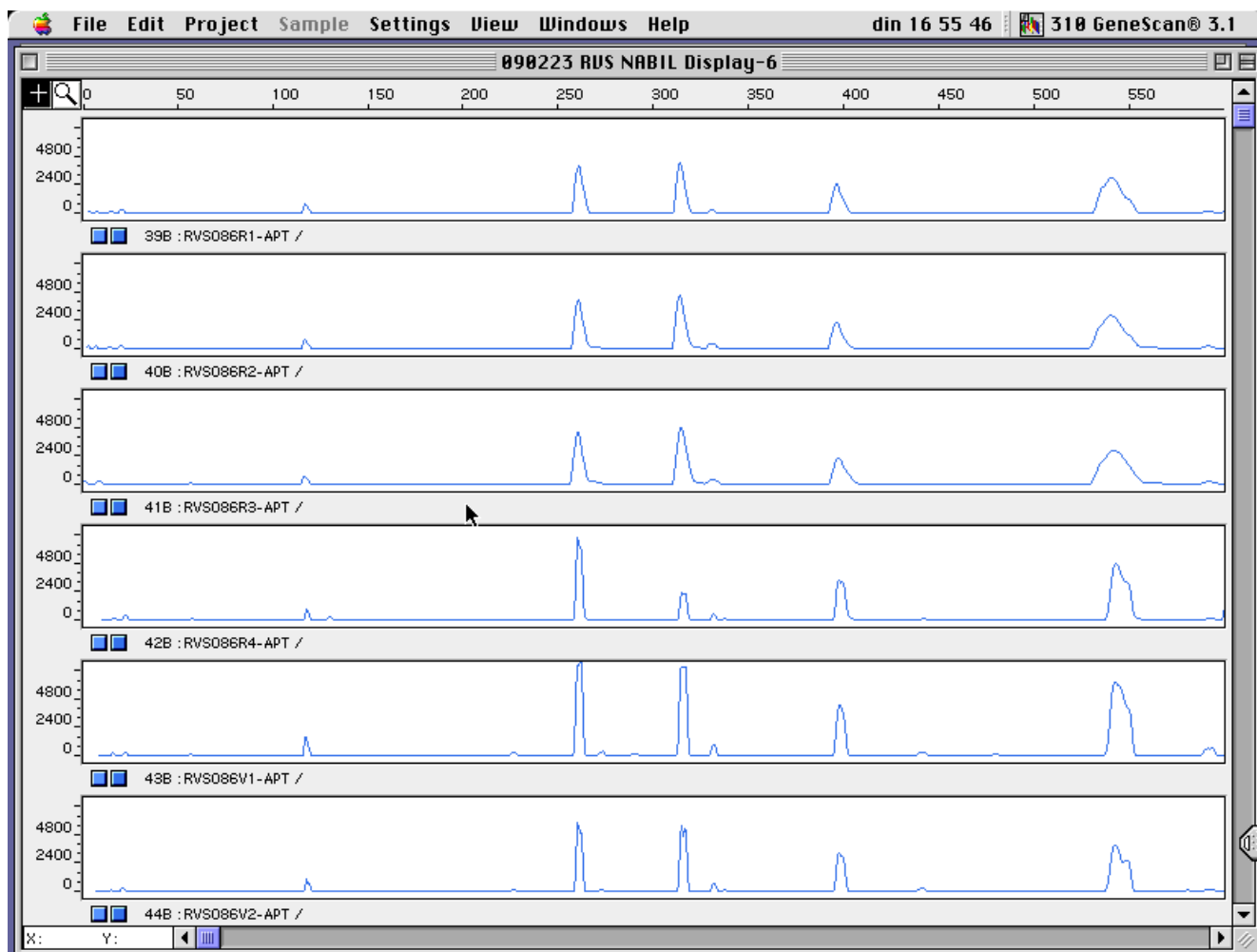


Figure 4
RAPD fingerprints of vaginal and rectal *E. faecalis* isolates of subject RVS086. Vaginal isolates: V1 and V2; rectal isolates: R1 - R4. x-axis: length of amplified DNA fragments expressed in bps. y-axis: peak height (fluorescence intensity of DNA-fragment as measured by ABI310 capillary electrophoresis).

The high transmissibility of strains was also established in the study of Marrazzo *et al.* [3] by the observation that many sexual partners carried genotypically identical strains. This hypothesis may be confirmed by long term follow up of individual women, e.g. during subsequent menstrual cycles, and by picking more isolates per subject and per site at multiple time points.

Conclusion

Although it has been claimed that the vaginal microflora originates from the rectal microflora, this is to our knowledge only the second study, besides the recent study of Marrazzo *et al.* [3] to address this in detail at the strain level. Several of the total of 63 species identified were found only vaginally (9, i.e. 14.3%) or only rectally (26, i.e. 41.3%), but 29 species (44.4%) were isolated from

both sites, indicating that many species can colonize the vagina from the rectum or migrate to the rectum from the vagina. For the 8 species for which isolates were present simultaneously in the same subject in vagina and rectum, we found considerable genotypic diversity within the species (i.e. on average 1.36 genotypes for on average 1.79 isolates per subject), both in rectum and vagina, as well as identical genotypes present simultaneously in rectum and vagina for 70% of the 50 species pairs studied. All these data indicate a strong correlation between vaginal and rectal microflora, not only at the species level but also at the strain level.

It is possible that the rectal colonization by lactobacilli may function as a reservoir for the maintenance of a normal vaginal flora and that this may be associated with a

Table 3: Vaginal and rectal occurrence of *Lactobacillus* species, expressed as percentage of subjects positive, according to different studies

Authors	Population studied	Species	Vagina only	Vagina all ^a	Vagina & Rectum	Rectum only	Rectum all ^a	Overall
Antonio et al. 1999	302 sexually active women	<i>L. crispatus</i>	32					
		<i>L. jensenii</i>	23					
		<i>L. gasseri</i>	5					
		<i>L. iners</i>	15					
		<i>L. vaginalis</i>	< 1					
Antonio et al. 2005	290 nonpregnant women	<i>L. crispatus</i>	17	31	14	1	15	33
		<i>L. jensenii</i>	17	23	6	4	10	27
		<i>L. gasseri</i>	3	5	2	8	10	13
		<i>L. iners</i>	15	16	1	0	1	16
		<i>L. vaginalis</i>	< 1	0	0	0	0	0
Marrazzo et al. 2009	237 women having sex with women	<i>L. crispatus</i>	49	93	44	5	49	98
		<i>L. jensenii</i>	8	10	2	1	3	11
		<i>L. gasseri</i>	10	16	6	6	12	21
		<i>L. iners</i>	1	1	0	0	0	1
		<i>L. vaginalis</i>	0	0	0	0	0	0
This study	132 pregnant women	<i>L. crispatus</i>	33	41	8	3	11	42
		<i>L. jensenii</i>	21	32	11	2	13	33
		<i>L. gasseri</i>	18	30	12	3	15	33
		<i>L. iners</i>	11	11	0	2	2	12
		<i>L. vaginalis</i>	10	10	0	0	0	10

a: The column entitled 'Vagina all' presents the sum of subjects with the species in the vagina only and those with the species in both vagina and rectum. The column entitled 'Rectum all' presents the sum of subjects with the species in the rectum only and those with the species in both vagina and rectum.

decreased incidence of BV-associated adverse effects, as has been suggested [3].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NE, RV, GC and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. IT, HV and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. BS, PC, GLSS and EDB participated in the analysis of the study samples and interpretation of the data. All authors read and approved the final manuscript.

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II.2. Publication 2

El Aila NA, Tency I, Claeys G, Saerens B, De Backer E, Verstraelen H, Temmerman M, Verhelst R, Vaneechoutte M.

Correlation of the vaginal and rectal occurrence and bacterial load of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae*, as established by culture and qPCR.

Submitted to Research in Microbiology.

Correlation of the vaginal and rectal occurrence and bacterial load of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae*, as established by culture and qPCR

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Abstract

Background

To further establish the potential role of the rectum as a reservoir for the vaginal microflora, both sites were sampled and the bacterial loads of six species were established by quantitative PCR (qPCR).

Methods

Vaginal and rectal occurrence of *Lactobacillus crispatus*, *L. jensenii*, *L. gasseri*, *L. iners*, *Gardnerella vaginalis* and *Atopobium vaginae* was detected by culture and qPCR, for 71 pregnant women at 35-37 weeks of gestation. Vaginal and rectal swabs were cultured anaerobically on Columbia CNA agar and MRS agar. DNA was extracted from the swabs and existing qPCR formats for the quantification of the six species mentioned, was carried out.

Results

Culture and qPCR results differed substantially with regard to the evaluation of the vaginal and rectal occurrence of the six species tested. The vaginorectal prevalence of *L. crispatus*, *L. jensenii*, *L. iners*, *L. gasseri*, *G. vaginalis* and *A. vaginae* as established by culture versus PCR was 32.3 vs 91.5%, 32.3 vs 77.4%, 12.6 vs 68.5%, 28.1 vs 91.5%, 12.6 vs 74.6% and 5.6 vs 69.0%, respectively.

Using qPCR, significant positive correlation was found between vaginal and rectal loads of *A. vaginae* ($r = 0.60$, $p = 0.002$), *L. crispatus* ($r = 0.80$, $p < 0.0001$), *L. gasseri* ($r = 0.45$, $p = 0.005$), *L. iners* ($r = 0.51$, $p = 0.003$) and *L. jensenii* ($r = 0.85$, $p < 0.0001$). Further significant positive correlation was found between the vaginal presence of *G. vaginalis* and that of *A. vaginae* ($r = 0.396$, $p = 0.0006$),

whereas the vaginal presence of *L. crispatus* was negatively correlated with that of *G. vaginalis* ($r = - 0.363$, $p = 0.001$) and that of *A. vaginae* ($r = - 0.261$, $p < 0.05$).

Conclusion

We established significant differences between detection of six bacterial species of the vaginal and rectal microflora using culture or qPCR. Using qPCR, significant correlations between quantities of vaginal and rectal lactobacilli and of *Atopobium vaginae* were established, indicating important correspondence of the vaginal and rectal microflora, not only in the occurrence of certain species in both niches, but also of cell densities per bacterial species, and confirming the possible role of the rectum as a reservoir for vaginal colonization.

Background

The vaginal microflora plays an active role in preventing genital infections [1-4]. This protective function, i.e., colonisation resistance, is especially related to the presence of bacteria belonging to the genus *Lactobacillus*, which are able to prevent the overgrowth of pathogenic and opportunistic organisms [5]. Four species of lactobacilli are now considered to be predominantly linked to the vaginal microflora: *Lactobacillus crispatus*, *L. jensenii*, *L. gasseri* and *L. iners*, with the latter only recently being recognized as it was long overlooked, also because it does not grow on de Man Rogosa Sharpe agar, the medium typically used to culture lactobacilli [6, 7]. Bacterial vaginosis (BV) is a common disorder that involves an alteration in the vaginal microflora, whereby the predominant lactobacilli are replaced by *Gardnerella vaginalis* and anaerobic bacteria [8-10]. Recently, *Atopobium vaginae* has been strongly associated with BV, independently by different groups [11-16]. Several bacterial species are known to colonize both the gastrointestinal and the reproductive tract, and the rectum has been suggested to play an important role as a source or reservoir for organisms that colonize the vagina [6, 17-20]. Recently, we showed that rectum and vagina not only harbour the same bacterial species in 36% of 132 pregnant women studied, but also the same genotypes for 34 of the 50 species pairs (68%) [21]. The ingestion of *Lactobacillus*-containing products for the treatment or prevention of vaginal infections has shown that vaginal colonization can be obtained with these strains within days after oral application [22, 23].

In this study, we compared culture with qPCR to detect and quantify four *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae* in the vagina and rectum of pregnant women.

Materials and Methods

Patients. The study was approved by the research ethics committee (IRB protocol nr 2007/096) of the Ghent University Hospital, Belgium. Participating women gave a written informed consent. Between April and December 2007, paired vaginal and rectal swabs were collected from 71 pregnant women at 35 – 37 weeks of gestation.

Sampling procedures. All specimens were collected using nylon flocked swabs that were submerged into 1 ml of liquid Amies transport medium (eSwab, Copan Diagnostics, Brescia, Italy). For rectal specimens, a swab was carefully inserted approximately 1.5 - 2 cm beyond the anal sphincter and then gently rotated to touch anal crypts. Vaginal samples were obtained through blind swabbing by which the swab was rolled around 360 degrees against the vaginal wall at the midportion of the vault. All study samples were collected by midwives and transported to the Laboratory for Bacteriology Research of the University of Gent, inoculated on culture media and submerged in DNA extraction buffer within 4 hours.

Culture and Gram staining

A total of 70 µl from the Amies liquid transport medium of each of the vaginal and rectal swabs was inoculated onto Columbia CNA agar with 5% sheep blood (Columbia CNA agar, Becton Dickinson, Erembodegem, Belgium) and de Man Rogosa Sharp Agar (MRS, Oxoid, Hampshire, UK) and then incubated at 37 °C in an anaerobic chamber (10% H₂, 10% CO₂, 80% N₂) (BugBox, LedTechno,

Heusden-Zolder, Belgium) for 72 h. Another 50 µl of the vaginal swab suspension was taken for Gram stain smear preparation.

Gram stain based grading was carried out according to modified Ison & Hay criteria [24], as described by Verhelst *et al.* [25]. Grade Ia specimens contained mainly *Lactobacillus crispatus* cell types, i.e., plump, quite homogeneous lactobacilli, grade Ib contained non-*L. crispatus* cell types, i.e., long or short, thin lactobacilli, grade Iab contained mixtures of *L. crispatus* and non-*L. crispatus* cell types, grade I-like contained irregular-shaped Gram positive rods, grade II contained a mixture of *Lactobacillus* cell types and bacterial vaginosis-associated bacteria (*Gardnerella*, *Bacteroides-Prevotella* and *Mobiluncus* cell types), whereas samples devoid of *Lactobacillus* cell types with the presence of only *Gardnerella*, *Bacteroides-Prevotella* or *Mobiluncus* cell types were classified as grade III. Finally, samples were classified as grade IV when Gram positive cocci were predominantly present and as grade 0 when no bacterial cells were present [25].

DNA-extraction from samples

DNA extraction from the samples was performed using the NucliSENS EasyMAG platform according to the manufacturer's instructions. Briefly, 200 µl of the Amies transport medium from each the vaginal and rectal swab was added to the easyMAG lysis buffer and incubated for 10 min at room temperature and stored at -80°C until extraction. Total nucleic acid extraction of the samples was carried out and nucleic acids were eluted in 100 µL of elution buffer.

DNA-extraction from isolates

DNA was extracted from cultured isolates by alkaline lysis as follows: One bacterial colony was suspended in 20 µl of lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and heated at 95 °C for 15 min. The cell lysate was diluted

by adding 180 µl of distilled water. The cell debris was spun down by centrifugation at 16,000 *g* for 5 min. Supernatants were used for tDNA-PCR or frozen at -20 °C until further use.

Identification of isolates by tRNA intergenic length polymorphism analysis (tDNA-PCR)

From 71 women, 8 colonies per subject, i.e., one colony of each of the two most abundant colony types from both Columbia CNA and MRS agar plates and for both rectal and vaginal swabs were picked, i.e., a total of 568 isolates, 284 vaginal and 284 rectal. Isolates were identified by means of tRNA intergenic length polymorphism analysis (tDNA-PCR) as described previously [13, 26, 27]. For some isolates, no clearcut identification was obtained by tDNA-PCR. These isolates were identified by 16S rRNA gene sequencing [13].

Primers for qPCR

qPCR primer sets targeting *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. iners*, *G.vaginalis*, and *A. vaginae* were used as described previously [28].

Construction of the standard curves for qPCR

To construct standard curves for the qPCR's, *A. vaginae* (PB2003/189-T1-4), *L. crispatus* (PB2003/125-T1-1), *L. gasseri* (PB2003/088-T2-1) and *L. jensenii* (PB2003/204-T1-1) were cultured on TSA + 5% sheep blood (Becton Dickinson) and *G. vaginalis* (BVS026) and *L. iners* (BVS11) were cultured onto Columbia CNA agar with 5% sheep blood (Columbia CNA agar, Becton Dickinson, Erembodegem, Belgium). A suspension was made in MRS Broth (Oxoid, Drongen, Belgium) and DNA was extracted with Roche extraction method. The DNA concentration of this stock was determined ten times by using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, USA) and the mean value was used

to determine the number of bacterial cells in the initial suspension, using the genome weight per cell, as calculated for each species on the basis of GC-content and genome size of these species. For, each strain a tenfold dilution series of the DNA was prepared by dilution of the stock in HPLC grade water. Dilutions were aliquoted and stored at -20°C.

Quantitative PCR (qPCR)

The qPCR Core Kit for SYBR Green I (Eurogentec) was applied and analysis was performed on the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR mixtures contained 2.5 µl of DNA extract, 2.5 µl of 10 × Reaction Buffer, 3.5 mM MgCl₂, 0.2 mM dNTP mixture, 0.625 U HotGoldStar *Taq* polymerase, 0.75 µl SYBR® Green I, diluted 10-fold in DMSO and primers at a concentration of 0.2 µM, except for *G. vaginalis* where the primer concentration was 0.1 µM and were adjusted with HPLC grade water to 25 µl. Each sample was analyzed in duplicate with each of the six different primer sets, i.e., one for each of the six species tested, and each run included a standard curve for the species tested. In case the result was not in the range of the standard curve, the samples were diluted tenfold and analyzed in duplicate again.

Statistical analysis

Data were analyzed using the Mann-Whitney U-test for two groups and with the Kruskal Wallis test for multiple groups. Correlations between the different species were determined by the Spearman (rank) test and reported as Spearman's rho value (r). All analyses were performed using Graphpad Prism software (<http://www.graphpad.com>)

Results

Culture results

Table 1 and Figure 1 present the occurrence of four *Lactobacillus* species and *G. vaginalis* and *A. vaginae* vaginally, rectally and both vaginally and rectally, as estimated by using culture and qPCR.

Based on culture data, all six species were recovered more frequently from the vagina (ranging from 4.2% for *A. vaginae* to 30.9% for *L. crispatus* and *L. jensenii*) than from the rectum (ranging from 1.4% for *A. vaginae* to 11.2% for *L. crispatus* and *L. jensenii*). For most women for whom a species could be cultured rectally, it could also be cultured from the vagina. The number of women with one of the six species only in the rectum was low (0-2.8%), compared to the number of women with these species only in the vagina (ranging from 4.2% for *A. vaginae* to 21.1% for *L. crispatus* and *L. jensenii*). *A. vaginae* was cultured least frequently, i.e., from only 1.4% of the women rectally and only 4.2% of the women vaginally. The species most abundantly present overall, according to culture, were *L. crispatus*, *L. jensenii* and *L. gasseri*, i.e., in approximately 30% of the women.

qPCR results

qPCR yielded a much higher number of women positive rectally and/or vaginally for any of the six species, compared to culture. The overall prevalence of *L. crispatus*, *L. jensenii*, *L. iners*, *L. gasseri*, *G. vaginalis* and *A. vaginae* as established by culture versus qPCR was 32.3 vs 91.5%, 32.3 vs 77.4%, 12.6 vs 68.5%, 28.1 vs 91.5%, 12.6 vs 74.6% and 5.6 vs 69.0%, respectively (Table 1).

The higher number of qPCR positive women is also reflected when looking at the number of women carrying the same species simultaneously vaginally and rectally: according to qPCR these percentages range from 30.9% for *A. vaginae* to

60.0% for *L. crispatus*, whereas, according to culture the percentage of positives ranges from 0% for *A. vaginae*, *G. vaginalis* and *L. iners* to 9.8% for *L. crispatus* and *L. jensenii*.

Moreover, when using qPCR, significant positive correlations were found between vaginal and rectal colonization, not only with respect to the presence of the same species rectally and vaginally, but also with respect to the bacterial load in both sites, i.e., there was a high degree of correspondence between vaginal bacterial load and rectal bacterial load for five of the six species: *A. vaginae* ($r = 0.60$, $p = 0.002$), *L. crispatus* ($r = 0.80$, $p < 0.0001$), *L. gasseri* ($r = 0.45$, $p = 0.005$), *L. iners* ($r = 0.51$, $p = 0.003$) and *L. jensenii* ($r = 0.85$, $p < 0.0001$). Only for *G. vaginalis*, we could not establish significant correlation between vaginal and rectal bacterial load ($r = 0.23$, $p = 0.172$). This correspondence between vaginal and rectal load for five of the six species tested is also apparent from Figures 2 (panels A-D and F).

Culture vs qPCR results

Figures 2, panels A-F, demonstrates that culture positive women mostly corresponded to women for which a high bacterial load was established when using qPCR. Women positive by culture for a certain species only vaginally or only rectally were usually found to carry this species in high numbers and in both sites, according to qPCR. Correspondingly, when women were culture positive, irrespective of whether this was vaginally only, rectally only or vaginorectally, they in general had high qPCR bacterial loads both vaginally and rectally. In addition, few culture negative women had high bacterial loads according to qPCR. This is especially clear for *L. crispatus* and *L. jensenii*, for which most culture positive women were found.

It can be noted that, when using culture, all species were recovered mostly only vaginally, whereas when using qPCR, most species were recovered from both vagina and rectum, except for *A. vaginae*, which was recovered mostly from the rectum.

Presence and load of different bacterial species according to category of vaginal microflora

In this study, Gram-stained smears of 71 vaginal swabs from pregnant women were examined microscopically and categorized as described previously [25]. Nineteen women had a vaginal microflora categorized as grade Ia, 7 as grade Iab, 22 as grade Ib, 5 as grade I-like, 12 as grade II and 6 as grade III.

We found a significantly higher level of vaginal *L. crispatus* in grade Ia (median value = 7.03 log₁₀ cells/ml), compared to its presence in the non grade I categories, i.e., 0 in grade II (p < 0.0001), 3.1 in grade III (p = 0.0025). Also, there was a higher level of *L. crispatus* in grade Iab (median = 7.6 log₁₀ cells/ml), compared to the non grade I categories, i.e., 0 in grade II (p = 0.0011), 3.1 in grade III (p = 0.035) and 0 in grade I-like (p = 0.0012). The *L. crispatus* load in grade Ib samples (median = 3.00 log₁₀ cells/ml) was not significantly different from that of the non grade I categories, i.e., 0 in grade II (p = 0.26) and 3.1 in grade III (p = 0.44). Accordingly, *L. crispatus* was also present in significantly higher amounts vaginally (p < 0.0001) in grade Ia (median = 7.03 log₁₀ cells/ml) and grade Iab (7.6) than in grade Ib (2.6).

L. iners was found in all grades, but at significantly higher concentrations in grade Iab (median = 7.8 log₁₀ cells/ml) and grade Ib (8.1), than in grade Ia (0), grade II (0) and grade III (5.0). The *L. iners* load in grade Ib was not significantly different from that of grade Iab. The load of *G. vaginalis* in grade I (median = 2.7

log₁₀ cells/ml) was significantly lower than that in grade II (8.7, $p < 0.05$) and grade III (9.1, $p < 0.0001$), but did not differ significantly between grades II and III. The load of *A. vaginae* in grade I (median = 0 log₁₀ cells/ml) was significantly lower than that in grade II (5.3, $p = 0.004$) and grade III (5.6, $p < 0.05$), but did not differ significantly between grades II and III.

Correlation between the presence and load of different species

Forty two vaginal samples (59%) were positive for *G. vaginalis* by qPCR and 18 (43%) of these were also positive for *A. vaginae*. Seven samples were positive for *A. vaginae* but negative for *G. vaginalis*. All six grade III samples were positive for *G. vaginalis* and four of these were also positive for *A. vaginae*. *G. vaginalis* and *A. vaginae* were present respectively in 10 and 7 of the 12 grade II samples.

Significant positive correlations were found between the vaginal bacterial load in the different grades for *G. vaginalis* and *A. vaginae* ($r = 0.396$, $p = 0.0006$), as determined by qPCR. The bacterial load of both species was low in normal vaginal microflora (grades Ia and Iab), and high in disturbed vaginal microflora (grades II and III). Negative correlations were found between *L. crispatus* and *G. vaginalis* ($r = -0.363$, $p = 0.001$) and also between *L. crispatus* and *A. vaginae* ($r = -0.261$, $p < 0.05$).

Significant positive correlations were found between the rectal bacterial load in the different grades for *G. vaginalis* and *A. vaginae* ($r = 0.40$, $p = 0.0001$), as determined by qPCR.

Discussion

In this study we compared the sensitivity of culture versus qPCR to detect, from the vagina and the rectum of pregnant women, six bacterial species that have been shown to be important components of the vaginal microflora.

qPCR has been applied previously for the description of changes in the vaginal microflora. Bradshaw *et al.* [29] applied qPCR for *A. vaginae* for a follow-up study of recurrent BV before and after treatment with oral metronidazole. Ferris *et al.* [30] applied qPCR for *A. vaginae* to samples of six BV patients before and after treatment with a topical metronidazole gel. Fredricks *et al.* [31] described the changes in vaginal bacterial concentrations following metronidazole therapy for BV and indicated that the detection of particular vaginal bacteria or consortia by PCR may be useful for the diagnosis or monitoring of the response to antibiotic therapy.

Our findings are in agreement with previous studies, which indicate that bacterial vaginosis is associated with a significant increase in *G. vaginalis* and *A. vaginae* and a drop in *Lactobacillus* species. Biagi *et al.* [32] showed that bacterial vaginosis is associated with a significant increase of *Prevotella*, *Atopobium*, *Veillonella* and *G. vaginalis* and a decrease in *Lactobacillus* species and with high variability over time and between individuals, whereas the vaginal microflora under normal conditions and that of patients developing candidiasis was homogeneous and stable over time. Zarrifard *et al.* [33] and Sha *et al.* [34, 35], who quantified lactobacilli in cervicovaginal lavage samples, using a qPCR format, which picked up only *L. crispatus* and *L. jensenii*, found a decline in the number of lactobacilli in BV. qPCR based quantification of *G. vaginalis* and *M. hominis* from CVL was reported to be significantly more sensitive than the Amsel criteria for diagnosing BV [34]. Menard *et al.* [36] reported that qPCR can be used to objectively diagnose BV, when using cutoff values for the concentrations of *A. vaginae* and *G. vaginalis*. Recently, De Backer *et al.* [28] performed a study on 71 vaginal samples with the same primer sets, used here. Major advantages of

qPCR over the two most commonly used methods for diagnosing BV, i.e. the Amsel criteria [37] and the Gram stain [38] are the high sensitivity of PCR, which can be optimized to pick up very low numbers of bacteria, and the fact that it can be applied for the detection of bacteria in a dormant state, as is the case in biofilms, which are present vaginally in cases of BV, as has been shown by means of FISH [39]. To our knowledge, qPCR has not yet been used to compare in a quantitative manner the rectal and vaginal microflora of women, and in comparison with culture, as done in this study.

Culture versus qPCR

We compared the performance of qPCR and culture for the detection of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae*.

A striking finding is that a high number of pregnant women are positive for most of the species studied, when using qPCR, compared to culture. Instead of due to the high sensitivity of PCR, this could also be due to low specificity of this approach. However, this latter explanation is contradicted by the finding that women that were culture positive, usually also had the highest bacterial loads according to qPCR. In addition, the finding that for five of the six species, except *G. vaginalis*, high vaginal load usually corresponded with high rectal load, according to qPCR, is also an argument to explain these findings as result of the high sensitivity of qPCR rather than to its possible low specificity. In summary, qPCR is much more sensitive than culture for determining vaginal and rectal presence of bacteria, and qPCR indicates a much higher correspondence between vaginal and rectal bacterial loads, than can be established by culture. It should be noted that we picked a total of 8 colonies for each woman, whereas in routine culture applications only one or few colonies are selected for further

characterization, i.e., the sensitivity of culture in routine laboratories can be considered as even lower. This is not unexpected, because e.g., *A. vaginae*, *G. vaginalis* and *L. iners* are fastidious species, which moreover may be easily overlooked or overgrown when dealing with complex intestinal microflora.

The finding that vaginal and/or rectal samples, which are culture positive for a certain species contain high loads of that bacterial species, as quantified by qPCR, indicates that culture detects *Lactobacillus* species, *G. vaginalis* and *A. vaginae* mostly only when these species are present in higher concentrations.

Bacterial load in vaginal microflora

In our study, significant positive correlations were found between the bacterial loads of *G. vaginalis* and *A. vaginae*, as determined by qPCR. The bacterial load of both species was low in normal vaginal microflora (grades Ia and Iab), and high in disturbed vaginal microflora (grades II and III). This confirmed our previous finding, i.e., a high correlation between vaginal bacterial loads for *A. vaginae* and *G. vaginalis* [28].

Negative correlations were found between *L. crispatus* and *G. vaginalis* and also between *L. crispatus* and *A. vaginae*. In this study, we established a mean vaginal bacterial load of 9.1 cells/ml for *G. vaginalis* in grade III, which corresponds well to the value of 9.2 cells/ml, which we reported previously [28] and also to the value of 9.6 cfu/g, reported by Zariffard *et al.* [33]. In this study, we confirmed the coexistence of *A. vaginae* and *G. vaginalis*, which was recently documented in 78%–96% of vaginal samples from women with BV, whereas this association was only present in 5%–10% of samples from women with normal vaginal microflora [13, 25, 29]. In agreement with these findings, Menard *et al.* [36] (2008) reported that qPCR based quantification of *A. vaginae* ($\geq 10^8$ copies/mL)

and *G. vaginalis* ($\geq 10^9$ copies/mL) had the highest predictive value for the diagnosis of BV.

The correspondence between vaginal and rectal bacterial load

In this study, using qPCR, significant positive correlations were found between vaginal and rectal colonization, i.e., there was high degree of correspondence between vaginal bacterial load and rectal bacterial load for five of the six species, i.e., *A. vaginae*, *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*. Only for *G. vaginalis*, we could not establish significant correlation between vaginal and rectal bacterial load ($r = 0.23$, $p = 0.172$), although Figure 2e indicates that also for *G. vaginalis* for most women the vaginal loads correspond largely with rectal loads. This correspondence between vaginal and rectal load for the different species is also apparent from Figure 2. Several studies have indicated the rectum as the reservoir for the vaginal microflora. The rectum seems to be the primary habitat of *Candida* spp. in patients with candidal vulvovaginitis [40]. Meyen *et al.* [41] showed that rectal colonisation with GBS was the most significant predictor of vaginal colonization, suggesting that the vagina becomes colonized with GBS as a result of transfer of the organism from the rectum to the vagina. This is not in contradiction with our previous genotyping study, which showed a high correspondence of vaginal and rectal colonisation with GBS, also at the strain level [20]. Halen *et al.* [18] reported the simultaneous occurrence of *Mobiluncus* spp. in the vagina and rectum. Holst *et al.* [19] reported that the reservoirs of *Mobiluncus* spp., *G. vaginalis* and *Mycoplasma hominis* are in the intestinal tract. We previously showed high correlation between vaginal and rectal microflora, not only with regard to species composition but also with regard to the presence of

genotypically identical strains [20, 21]. To our knowledge, this study is the first one that also indicates a clear quantitative correspondence between the bacterial loads present in vagina and rectum, for at least five of the six species studied.

The prevalence and role of *L. iners* as part of the vaginal microflora remains intriguing. In this study, using qPCR, *L.iners* was detected vaginally and rectally in high numbers, when compared to culture, and when compared to other studies of the rectal microflora [6, 42]. In our study, the prevalence of *L. iners* in the rectum was 2.8% using culture versus 52.0%, using qPCR. In previous studies [17, 42], *L. iners* was not isolated from the rectum only but in our study, using qPCR, we could detect it from the rectum only in 9.8% and from both the vagina and the rectum in 42.2% of the subjects.

Establishing the prevalence of *L. iners* has been cumbersome thus far, when relying on culture and Gram-stain, because *L. iners* cannot be propagated on conventional *Lactobacillus*-selective media such as Rogosa or MRS agar [7] and because the cell morphology of *L. iners* is Gram-negative coccobacillar rather than Gram positive bacillar, as for regular lactobacilli [28]. Since it is the predominant *Lactobacillus* species in BV microflora [28], even present in high numbers, this has led to the false assumption that the BV microflora is devoid of lactobacilli. Moreover, because also the Gram-positive *G. vaginalis* stains Gram-negative, this has lead to the description of BV microflora as dominated by 'anaerobic Gram-negatives'.

More recently, several findings indicate some role for *L. iners* in the equilibrium between balanced and imbalanced vaginal microflora. In a previous qPCR based study, we found an inverse relationship between *L. iners* and *L. gasseri*, with *L. iners* predominant in grade III microflora, whereas *L. gasseri* was predominant in

grade II microflora [28]. Jakobsson and Forssum [43] reported pyrosequencing results that indicated that *L. iners* is a dominant part of the vaginal microflora when the microflora is in a transitional phase between abnormal and normal, either because of treatment or because of physiological changes such as varying estrogen levels. Ferris *et al.* [30] found that *L. iners* was predominant in 5 patients with BV after metronidazole treatment, whereas for the sixth patient, the only one with a complete treatment failure, *L. iners* was present but not predominant.

Conclusions

We established significant differences between detection of six bacterial species of the vaginal and rectal microflora using culture or qPCR. The carriage rate was 3-fold (for *L. crispatus*) to 17-fold (for *A. vaginae*) higher according to qPCR than according to culture, despite the fact that for each subject 8 colonies were picked and identified. Women were positive for the six species in 4.0 to 32.3% of cases according to culture, compared to 68.5 to 91.5% of cases according to qPCR. In addition and most importantly, using qPCR, significant correlation between quantities of vaginal and rectal lactobacilli and of *A. vaginae* were established, indicating important correspondence of the vaginal and rectal microflora, not only with regard to the simultaneous occurrence of these species in both niches, but also with regard to cell densities per bacterial species, and confirming the possible role of the rectum as a reservoir for vaginal colonization. Using qPCR, we could detect *L. iners* in high numbers vaginally and rectally when compared to other studies of the rectal microflora [6, 42]. Several recent studies, reviewed

here, indicate that the role of this peculiar *Lactobacillus* species in the transition between balanced and imbalanced vaginal microflora needs further elucidation.

Competing interests

The author (s) declare that they have no competing interests.

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Authors' contributions

NAE, RV and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. IT, HV and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. BS and EDB participated in the analysis of the study samples and interpretation of the data. All authors read and approved the final manuscript.

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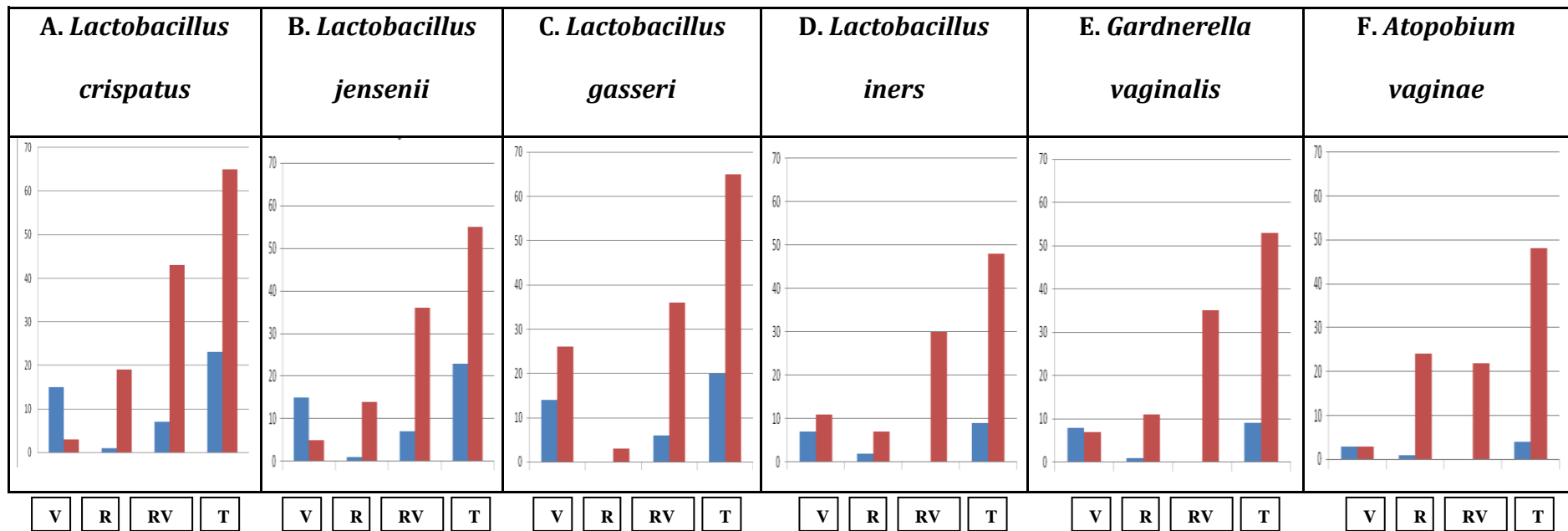
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Figures

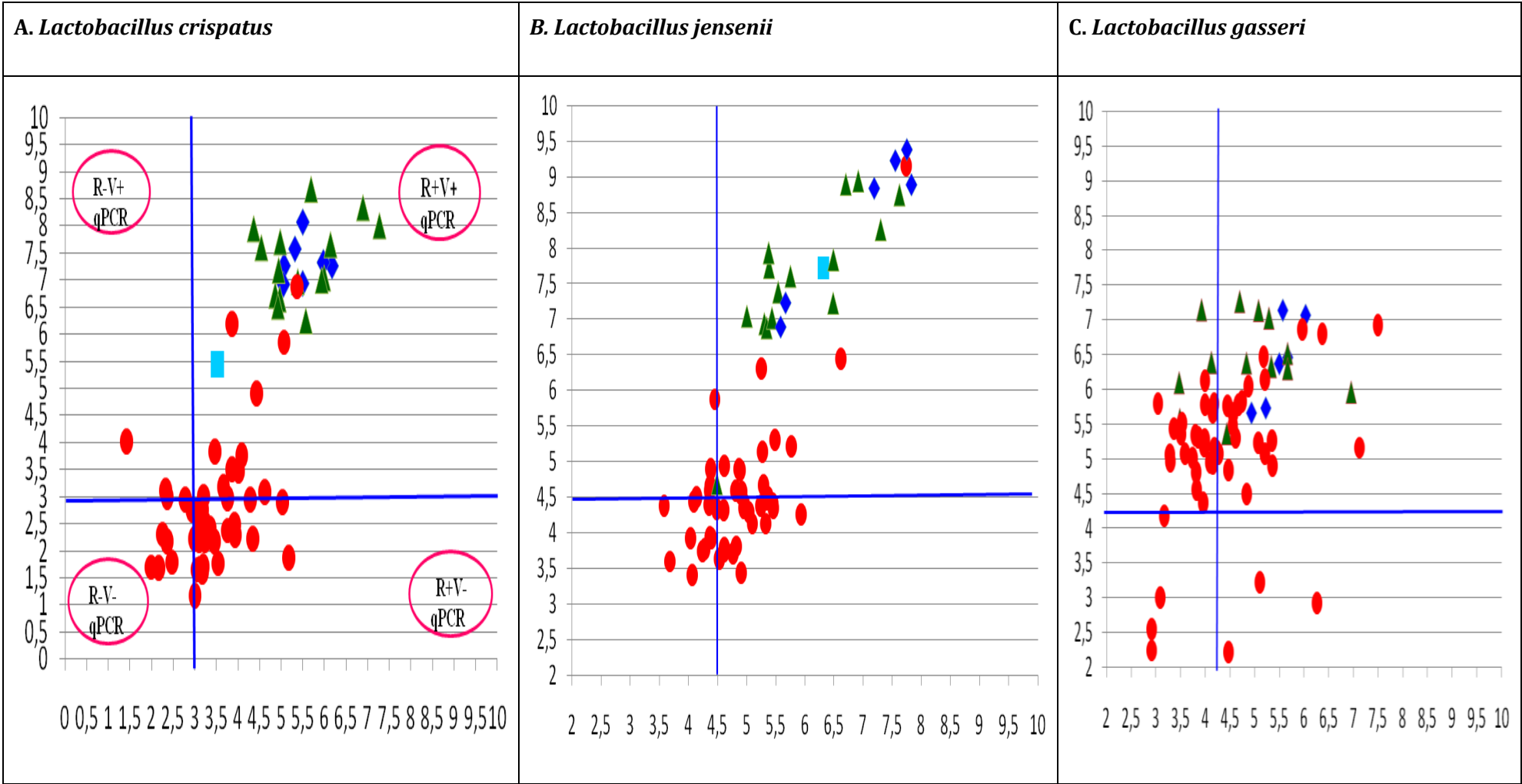
Figure 1. Number among 71 women carrying four *Lactobacillus* species (A-D) or *Gardnerella vaginalis* (E) or *Atopobium vaginae* (F)



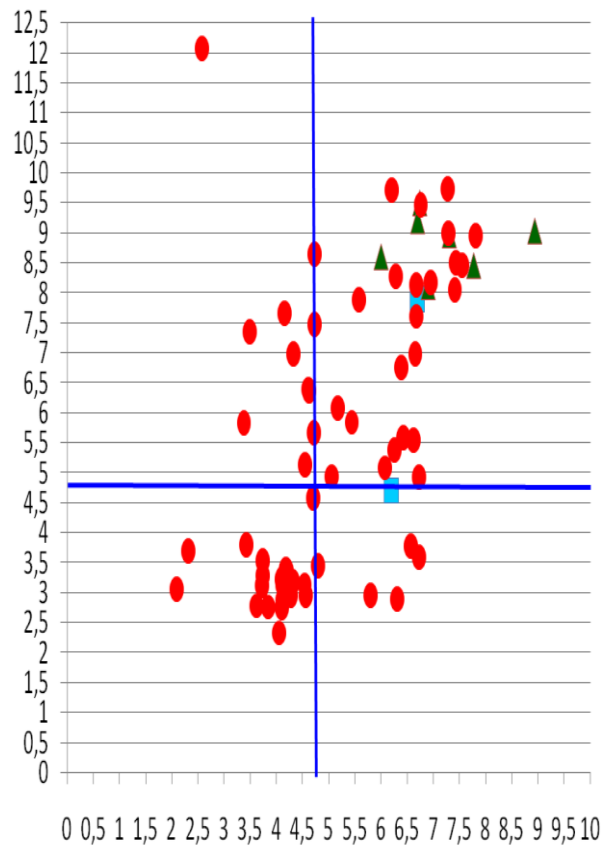
Legend

Y axis: Number of pregnant women. X axis: culture (■) and qPCR (■) results for V) vaginal only, R) rectal only, RV) both vaginal and rectal and T) overall (total) presence determinations.

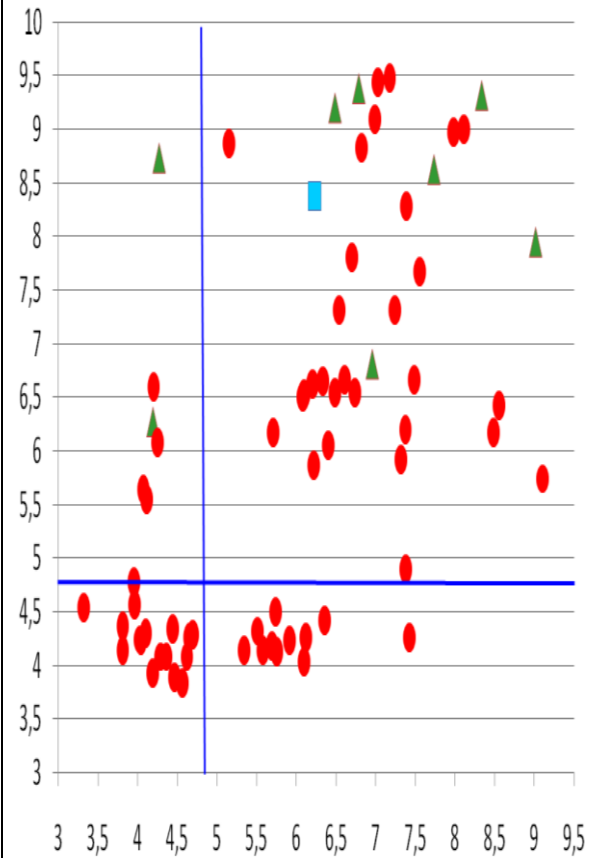
Figure 2. qPCR results, expressed as log10 concentration of *Lactobacillus* species (A-D) or *Gardnerella vaginalis* (E) or *Atopobium vaginae* (F), in relation to culture results



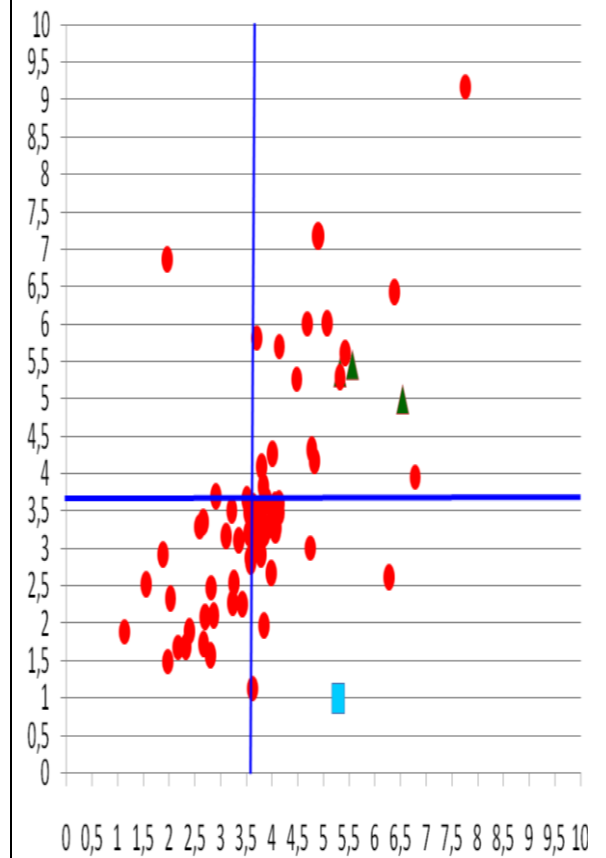
D. *Lactobacillus iners*



E. *Gardnerella vaginalis*



F. *Atopobium vaginae*



Legend:

X axis: Log 10 concentration of the bacteria in rectal samples as estimated by qPCR

Y axis: Log 10 concentration of the bacteria in vaginal samples as estimated by qPCR

Blue horizontal line: The lower limit of qPCR based detection of the bacteria in rectal samples

Blue vertical line: The lower limit of qPCR based detection of the bacteria in vaginal samples

◆ R+V+ ▲ R-V+ ■ R+V- ● R-V-

R+ V+: Both rectally and vaginally culture positive;

R- V+: Rectally culture negative and vaginally culture positive;

R+ V-: Rectally culture positive and vaginally culture negative;

R- V-: Both vaginally and rectally culture negative.

Table 1. Vaginal and rectal occurrence of six bacterial species, as estimated by culture and qPCR, expressed as percentage positive of 71 pregnant women

Species	Vagina all ^a		Vagina only		Vagina & Rectum		Rectum only		Rectum all ^a		Overall	
	Culture	qPCR	Culture	qPCR	Culture	qPCR	Culture	qPCR	Culture	qPCR	Culture	qPCR
<i>L. crispatus</i>	30.9 ^b	64.7	21.1	4.2	9.8	60	1.4	26.7	11.2	87.3	32.3	91.5
<i>L. jensenii</i>	30.9	57.7	21.1	7	9.8	50.7	1.4	19.7	11.2	70.4	32.3	77.4
<i>L. iners</i>	9.8	57.7	9.8	15.4	0	42.2	2.8	9.8	2.8	52.0	12.6	68.5
<i>L. gasseri</i>	28.1	87.3	19.7	36.6	8.4	50.7	0	4.2	8.4	54.9	28.1	91.5
<i>G. vaginalis</i>	11.2	59.0	11.2	9.8	0	49.2	1.4	15.4	1.4	64.7	12.6	74.6
<i>A. vaginae</i>	4.2	35.1	4.2	4.2	0	30.9	1.4	33.8	1.4	64.7	5.6	69.0

a: 'Vagina all' presents the sum of both the subjects with the species in the vagina only and the subjects with the species in both vagina and rectum. The column entitled 'Rectum all' presents the sum of both the subjects with the species in the rectum only and the subjects with the species in both vagina and rectum.

b: Although the numbers of women that are culture positive for *L. crispatus* are identical to those positive for *L. jensenii*, for all sites, this is coincidental, because different women are culture positive for both species.

II.3. Publication 3

El Aila NA, Tency I, Claeys G, Saerens B, De Backer E, Temmerman M, Verhelst R, Vaneechoutte M.

Genotyping of *Streptococcus agalactiae* (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy.

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Research article

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Genotyping of *Streptococcus agalactiae* (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy

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Abstract

Background: Group B streptococci (GBS), or *Streptococcus agalactiae*, are the leading bacterial cause of meningitis and bacterial sepsis in newborns. Here we compared different culture media for GBS detection and we compared the occurrence of different genotypes and serotypes of GBS isolates from the vagina and rectum.

Methods: *Streptococcus agalactiae* was cultured separately from both rectum and vagina, for a total of 150 pregnant women, i) directly onto Columbia CNA agar, or indirectly onto ii) Granada agar resp. iii) Columbia CNA agar, after overnight incubation in Lim broth.

Results: Thirty six women (24%) were colonized by GBS. Of these, 19 harbored GBS in both rectum and vagina, 9 only in the vagina and 8 exclusively in the rectum. The combination of Lim broth and subculture on Granada agar was the only culture method that detected all GBS positive women. Using RAPD-analysis, a total of 66 genotypes could be established among the 118 isolates from 32 women for which fingerprinting was carried out. Up to 4 different genotypes in total (rectal + vaginal) were found for 4 women, one woman carried 3 different genotypes vaginally and 14 women carried two 2 different genotypes vaginally. Only two subjects were found to carry strains with the same genotype, although the serotype of both of these strains was different.

Eighteen of the 19 subjects with GBS at both sites had at least one vaginal and one rectal isolate with the same genotype.

We report the presence of two to four different genotypes in 22 (61%) of the 36 GBS positive women and the presence of identical genotypes in both sites for all women but one.

Conclusion: The combination of Lim broth and subculture on Granada medium provide high sensitivity for GBS detection from vaginal and rectal swabs from pregnant women. We established a higher genotypic diversity per individual than other studies, with up to four different genotypes among a maximum of 6 isolates per individual picked. Still, 18 of the 19 women with GBS from both rectum and vagina had at least one isolate from each sampling site with the same genotype.

Background

Streptococcus agalactiae, group B *Streptococcus* (GBS), is a leading cause of neonatal morbidity and mortality in the US, Western Europe and Australia. Maternal carriage has been recognized as the most important risk factor for GBS neonatal infection [1,2] and indeed vertical transmission before or during delivery has been shown [3,4]. Mother-to-child transmission may lead to neonatal infection in 1 to 2 infants per 1,000 live births [5] with mortality rates ranging from 10 to 20% [6]. Among pregnant women, the prevalence of colonization with GBS ranges from 3.2 to 36% [7-9]. Screening consists of obtaining vaginal and rectal specimens for culture at 35 to 37 weeks of gestation. Recently, several molecular techniques have been applied to study the genetic diversity of GBS, such as restriction fragment length polymorphism analysis (RFLP) [10], ribotyping [10,11], pulsed-field gel electrophoresis (PFGE) [3,12-17] multilocus enzyme electrophoresis (MLEE) [18] randomly amplified of polymorphic DNA-analysis (RAPD) [9,19,20], amplified *cps* restriction polymorphism analysis [21] and multilocus sequence typing (MLST) [13,22-25].

To our knowledge, only one study [12], addressed the genotypic and serological diversity of GBS within individual women. Therefore, the aim of this study was to compare the genotypes of the GBS isolates from separate vaginal and rectal swabs using a simple genotypic approach (RAPD analysis with primer OPM1, followed by capillary electrophoresis) and also to study the correlation between serotype and genotype of the GBS isolates.

Methods

Study design

The study was approved by the research ethics committee (IRB protocol nr 2007/096) of Ghent University Hospital, Flanders, North region of Belgium, and all the women gave written informed consent. Between April and December 2007, 150 paired vaginal and rectal swabs were collected from pregnant women at 35 - 37 weeks of gestation.

Collection and culture of specimens

All specimens were collected using nylon flocked swabs that were submerged into 1 ml of liquid Amies transport medium (eSwab, Copan Diagnostics, Brescia, It.). For rectal specimens, a swab was carefully inserted approximately 1.5 - 2 cm beyond the anal sphincter and then gently rotated to touch anal crypts.

Vaginal samples were collected by inserting a swab into the vagina. The swab was rolled round through 360 degrees against the vaginal wall at the midportion of the vault. At Ghent University Hospital, the routine GBS-screening of pregnant women is always performed during

the prenatal consultation at 35-37 weeks' gestation. All study samples were collected by midwives and transported to the Laboratory of Bacteriology Research within 4 hours.

A total of 70 μ l from the Amies liquid transport medium of each of the vaginal and rectal swabs was seeded on Columbia CNA agar with 5% sheep blood (Columbia CNA agar, Becton Dickinson, Erembodegem, Belgium) and 200 μ l was inoculated into 5 ml of Lim Broth (Todd-Hewitt broth, 1% yeast extract, 15 μ g nalidixic acid/ml and 10 μ g colistin/ml) (Lim Broth, Becton Dickinson) [26]. Both media are selective for Gram positive bacteria.

The Columbia CNA agar plates were incubated at 37°C in 5% CO₂ for 24-48 h.

The Lim Broth was incubated aerobically at 37°C for 18-24 hours and then subcultured onto Granada agar (Becton Dickinson) [27] and onto Columbia CNA agar. Granada agar was incubated at 37°C in an anaerobic chamber (BugBox, LedTechno, Heusden-Zolder, B.) for 24-48 h and Columbia CNA agar was incubated at 37°C in 5% CO₂ for 24-48 h.

Granada agar was examined for yellow-orange pigment colonies that confirm the presence of GBS, whereas β -haemolytic and non-haemolytic colonies were picked from Columbia CNA agar for further identification as *S. agalactiae* by the CAMP test.

Identification of the isolates as *Streptococcus agalactiae*

The isolates were identified as *S. agalactiae* by the following criteria: growth and orange pigment formation on Granada agar, positive for the CAMP test on blood agar and molecular identification by tDNA-PCR [28].

Antibiotic susceptibility testing

Forty isolates of group B streptococci of 8 pregnant women were tested by disk diffusion for susceptibility to clindamycin and erythromycin. Colonies taken from Trypticase Soy Agar (TSA) + 5% sheep blood (Becton Dickinson) were suspended in 5 ml of saline and the inoculum was adjusted to the turbidity of a 0.5 McFarland standard. This suspension was streaked onto TSA + 5% sheep blood to obtain confluent growth, disks were added and the plates were incubated overnight at 37°C with 5% CO₂. Strains were considered resistant to clindamycin and erythromycin when the inhibition zones were less than 15 mm.

DNA-extraction from isolates

DNA was extracted from cultured isolates by alkaline lysis as follows: One bacterial colony was suspended in 20 μ l of lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N

NaOH) and heated at 95°C for 15 min. The cell lysate was diluted by adding 180 µl of distilled water. The cell debris was spun down by centrifugation at 16,000 g for 5 min. Supernatants were used for PCR or frozen at -20°C until further use.

Genotyping of isolates

The cultured vaginal and rectal GBS isolates were genotyped using RAPD-analysis with the RAPD Ready-to-Go beads (GE Healthcare, Buckinghamshire, UK) as described previously [29] with primer OPM1 (5' GTT GGT GGCT) at a final concentration of 2 µM, including 0.2 µM of fluorescent TET-labeled OPM1 primer. After 5 min at 94°C, 5 min at 35°C and 5 min at 72°C, reaction mixtures were cycled 30 times in a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, Ca.), with the following conditions: 30 s at 94°C, 1 min at 35°C, and 1 min at 72°C, with a final extension period of 5 min at 72°C. Reaction vials were then cooled to 10°C and kept on ice until electrophoresis.

Capillary electrophoresis

A volume of 11.9 µl of deionized formamide (ACE formamide, Lucron, De Pinte) was mixed with 0.6 µl of an internal size standard mixture containing 0.3 µl of the ROX-400 high-density size standard (Applied Biosystems, Foster City, Ca.) and 0.3 µl of Map marker 1000 size standard (BioVentures, Murfreesboro, Tn.). One microliter of OPM1-PCR product was added. The mixtures were denatured by heating at 95°C for 3 min and placed directly on ice for at least 10 min. Capillary electrophoresis was carried out using an ABI-Prism 310 genetic analyzer (Applied Biosystems) at 60°C, at a constant voltage of 1.5 kV, and at a more or less constant current of approximately 10 mA. Capillaries with a length of 47 cm and diameter of 50 µm were filled with performance-optimized polymer 4. Electropherograms were normalized using Genescan Analysis software, version 2.1 (Applied Biosystems).

Data analysis

OPM1-PCR fingerprints were obtained as table files from the Gene Scan Analysis software and used in a software program developed at our laboratory [30]. Using these sample files composed of numbers, representing the length of the amplification fragments in base pairs, a distance matrix was calculated with the in-house software

using the differential basepairs (dbp) and the Dice algorithm [30]. Clustering analysis was done with the Phylip software <http://evolution.genetics.washington.edu/phylip.html>, using the Neighbor Joining algorithm.

Serotyping

A total of 122 GBS isolates from 36 pregnant women were serotyped, using the latex co-agglutination kit of Essum AB (Umea, Sweden), according to the manufacturer's instructions. This kit enables to differentiate between serotypes Ia, Ib, II, III, IV and V.

Statistical methods

The McNemar test for correlated percentages was used to compare the sensitivity of the culture media.

Results

For a total of 150 women, culture was carried out separately for both rectal and vaginal sites, using three culture methods, i.e. directly onto Columbia CNA agar, or indirectly, by subculturing onto Columbia CNA agar resp. Granada agar, following overnight incubation in LIM broth.

Comparison of culture techniques

A total of 36 out of 150 pregnant women studied (24%) were colonized by GBS. Of 55 samples from which GBS was isolated, 22 were positive by direct inoculation on Columbia CNA agar, 55 by Lim broth enrichment with subculture on Granada agar and 45 by Lim broth with subculture on Columbia CNA agar, resulting in sensitivities of 40, 100 and 81% respectively (Table 1).

Culture of vaginal specimens by direct plating on Columbia CNA agar was significantly less sensitive than culture in Lim broth with subculture on Granada agar or subculture on Columbia CNA agar (McNemar test, $p < 0.0001$). In addition, the culture of rectal specimens, direct plating onto Columbia CNA agar was significantly less sensitive than culture in Lim broth with subculture on Granada agar ($p < 0.0001$), which was more sensitive than culture on Lim broth with subculture on Columbia CNA agar ($p = 0.0313$).

Table 1: Number of GBS-positive cultures detected by different culture media in separate vaginal and rectal specimens obtained from 150 pregnant women

Specimen	No. of positive GBS cultures detected by			Total no. of women colonized
	Columbia CNA agar	Lim broth + Granada agar	Lim broth + Columbia CNA agar	
Vaginal	11	28	24	28
Rectal	11	27	11	27
Total	22	55	45	36

Carriage of GBS and genotyping

For the 36 women that were found positive, 9 (25%) carried GBS only in the vagina, 8 (22%) only in the rectum and 19 (53%) in both sampling sites (Table 1). Using three culture methods for two sampling sites, a maximum of 6 colonies per woman was picked, one from each of the positive culture plates. A total of 122 isolates were obtained (Table 2) of which 118 were genotyped using RAPD with primer OPM1 and analysis by capillary electrophoresis. The single isolates from four women were not fingerprinted, because there were no other isolates from the same subject to compare with. A tree was constructed after distance matrix calculation, and this revealed the presence of a total of 66 genotypes among the 118 isolates

from 32 women for which fingerprinting was carried out. Only two subjects (RVS033 and RVS062) were found to carry strains of which the RAPD genotype was indistinguishable (Figure 1).

For 5 out of the 19 women from which both vaginal and rectal isolates were obtained, all the rectal and vaginal isolates were identical. For another 13 of these 19, at least one of the vaginal isolates had a similar genotype as one of the rectal isolates. Four women had two genotypes, seven had three genotypes and three had four genotypes. One of the five women with only rectal isolates had one genotype, whereas the other four had two genotypes. For the eight women with only vaginal isolates, four of them

Table 2: Overview of genotyping and serotyping results for 36 women positive for GBS.

Number	Number	V DC	V LG	V LC	R DC	R LG	R LC
1	RVS143	-	+ ^a /V ^b	-	-	-	-
2	RVS038	-	A/III	A/V	-	-	-
3	RVS071	-	A/IV	A/IV	-	-	-
4	RVS073	-	A/III	A/V	-	-	-
5	RVS109	-	A/V	A/V	-	-	-
6	RVS034	A/III	B/Ib	-	-	-	-
7	RVS035	B/III	A/II	A/II	-	-	-
8	RVS076	A/Ib	B/Ib	B/Ib	-	-	-
9	RVS148	-	A/III	B/III	-	-	-
10	RVS004	-	-	-	-	+/Ia	-
11	RVS051	-	-	-	-	+/V	-
12	RVS080	-	-	-	-	+/IV	-
13	RVS084	-	-	-	-	A/NT	A/NT
14	RVS021	-	-	-	B/Ia	A/Ib	A/Ib
15	RVS061	-	-	-	B/V	A/NT	A/NT
16	RVS120	-	-	-	-	A/Ia	B/Ia
17	RVS145	-	-	-	-	A/Ia	B/V
18	RVS017	-	A/IV	A/IV	-	A/IV	-
19	RVS047	-	A/IV	A/IV	A/IV	A/IV	A/IV
20	RVS106	A/II	A/II	-	A/II	A/II	-
21	RVS127	A/IV	A/IV	A/IV	-	A/IV	A/IV
22	RVS069	-	A/NT	-	-	A/NT	-
23	RVS039	-	A/Ia	A/Ia	-	B/Ia	A/Ia
24	RVS083	-	A/III	B/Ib	A/III	A/III	A/III
25	RVS114	-	B/V	A/V	-	A/V	A/V
26	RVS123	-	A/NT	A/NT	-	A/NT	B/NT
27	RVS027	A/V	A/V	C/V	A/V	A/V	B/V
28	RVS033	A/III	C/III	A/III	A/III	B/III	A/III
29	RVS064	A/III	B/III	A/III	A/III	C/III	A/III
30	RVS075	-	B/II	A/II	-	C/II	A/II
31	RVS110	A/V	A/V	A/V	C/V	B/V	A/V
32	RVS126	A/NT	C/IV	A/NT	-	B/IV	A/NT
33	RVS136	-	A/NT	B/III	-	A/NT	C/NT
34	RVS031	-	D/III	A/III	B/NT	A/III	C/II
35	RVS062	-	D/II	A/II	-	B/II	C/II
36	RVS074	B/Ib	A/Ib	D/Ib	B/Ib	A/Ib	C/Ib

Column headings: V DC: Vaginal sample, cultured directly on Columbia Agar, V LG: after Lim broth enrichment culture, plated onto Granada agar, V LC: after Lim broth enrichment culture, plated onto Columbia agar; R DC: Rectal sample, idem as for vaginal samples.

-: No GBS isolated.

^a: Genotype: +: strain present, but not genotyped, A, B, C, D: genotypes numbered per individual.

^b: Serotype: serotype number as determined by the Essum AB kit (Umea, Sweden). The following serotypes can be detected by this kit: Ia, Ib, II, III, IV and V. NT: nontypeable.

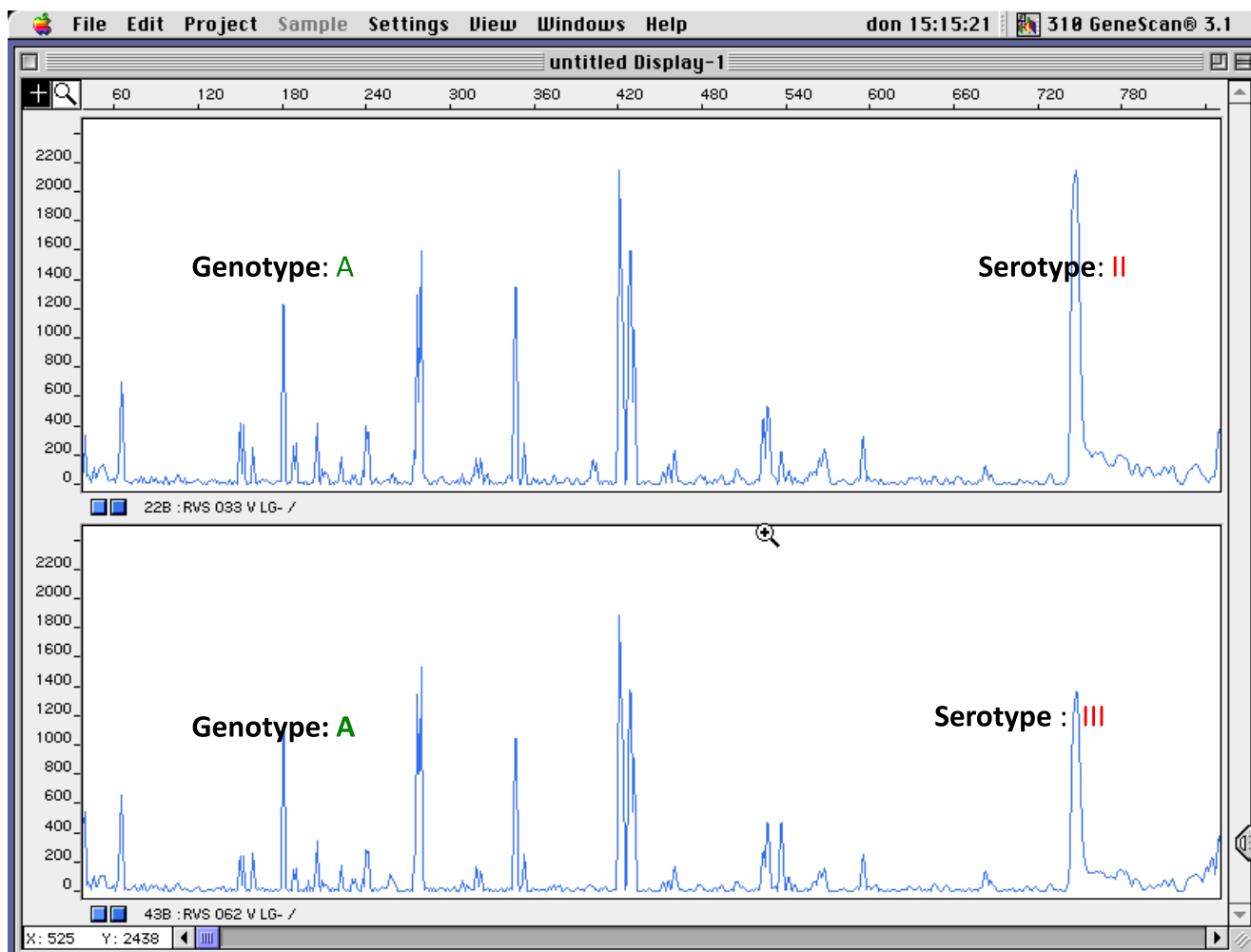


Figure 1
Fingerprints of two isolates from two different subjects (RVS033 and RVS062) showing the same genotype and different serotypes. x-axis: length of amplified DNA fragments expressed in bps. y-axis: peak height (intensity of DNA-fragment).

had only one genotype and the other four had two genotypes.

Serotyping

In addition, all isolates were serotyped (Table 2). Seventeen isolates, from 7 subjects, were nontypeable. Furthermore, a rather equal distribution of the serotypes was found among the strains (Table 2). Eight women were found to carry isolates of the same genotype and serotype. An example is shown in Figure 2. In 11 subjects, only one serotype was present, although 2 to 4 genotypes could be found per subject. Nine subjects were found to carry isolates with different genotype and different serotype (Figure 3). The two isolates with the same genotype from the two different subjects belonged to different serotypes.

The frequency of different serotypes was as follows Ia: 10.4%, Ib: 10.4%, II: 10.4%, III: 20.8%, IV: 12.5, V: 20.8 and nontypeable: 14.5%. Overall serotype distribution among vaginal vs rectal isolates was comparable, with a rectal predominance of Ia, i.e. 1 vaginal isolate vs 5 rectal isolates and a vaginal predominance of III, i.e. 10 vaginal isolates vs 4 rectal isolates. The presence of *L. crispatus*, generally accepted to confer vaginal colonisation resistance to pathogenic organisms [31,32], did not seem to protect against vaginal *S. agalactiae* colonization, since a comparable number of women colonized only vaginally by *S. agalactiae* resp. only rectal by *S. agalactiae* were colonized vaginally by *L. crispatus*, i.e. 3/9 resp. 2/8. Six of the 19 women colonized by *S. agalactiae* both rectally and vaginally carried *L. crispatus* vaginally.

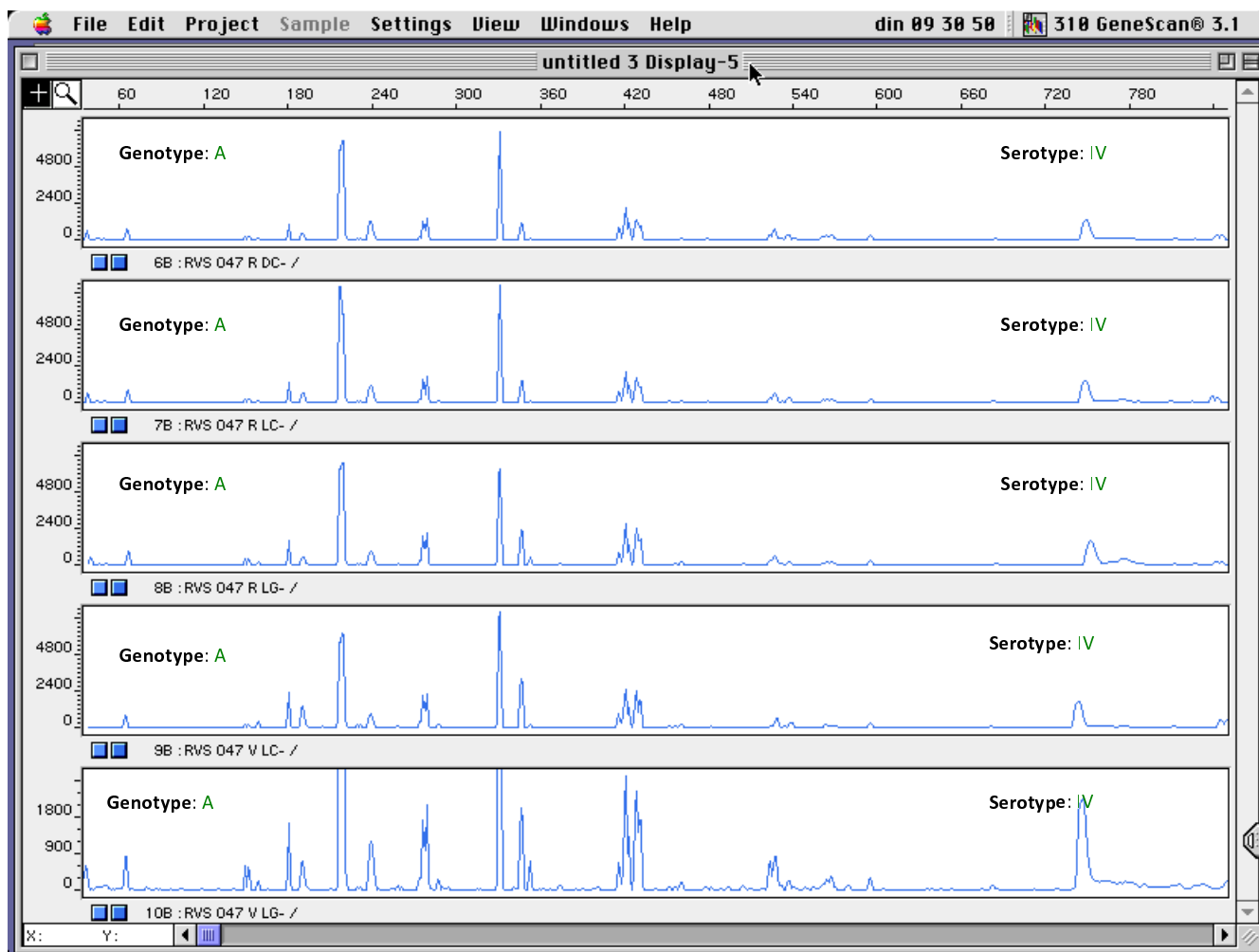


Figure 2
Fingerprints of different isolates of subject RVS047 with the same genotype and the same serotype. x-axis: length of amplified DNA fragments expressed in bps. y-axis: peak height (intensity of DNA-fragment).

Antibiotic susceptibility testing

GBS is considered homogenously susceptible to penicilline and amoxicillin. In case of allergy, second choice antibiotics are clindamycin or erythromycin. According to CLSI erythromycine can be tested with a simple disk test, although this is not done in our routine laboratory. Here we checked 40 isolates from 8 patients and found 26 isolates to be susceptible to both clindamycine and erythromycine, 3 to be clindamycine resistant and 11 to be resistant to both antibiotics. The susceptibility pattern of all strains was homogeneous for six subjects, despite genotypic differences among the isolates, whereas two subjects carried clindamycin resistant strains besides isolates susceptible to both antibiotics.

Discussion

Sensitivity and specificity of different culture techniques for the detection of GBS

In our study, only the combination of Lim broth and subculture on Granada agar enabled detection of all carriers, whereas direct inoculation onto Columbia CNA agar achieved a sensitivity of 40% and subculture of the Lim broth onto Columbia CNA agar detected 81% of the carriers.

Gil *et al.* [33] showed that in different studies the sensitivity of Granada agar ranged from 88.5 to 91.1%, that of Columbia CNA agar from 83.9 to 94.3% and that of Lim broth (to which 5% horse serum was added) was 63.5% when subcultured on Granada agar and 75% when subcultured on Columbia CNA agar. Bosch-Mestres *et al.* [34] showed that the use of direct inoculation onto Granada

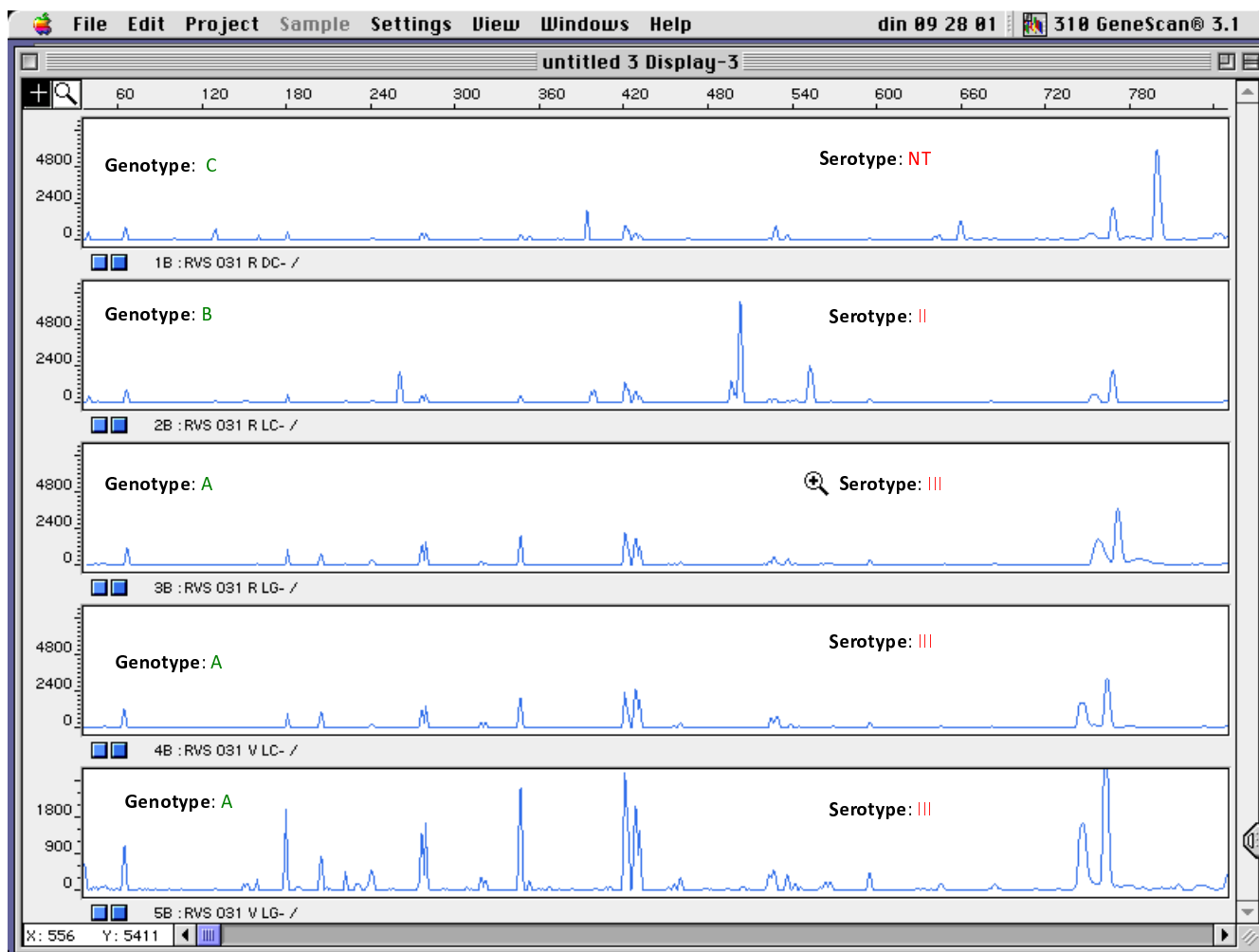


Figure 3
Fingerprints of different isolates of subject RVS031 with different genotypes and different serotypes. x-axis: length of amplified DNA fragments expressed in bps. y-axis: peak height (intensity of DNA-fragment).

agar allowed fast detection for about 87% of carriers, whereas the combination of Todd-Hewitt broth and subculture on Granada agar or Columbia CNA agar allowed detection of more than 99% of GBS carriers. Elsayed *et al.* [35] reported 79% sensitivity for direct inoculation onto Columbia CNA agar and incubation during 48 hours compared to 100% sensitivity for Lim broth with subculture on blood agar. It can be concluded from this study and others that the combination of broth enrichment (Todd-Hewitt or LIM broth) with subculture on solid medium, yields higher sensitivity than direct inoculation onto solid media.

Although no definite conclusions can be drawn with regard to the sensitivity of the different solid media, Granada agar has the advantage that it makes possible to provide overnight results by visual inspection of the presence

of red orange colonies, that are produced exclusively by GBS. The red orange colonies are easily observed, even when few colonies are present or when GBS is mixed with other microorganisms, which are mainly other streptococci [33]. The colonies are so characteristic and unique that identification by antigen detection or the CAMP test is unnecessary. In this study, all red orange colonies were CAMP positive (data not shown). Claeys *et al.* [36] missed only two CAMP positive isolates on a total of 310 tested, which were not red orange pigmented on Granada. Blanckaert *et al.* [37] used a combination of Granada and Columbia blood agar for GBS screening and demonstrated that 6% of the samples positive for GBS on Columbia blood agar lacked red orange colonies on Granada agar. Notably, Granada agar does not detect non-pigmented isolates, and on blood-agar these non-hemolytic isolates are difficult to detect as well. As a result of that,

non hemolytic, nonpigmented strains may have been missed. In conclusion, in our hands, the use of combination of Lim broth and subculture on Granada agar provided high sensitivity and specificity for detecting GBS in vaginal and rectal swabs from pregnant women.

Epidemiology

The high prevalence of *S. agalactiae* colonization, i.e. 24%, as established in this study, is in accordance with results from other European studies that report colonization rates between 10% and 36% [38-43]. Thinkhamrop *et al.* [44] reported a prevalence of 7.1% (Myanmar) to 19.1% (Philadelphia), using Lim broth culture. Toresani *et al.* [9] found a prevalence of only 3.2% among 531 Argentinian pregnant women. Brimil *et al.* [38] showed equal carrier rates of 16% for GBS among pregnant and nonpregnant women and, based on the prevalence of GBS carriage, these authors concluded that strict adherence to the guidelines for prevention of GBS neonatal infection results in periparturient antibiotic prophylaxis in up to 20% of all deliveries.

Our data, are also in correspondence with other results on GBS prevalence in our country. Rectovaginal colonization with group B streptococci in Belgium is 13-25%. These data are based on different studies carried out by the Belgian reference laboratory for GBS in collaboration with the section of epidemiology of the Scientific Institute for Public Health (ISP-WIV, Brussels) [45]. For example, Blanckaert *et al.* [37] compared the results of GBS screening on Granada agar with those obtained using standard Columbia blood agar at two participating centers in Belgium. They reported GBS-positive culture results of 10-30% of pregnant women. The Flemish Study Centre for Perinatal Epidemiology evaluated GBS prevalence in Flanders and found an average colonization rate of 16% among Flemish pregnant women [46].

Manning *et al.* [41] found that the prevalence of GBS colonization was equally high among 241 women (34%) and 211 men (20%) living in a college dormitory. Sexually experienced subjects had twice the colonization rates of sexually inexperienced participants. Van der Mee-Marquet *et al.* [47] reported that the prevalence of carriage was 27% in women and 32% in men. The major positive body site was the genital tract (23% in women and 21% in men) and skin, throats, and anal margins were also positive in 2%, 4%, and 14%, respectively.

Comparison between vaginal and rectal carriage

Brimil *et al.* [38] reported that for a total of 34 GBS positive pregnant women, 32% carried GBS only vaginal, 24% only rectal and 44% both rectal and vaginal, which compares well with our results, i.e. resp. 24%, 22% and 53%.

Serotypic and genotypic diversity among GBS isolates

We studied the rectal and vaginal colonization with GBS of pregnant women attending the Ghent University hospital (Belgium) and the serotypic and genotypic diversity among the GBS isolates. Strong genotypic and serotypic heterogeneity was observed between women and for individual women, e.g., we found a total of 66 genotypes among 118 isolates (from 32 women), of which 4 women had up to 4 different genotypes in total (rectal + vaginal) and 14 women had up to 2 different genotypes vaginally.

Manning *et al.* [48] genotyped GBS isolates from vaginal-rectal swabs of women at two visits and documented a turnover in 8.3% of 126 women colonized both at 35-37 weeks of gestation and 6 weeks after delivery. Unfortunately, only one isolate was genotyped per visit. Taking into consideration the genotypic diversity per subject, as observed in our study, a turnover of 8.3% might be an overestimation. When the same subject, carrying e.g. genotypes a, b and c, is sampled at two different moments, and whereby on each occasion only one colony is picked, the detection of a different genotype may be interpreted as turn over, but it may be that a genotype a strain has picked at the first visit but a genotype b or c strain at the second visit.

Moreover, their conclusion that some clones are more likely to be lost should be interpreted with care, since their apparent disappearance might be explained by the presence of these clones in relatively lower numbers (compared to other clones), which reduces their chance of being picked at two separate occasions. To our knowledge, only one group studied genotypic diversity within individual women, by genotyping 15 randomly picked isolates for each of 30 women, albeit from mixed vaginal-rectal specimens [12]. In opposition to our results, these authors found a high degree of genotypic and serotypic homogeneity, i.e. for 29 of 30 women, all 15 isolates from each woman had the same serotype, and for 27 of 30 women, all 15 isolates had the same chromosomal *Sma*I-DNA restriction digest fingerprint. For the three women with different PFGE types, one had three different genotypes and two different serotypes. Another PFGE based study reported high stability of the GBS type for each woman, followed up to two years [40]. In accordance with our results, these authors found 30 different GBS isolates among 32 women, with only two women carrying isolates with the same genotype.

The high level of heterogeneity established in our study, compared to other studies, may be due to the different culture media that were used and to the use of separate swabs for sampling vagina and rectum for each individual woman. We found that strains of the same serotype recovered from different women were heterogeneous in DNA

profiles. In accordance, *SmaI*-restriction digestion of chromosomal DNA and PFGE revealed high genotypic heterogeneity among both Zimbabwean serotype III and serotype V isolates [16], another study also reported different *SmaI* restriction types within serotypes II and V [12], and Fasola *et al.* [14] and Savoia *et al.* [49] showed that several genotypic lineages are present within the different serotypes.

Serotype switching is believed to occur within genotypic lineages [7,24,25], presumably by horizontal transfer of genes of the *cps* locus, i.e. of genes encoding the GBS capsular polysaccharide structure [50], and may be an explanation for our observation of the presence of two isolates in two different women with the same genotype, but with a different serotype.

Although MLST has become the standard method to study the population structure of GBS [22,24], more rapid and less expensive and laborious methods remain useful for carrying out single centre studies. We used RAPD-analysis in combination with high resolution capillary electrophoresis, which also makes possible immediate digitization of the fingerprints. Chatellier *et al.* [51] found that the simplest typing scheme of *S. agalactiae* was obtained by the combination of RAPD typing and serotyping (discriminatory index 0.97). Zhang *et al.* [20] found congruence between RAPD analysis and serotyping, on a limited number of strains. The findings of Toresani *et al.* [9], who found a total 16 RAPD profiles among 21 GBS isolates, from 17 women, and of Chatellier *et al.* [51], who identified 71 RAPD types among 54 unrelated *S. agalactiae* strains isolated from cerebrospinal fluid samples from neonates, point to the same genotypic diversity as observed in our study.

General serotype distribution

The capsule of *S. agalactiae* has long been recognized as one of the most important virulence factors. Variations of the capsular polysaccharide structure allow the antigenic distinction of 13 different *S. agalactiae* serotypes, of which 9 are of clinical importance (Ia, Ib, II-VIII). Studies from the US and Europe show that the serotypes Ia, II, III, and V are found in 80-90% of all clinical isolates [4,48].

Serotype distribution among GBS isolates from pregnant women in our study was compared with that reported by others. Several studies indicate that serotype III is globally the most prevalent serotype, e.g. 29.2% of Israeli isolates [7], 28% of German isolates [38], 24.3% of Swedish isolates [39] and 33.2% of Czech isolates [42]. In accordance, we observed a frequency of 20.8% for serotype III isolates in our study, as the most frequent serotype.

Antibiotic susceptibility

The finding that two out of 8 women carried isolates with different susceptibility to clindamycin indicates that when testing susceptibility for clindamycin several colonies should be tested, since colonies with different susceptibility may be simultaneously present.

Conclusion

In summary, our study, including 150 pregnant women, confirmed the European prevalence of around 20% of GBS among pregnant women and the predominance of serotypes III and V among these women, but we established a higher genotypic diversity per individual than other studies, with up to four different genotypes among a maximum of 6 isolates per individual picked. Still, 18 of the 19 women with GBS from both rectum and vagina had at least one isolate from each sampling site with the same genotype. In our hands, the combination of Lim broth and subculture on Granada medium provided higher sensitivity than direct culture on Columbia CNA or Lim broth and subculture on Columbia CNA for GBS detection from vaginal and rectal swabs from pregnant women.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NAE, RV, GC and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. IT and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. BS and EDB participated in the analysis of the study samples and interpretation of the data. All authors read and approved the final manuscript.

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II.4. Publication 4

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Comparison of different sampling techniques and of different culture methods for detection of Group B streptococci carriage in pregnant women.

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RESEARCH ARTICLE

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Comparison of different sampling techniques and of different culture methods for detection of group B streptococcus carriage in pregnant women

Nabil A El Aila¹, Inge Tency^{2†}, Geert Claeys³, Bart Saerens¹, Piet Cools¹, Hans Verstraelen², Marleen Temmerman², Rita Verhelst¹, Mario Vaneechoutte^{1*}

Abstract

Background: *Streptococcus agalactiae* (group B streptococcus; GBS) is a significant cause of perinatal and neonatal infections worldwide. To detect GBS colonization in pregnant women, the CDC recommends isolation of the bacterium from vaginal and anorectal swab samples by growth in a selective enrichment medium, such as Lim broth (Todd-Hewitt broth supplemented with selective antibiotics), followed by subculture on sheep blood agar. However, this procedure may require 48 h to complete. We compared different sampling and culture techniques for the detection of GBS.

Methods: A total of 300 swabs was taken from 100 pregnant women at 35-37 weeks of gestation. For each subject, one rectovaginal, one vaginal and one rectal ESwab were collected. Plating onto Columbia CNA agar (CNA), group B streptococcus differential agar (GBSDA) (Granada Medium) and chromID Strepto B agar (CA), with and without Lim broth enrichment, were compared. The isolates were confirmed as *S. agalactiae* using the CAMP test on blood agar and by molecular identification with tDNA-PCR or by 16S rRNA gene sequence determination.

Results: The overall GBS colonization rate was 22%. GBS positivity for rectovaginal sampling (100%) was significantly higher than detection on the basis of vaginal sampling (50%), but not significantly higher than for rectal sampling (82%). Direct plating of the rectovaginal swab on CNA, GBSDA and CA resulted in detection of 59, 91 and 95% of the carriers, respectively, whereas subculturing of Lim broth yielded 77, 95 and 100% positivity, respectively. Lim broth enrichment enabled the detection of only one additional GBS positive subject. There was no significant difference between GBSDA and CA, whereas both were more sensitive than CNA. Direct culture onto GBSDA or CA (91 and 95%) detected more carriers than Lim broth enrichment and subculture onto CNA (77%). One false negative isolate was observed on GBSDA, and three false positives on CA.

Conclusions: In conclusion, rectovaginal sampling increased the number GBS positive women detected, compared to vaginal and/or rectal sampling. Direct plating on CA and/or GBSDA provided rapid detection of GBS that was at least as sensitive and specific as the CDC recommended method of Lim broth subcultured onto non chromogenic agar.

Background

Streptococcus agalactiae (group B streptococcus, GBS) is a significant cause of perinatal and neonatal infections worldwide. Rectovaginal colonization occurs in 10 to 30% of pregnant women [1-3] and is responsible for 1.8 neonatal infections per 1,000 live births per year [4]. In

Belgium, 13 to 25% of pregnant women are colonized with GBS. GBS is responsible for 38% of early neonatal infections [5].

GBS can be acquired during labor or in utero by transmission from maternal vaginal or anorectal-colonized mucosa. Prematurity is also a risk factor for GBS neonatal sepsis, and mortality due to GBS is higher in preterm than in term newborns [6]. Because results at 35-37 weeks correlate more closely with GBS colonization at term delivery, the Centers for Disease Control

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and Prevention (CDC) has recommended that all pregnant women be screened for carriage of GBS at between 35 and 37 weeks of gestation [7], so that GBS positive women can receive antibacterial treatment (chemoprophylaxis) prior to delivery, to reduce mother-to-child transmission.

To maximize GBS carriage detection rates, both the anatomic site of sampling and the culture methods used are important. Rectovaginal swabs have been reported to provide high bacterial yields, as the gastrointestinal tract is a natural reservoir for GBS and a potential source of vaginal colonization [7-11]. In the present study, we compared three sampling techniques, i.e. rectovaginal swabbing, vaginal swabbing only and rectal swabbing only, using the ESwab, a nylon flocked swab in liquid Amies transport medium (Copan, Brescia, Italy), which, according to the manufacturer, is a liquid-based multi-purpose collection and transport system that maintains viability of aerobic, anaerobic and fastidious bacteria for up to 48 hours at room and refrigerator temperature, and suitable for automation, gram stains, and traditional culture, and which compares well to other swabs with regard to species recovery [12,13].

With regard to bacterial culture, the CDC recommends isolation of the bacterium from vaginal and rectal swabs by growth in a selective enrichment medium, such as Lim broth, i.e. Todd-Hewitt broth with colistin and nalidixic acid, followed by subculture on sheep blood agar. In the same guidelines, the CDC identified various research priorities, including 'the development of media with a reliable color indicator to signal the presence of GBS to improve accuracy of prenatal culture results and facilitate prenatal culture processing at clinical laboratories with limited technical capacity' [14].

Therefore, in the present study, we also determined the sensitivity and specificity of two types of color indicator based media that are commercially available for detecting GBS carriage in pregnant women, with that of the CDC recommended method, i.e. Lim broth enrichment with subculture onto sheep blood agar.

The first type, designated Granada Medium (GM) [15], is an adaptation of Islam's medium [16], and exploits the ability of GBS to synthesize - under anaerobic conditions and on media containing starch and serum - an orange pigment, recently identified as grana-daene [17].

This method is very specific and simple, thereby allowing identification of GBS in a single step within 24 h. Later, a modification of GM was described as new GM [15]. In this study, we used group B streptococcus differential agar (GBSDA, Becton Dickinson), which is itself a modification of new GM and of which the manufacturer claims that it has improved selectivity and stability compared to new GM, without further specification.

The second type of medium used in this study, recently developed by bioMérieux as chromID™ Strepto B agar (CA), is a selective chromogenic medium, of which the constituents are not specified by the company, and which enables the recognition of *S. agalactiae* as pink to red, round and pearly colonies, without the need of anaerobic incubation. Most other bacterial species are either inhibited or the colonies produced have a different colour (e.g. violet, blue, colourless) [18-20].

In summary, in addition to comparing three sampling methods, we compared six different culture methods, i.e. direct culture onto Columbia Colistine Nalidixic Acid Agar (CNA), GBSDA and CA, and Lim broth enrichment with subculture on these three agars.

Methods

Study design

The study was approved by the research ethics committee (IRB protocol nr 2007/096) of the Ghent University Hospital, Flanders, Belgium, and all the women gave written informed consent. Between June 2009 and January 2010, 100 vaginal samples, 100 rectal and 100 rectovaginal ESwab samples were collected from 100 pregnant women at 35 - 37 weeks of gestation, i.e. three different samples per subject.

Collection and culture of specimens

Rectovaginal, vaginal and rectal samples were collected using nylon flocked swabs that were submerged into 1 ml of ESwab transport medium (ESwab, Copan Diagnostics, Brescia, Italy).

Rectovaginal sampling was carried out by rotating an ESwab against the vaginal wall at the midportion of the vault. Subsequently, the swab was carefully withdrawn to prevent contamination with microflora from the vulva and introitus and the swab was inserted 1.5 to 2 cm beyond the anal sphincter and gently rotated to touch the anal crypts. Next, vaginal sampling was carried out by inserting the ESwab following the same procedure described above for swabbing the vaginal wall. Finally, an ESwab was used for rectal sampling as described above for the anal procedure of the rectovaginal sampling.

All samples were collected by midwives and transported to the Laboratory of Bacteriology Research within 4 hours. Direct plating was carried out only for the rectovaginal ESwab, by inoculating 50 µl from the ESwab transport medium onto Columbia agar with 5% sheep blood and with 10 mg/ml colistin and 15 mg/ml nalidixic acid (CNA, Becton Dickinson, Erembodegem, Belgium), 50 µl onto group B streptococcus differential agar (GBSDA, Becton Dickinson) and 50 µl onto chromID™ Strepto B agar (CA, BioMérieux, Marcy l'Etoile, France). The CNA plates were incubated at 37°C in 5%

CO₂ for 24-48 h, the GBSDA plates were incubated at 37°C in an anaerobic chamber (BugBox, LedTechno, Heusden-Zolder, Belgium) for 24-48 h, and the CA plates were incubated at 37°C for 18-24 hours in aerobic conditions in the dark. Volumes of 200 µl from the ESwab transport medium of the rectovaginal, vaginal and rectal ESwabs were inoculated into separate tubes with 5 ml of Todd-Hewitt broth with 1% yeast extract, 15 µg/ml nalidixic acid and 10 µg colistin/ml (Lim broth, Becton Dickinson), which were incubated aerobically at 37°C and subcultured onto CNA, GBSDA and

CA after overnight incubation. GBSDA was examined for yellow-orange pigment colonies indicative of the presence of GBS, whereas CA was examined for pale pink to red, round and pearly colonies. β-haemolytic and non-haemolytic colonies were picked from CNA for further identification (Figure 1). The isolates were confirmed as *S. agalactiae* using the CAMP test on sheep blood agar. GBS colonies with discrepant results (either false positive on CA or false negative on GBSDA) were identified using tDNA-PCR, as described previously [21], and by 16S rRNA gene sequence determination.

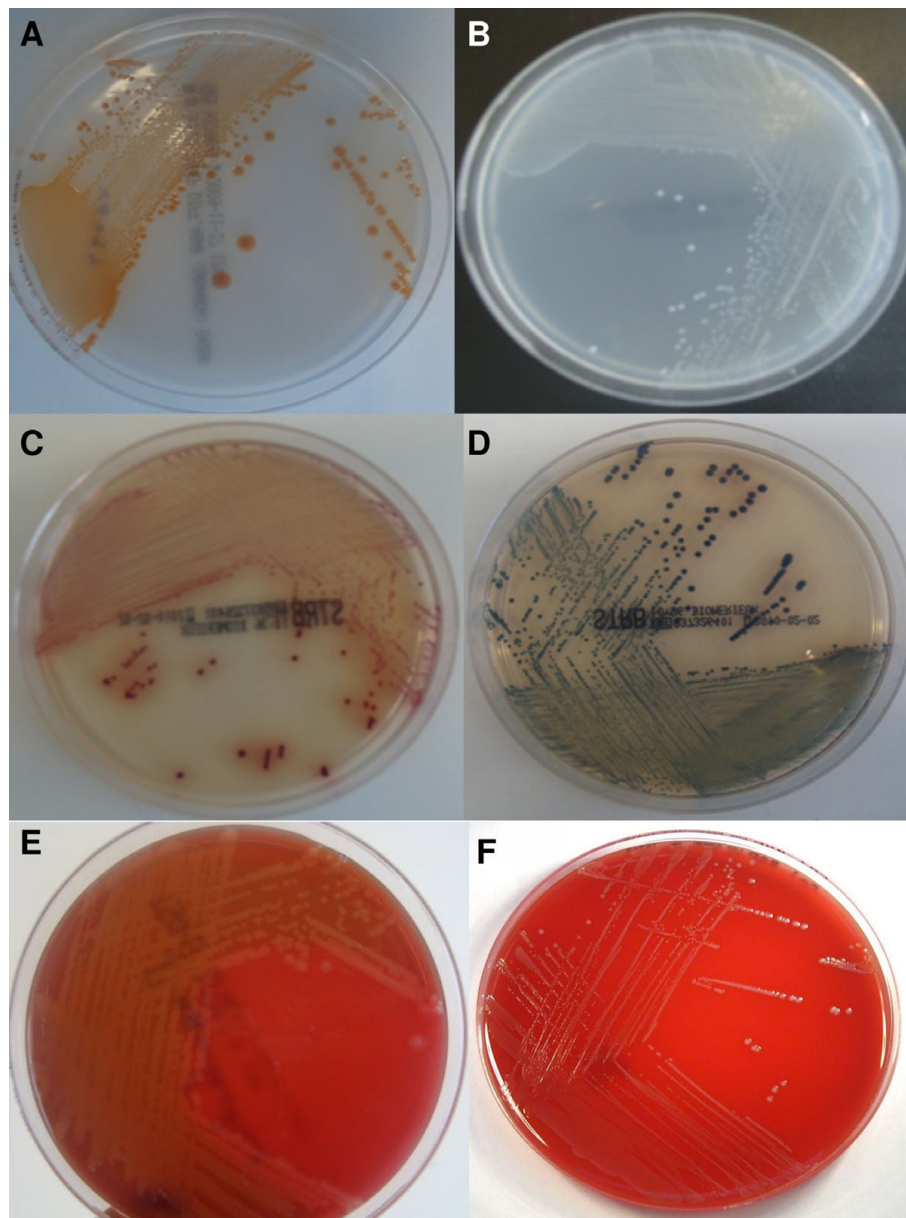


Figure 1 Appearance after 24 h incubation of (A): *Streptococcus agalactiae* (Group B *Streptococcus* (GBS) and (B) *Enterococcus faecalis* on GBSDA, (C) GBS and (D) *Enterococcus faecalis* on Strepto B ID® chromogenic agar and (E) GBS and (F) *Enterococcus faecalis* on CNA.

Statistical methods

The McNemar test for correlated percentages was used to compare the sensitivity of the culture methods. The total number of positive subjects (22) was taken as 100%, to calculate sensitivities, specificities, positive and negative predictive values of the different sampling and culture methods.

Results

A total of 300 swabs was taken from 100 pregnant women at 35-37 weeks of gestation, and 49 of these swabs were positive for a total of 22 subjects. Data for each subject, sampling site and method and culture method are listed in additional file 1, Table 1. GBS could be cultured from all 22 rectovaginal swabs, although one was only positive after Lim broth enrichment. Of the 22 GBS positive subjects, GBS could be cultured for nine from both the rectal and vaginal swabs, for two only from the vaginal swab, and for nine exclusively from the rectal swabs. For another two women, only the rectovaginal swab was positive.

The GBS detection rate on the basis of rectovaginal samples (22 GBS positive women) were significantly higher than the detection rate on the basis of vaginal samples (11 positive) ($P = 0.01$), but not significantly higher than that on the basis of rectal samples (18 positive) ($P = 0.12$).

Direct plating of the rectovaginal swabs on CNA, GBSDA and CA resulted in detection of 59, 91 and 95% respectively of the total number of carriers detected on all samples and media, whereas Lim broth with subculture onto on CNA, GBSDA and CA resulted in positivities of 77, 95 and 100% respectively (Table 1). For all sampling methods, and for the rectovaginal swab with and without enrichment, GBSDA and CA detected more positive women than CNA. In addition, detection of GBS from rectovaginal specimens by direct plating onto GBSDA or CA was equally sensitive as detection by Lim broth enrichment with subculture on these agars ($P = 1$) and only one pregnant woman (RVS001) was identified as GBS-positive only after Lim broth enrichment. It should be noted that the inoculum of GBS in this subject was low, as only a few colonies were observed on the agars, even after the enrichment incubation. One

false-negative result on GBSDA, i.e. a colony without orange pigmentation, corresponded to a non-haemolytic and non-pigmented GBS strain that was correctly identified on CA. Three false positives for three different women were observed on CA, because the red colonies isolated from CA were all three identified as *Streptococcus anginosus*, by means of 16S rRNA gene sequence determination and/or tDNA-PCR [21].

Discussion

This study intended to compare the sensitivity of different sampling and culture procedures to establish the presence of GBS in pregnant women. We compared i) rectovaginal, vaginal and rectal sampling, and ii) culture on Columbia CNA agar (CNA), on group B streptococcus differential agar (GBSDA) and on Chromogenic Strepto B ID Agar (CA), iii) directly and after Lim broth enrichment. Other groups studying chromogenic agar did so only for vaginal samples [18-20,22], whereas we also included the CDC recommended rectovaginal sampling method, or they did not compare to Granada agar and used miscellaneous samples [23].

A limitation of this study may be the limited sample size of one hundred subjects, although it should be noted that each subject was studied intensively, i.e. three different sampling sites and six different culture methods were compared for each subject, in a strictly designed study setup that enabled to compare the culture results for each subject in a direct and unambiguous manner.

Comparison of rectovaginal, vaginal and rectal sampling

We found that rectovaginal swabbing was the best sampling method to detect GBS colonization of pregnant women, because all 22 GBS positive women in this study were detected by means of rectovaginal sampling and because two subjects were GBS positive only on the basis of the rectovaginal swab. Our results correspond with previous reports that GBS colonization of rectal samples is 18% to 24% higher than that of vaginal samples [24,25] and with those of other studies that find rectovaginal sampling more appropriate than vaginal sampling only [7,26,27]. For example, in an analysis of 651 specimens, the combination of separate rectal and

Table 1 Number of GBS culture positive samples detected by different culture media in rectovaginal, vaginal and rectal specimens obtained from 49 GBS positive samples from 22 GBS positive women

Specimen	CNA	GBSDA	CA	Lim-CNA	Lim- GBSDA	Lim- CA	Estimated number of women colonized
Rectovaginal ESwab	13	20	21	17	21	22	22
Vaginal ESwab	NT	NT	NT	9	11	11	11
Rectal Eswab	NT	NT	NT	13	17	18	18

CA: Chrom ID Strepto B agar (BioMérieux), CNA: colistin nalidixic acid Columbia agar, GBSDA: GBS Differential Agar (Becton Dickinson), Lim: Lim broth. NT: Not tested.

vaginal sampling enabled detection of 97.8% of GBS carriers, compared to 31.8% of positives as established by vaginal sampling only [28]. Although our results are in correspondence with the CDC recommendations to carry out rectovaginal sampling, it should be noticed that Nomura *et al.* [29] found no significant difference in detection rates between vaginal and rectal samples and Gupta & Briski [30] reported a similar detection rate of 23.8% of GBS when using rectovaginal and vaginal sampling. Votava *et al.* [11] even found that the GBS detection rate using rectovaginal samples was only 16.9%, whereas the use of separate vaginal and rectal swabs yielded 22.7 and 24.1% GBS positive women, respectively. Also, several obstetric departments still use vaginal sampling only to assess GBS positivity.

It is worthwhile mentioning that we also compared vaginal sampling using the recently marketed ESwab with vaginal sampling using the classical cotton swab in Amies gel transport medium (Nuova Aptaca, Canelli, Italy) for the detection of GBS (data not presented to increase readability of the manuscript). Both swabs were introduced simultaneously into the vagina and culture was carried out in an identical manner for both swabs. The classical swab could detect two more GBS positive subjects than the ESwab, raising the number of women positive for GBS in the vagina from 11 to 13.

Different types of selective media formulations

Different selective media for the improved isolation of GBS have been described. Islam *et al.* [16] showed that adding horse serum and starch to agar based media increased the orange/red pigment formation, that was already present to some degree on Columbia agar and that was typical for GBS, being absent for all other serotypes. De la Rosa *et al.* [31] improved this medium adding horse serum at 90 to 95°C instead of at 55°C, making the agar opaque and increasing the natural pigmentation of the colonies, specific for *S. agalactiae*. They showed that pigmentation depends strongly on the use of the correct starch and of proteose peptone n°3 and that it was further increased by the addition of the folate inhibitor trimethoprim (15 µg/ml), in combination with anaerobic incubation. This medium was designated Granada Medium (GM). The same authors later improved GM, which they designated as New Granada medium (NGM) [15], by replacing trimethoprim with 6 µg/ml methotrexate, which is also a folate synthesis inhibitor, but further increases orange to salmon pigmentation of the GBS colonies, and by adding 0.2 µg/ml crystal violet, 5 µg/ml colistin sulphate and 10 µg/ml metronidazole as selective agents. It should be noted that horse serum was added again at 55°C. The commercially available group B streptococcus differential agar (GBSDA), that was used in this study, is a modification of NGM with improved stability and selectivity,

not further specified by the company. Its usefulness has been evaluated in several studies [9,32-34]. Bou *et al.* [32] found that the intensity of colony pigmentation on GBSDA is stronger than on GM and that the commensal microflora is more suppressed.

Because different modifications have been used, sometimes also designated as GM, or because the media were home made [35] or prepared by other companies than the one that supplied the GBSDA in this study [30,36], or because other selective media were used [8,37,38], it is difficult to compare the outcome of several of the previous studies with this study. Therefore, we largely limit our comparison to studies that explicitly used CA from BioMérieux and/or GBSDA from Becton Dickinson.

Direct plating vs broth enrichment culture

Possibly, the use of different (commercial) preparations, modifications and designations, as mentioned above, may explain why some studies found comparable sensitivity of direct plating on 'Granada Medium' compared with Lim broth enrichment [11,15,39,40], whereas other studies found direct plating on chromogenic and/or selective media significantly less sensitive [30,36]. Blanckaert *et al.* [41] suggested to use a combination of Granada and Columbia blood agar and an adequate sample (rectovaginal swab in transport medium) for optimal GBS screening.

In our study, the sensitivity of direct plating on CA and GBSDA was comparable to that of plating on CA and GBSDA after Lim broth enrichment, whereby the latter enabled the detection of only one additional sample, leading to 22 GBS (22%) positive women. Our data suggest that CA and GBSDA are not only faster and easier to use than the CDC recommended method, but that they are also at least as sensitive for the detection of GBS, in agreement with several other recent studies. Also Tazi *et al.* [18] found that, compared to CA and GBSDA, Lim broth enrichment enabled the detection of only two additional samples leading to 34 (17%) GBS positive cultures. Also in the study of Bou *et al.* [28], only one swab was only positive following subculture in Lim broth and was missed on direct GBSDA [32]. Adler *et al.* [34] reported that GBSDA is not only faster and easier to use than Lim broth combined with antigen detection or with subculture on blood agar, but is also at least as sensitive for the detection of GBS from vaginal swabs. In addition, Dunne *et al.* [8] reported that direct plating on neomycin nalidixic acid agar reduced the potential of enterococci competitively overgrowing and masking the presence of GBS in the Lim broth, and this ultimately increased the sensitivity of the direct assay by 14%.

In conclusion, although we missed one out of 22 carriers by direct plating on CA and GBSDA, in our hands

direct plating on CA and GBSDA provided high sensitivity for GBS detection among pregnant women.

CA and GBSDA vs colistin nalidixic acid selective agar (CNA)

We found that CA and GBSDA had comparable sensitivity and provided superior recovery of GBS when compared with CNA. This difference was even more apparent for direct plating. Also, the single subject (RVS072) positive only for the rectovaginal swab was so only on CA and GBSDA, but not on CNA. This is in agreement with another study [30] that showed that selective media producing pigmented colonies are more sensitive in GBS detection than enriched media like blood agar or selective media like CNA.

Direct plating on CA and GBSDA offers the advantage of reducing workload and providing an identification of GBS 24 h sooner than the Lim broth enrichment method. In this study, all GBS isolated from CA and GBSDA were identified within 1-2 days of specimen receipt, whereas all Lim broth enrichment cultures required a minimum of 2-3 days for the identification of specimens positive for GBS. In addition, CA offers an additional advantage with respect to GBSDA, because culture on CA can be carried out aerobically, not requiring special equipment and extra costs and workload associated with anaerobic culture needed for GBSDA.

Sensitivity, specificity, positive and negative predictive value of GBS detection with CA versus GBSDA

The sensitivity and positive predictive value of direct plating of CA for GBS detection were 100 and 87, respectively (Table 2), whereas these values for direct plating on GBSDA were 95 and 100, respectively. In this study, one false negative isolate, lacking the orange pigment, was found on GBSDA, for subject RVS041. This isolate was not missed on CA and was confirmed as GBS by means of the CAMP test. Also Tazi *et al.* [18] found two false negative isolates on Granada medium. Non-haemolytic and non-pigmented GBS have been reported to occur in 1 to 4% among pregnant women [11,30,36,39,40]. Pigment is produced by 93 to 98.5% of GBS clinical isolates. There is a high correlation between the capacity to produce pigment and the

capacity to release hemolysin [42,43], since the genes that determine these properties are in contiguous loci on the chromosome [40].

In our study, we found three false positive results with CA after 24 hrs of incubation, whereby all three isolates were identified as *Streptococcus anginosus*. Tazi *et al.* [18] found two false positive results on Chromagar and showed that these corresponded either to *Streptococcus pyogenes* or *S. porcinus*. These observations indicate that colonies that grow on CA and are suspected to be GBS must be confirmed by additional tests such as CAMP, latex agglutination or molecular techniques, or by positivity on GBSDA. It may be suggested that the combination of direct plating onto CA with aerobic incubation (95% sensitivity), with confirmation of CA positive isolates by means of the CAMP test (100% specificity), may be a highly sensitive, specific and cost effective manner to detect GBS from pregnant women.

Conclusions

In conclusion, to detect GBS carriage among pregnant women, our results indicate i) that rectovaginal sampling is the preferred sampling method, ii) that the ESwab is not superior to the classical cotton swab for sampling, iii) that the inoculation of rectovaginal specimens directly onto CA (21 positives/22) and/or GBSDA (20 positives) has comparable sensitivity as enrichment by Lim broth (22 and 21 positives after subculture onto CA and GBSDA, respectively), and iv) that direct inoculation onto CA or GBSDA is at least as sensitive as the recommended CDC method, i.e. overnight Lim broth enrichment followed by plating onto sheep blood agar (which in this study was replaced by CNA: 17 positives).

Direct inoculation offers several advantages such as decreased workload, because no subculture is needed, and decreased time to detection, i.e. at least 24 hours faster than the standard method. Reagent costs of using CA or GBSDA may be comparable to Lim broth enrichment and subculture on blood agar, also because additional testing is rarely needed for the former approach. Plating on CA in addition does not require anaerobic incubation as is the case for GBSDA. The specificity

Table 2 Sensitivities, specificities, positive and negative predictive values for the six different culture methods, based on all 300 samples, and calculated for a number of 22 positive subjects on a total number of 100 subjects included

Culture Medium	% Sensitivity	% Specificity	% Positive predictive value	% Negative Predictive value
CNA ^a	56	84	52	86
GBSDA	95	100	100	99
CA	100	96	87	100
Lim broth + CNA	78	84	60	93
Lim broth + GBSDA	96	100	100	99
Lim broth + CA	100	96	88	100

a: False positive on CNA: beta-hemolytic colonies which were CAMP negative.

problems associated with the use of CA can be resolved by confirmation of CA positive isolates with the CAMP test.

Additional material

Additional file 1: Table 1: Detection of GBS by means of culture on different media for vaginal, rectal and rectovaginal samples from 22 GBS positive pregnant women. Three samples were collected from pregnant women (vaginal, rectal and rectovaginal). The rectovaginal samples were cultured directly and after Lim broth enrichment on the following media (CNA, GBSDA and CA) whereas vaginal and rectal samples were cultured only after Lim broth enrichment on the same three media.

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Authors' contributions

NAE, RV, GC and MV participated in the development of the study design, the analysis of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. IT, HV and MT participated in the development of the study design, the collection of the study samples, the collection, analysis and interpretation of the data, and in the writing of the report. BS and PC participated in the analysis of the study samples and interpretation of the data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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**Comparison of culture with two different qPCR assays for
detection of rectovaginal carriage of Group B streptococci in
pregnant women**

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Abstract

Objectives: Comparison of culture with two different qPCR assays for detection of Group B streptococci in pregnant women.

Methods

For a total of 100 pregnant women at 35-37 weeks of gestation, one rectovaginal ESwab was collected. Eswab was inoculated into Lim broth, incubated for 24 hours and plated onto chromID™ Strepto B agar (ChromAgar). DNA was extracted with the bioMérieux easyMAG platform, either directly from the rectovaginal ESwab or from the Lim broth enrichment culture. Two different qPCR formats were compared, *i.e.* the hydrolysis probe format (Taqman, Roche), targeting the *sip* gene and the hybridization probe format (Hybprobe, Roche), targeting the *cfb* gene.

Results

Both qPCR techniques identified 33% of the women as GBS positive. Only one culture-positive sample was qPCR-negative. qPCR, directly on the sample, already significantly increased the number of women found to be GBS positive (27%), compared to culture (22%). Moreover, the sensitivity of qPCR after Lim broth enrichment (33%) was again significantly higher than qPCR after DNA extraction directly from the rectovaginal swabs (27%).

Conclusions

When detecting GBS from rectovaginal swabs, the application of qPCR, irrespective of the target (*sip* or *cfb*) has increased the number of GBS positive women in comparison with culture.

Key words:

Group B streptococci, qPCR, Lim broth, Chromagar, pregnant women

Introduction

Group B streptococci (GBS), *i.e.* *Streptococcus agalactiae*, have remained the leading cause of bacterial sepsis and meningitis in neonates for the last two decades, despite a broadly implemented screen-and-treat policy. In Belgium, 13-25% of pregnant women are colonized with GBS and GBS is responsible for 38% of the early neonatal infections [1]. The incidence of invasive neonatal GBS infection is currently reported to amount from 0.5 to 3.0 per 1000 live births, with a 4% to 10% mortality, associated with early-onset infections within the first week of life [2, 3]. GBS disease has two clinical presentations, both of which can cause significant morbidity and mortality: 1) early-onset disease, which is defined by onset within the first 6 days of life, accounting for 85% of neonatal GBS infections, and 2) late-onset disease, which occurs after the 6th day of life with most of the infections occurring before 3 months of age [4]. Early-onset neonatal infection usually results from vertical transmission during delivery, or acquisition *in utero* just before labour, whereas late-onset disease is regarded as being caused predominantly by horizontal transmission. Of the colonized neonates, 1-3% develop disease, almost always in the form of early-onset infection within 24 h of birth [5]. Administration of intravenous intrapartum antibiotic prophylaxis is effective in reducing the incidence of early-onset neonatal GBS infections [6]. Guidelines from the Centers for Disease Control and Prevention (CDC) recommend that all women should be screened at 35–37 weeks of gestation and that those women found to be colonized with group B streptococci should receive intrapartum antibiotic prophylaxis either with penicillin G or ampicillin [2]. The standard method for the diagnosis of group B streptococcal colonization comprises culture of combined vaginal and rectal samples in a selective broth medium that

inhibits the growth of other microorganisms, with subculture onto solid media. Although this method has been shown to be sensitive and specific, it is time consuming, requiring 48-72 h [7].

Recently, alternative methods have become commercially available for the detection of GBS colonization. These include a novel chromogenic agar, *i.e.* chromID Strepto B (formerly Strepto B ID) agar or ChromAgar, which highlights GBS as red colonies, after aerobic incubation [8-10], which distinguishes it from other media, such as Granada agar, which rely on pigment production that is only revealed under anaerobic conditions [11]. Also qPCR assays have been described and commercialized. Ke *et al.* [12] developed a qPCR method, based on amplification of a *cfb* gene fragment that is present in virtually every strain of GBS and Bergh *et al.* [13] established a qPCR targeting the *sip* gene, present across all serotypes of GBS. In this study, we compared the sensitivity of both qPCR formats, directly from samples and after Lim broth enrichment, with that of an optimized culture method, as published previously [14].

Materials and methods

Study design

The study was approved by the research ethics committee (IRB protocol nr 2007/096) of the Ghent University Hospital, Flanders, Belgium, and all women gave written informed consent. Between June 2009 and January 2010, a rectovaginal ESwab was collected from each of 100 pregnant women at 35 – 37 weeks of gestation.

Collection and culture of specimens

Rectovaginal samples were collected using nylon flocked swabs that were submerged into 1 ml of ESwab transport medium (ESwab, Copan Diagnostics, Brescia, Italy).

Rectovaginal sampling was carried out by rotating an ESwab against the vaginal wall at the midportion of the vault. Subsequently, the swab was carefully withdrawn to prevent contamination with microflora from the vulva and introitus and the swab was inserted 1.5 to 2 cm beyond the anal sphincter and gently rotated to touch the anal crypts. All samples were collected by midwives and transported to the Laboratory of Bacteriology Research within 4 hours.

Direct plating was carried out by inoculating 50 µl from the ESwab transport medium onto ChromAgar, *i.e.* chromID™ Strepto B agar (BioMérieux, Marcy l'Etoile, France). The ChromAgar plates were incubated at 37 °C for 18-24 hours in aerobic conditions in the dark. Another 200 µl was inoculated into tubes with 5 ml of Todd-Hewitt broth with 1% yeast extract, 15 µg/ml nalidixic acid and 10 µg colistin/ml (Lim Broth, Becton Dickinson), which were incubated aerobically at 37 °C and subcultured onto ChromAgar after overnight incubation. ChromAgar was examined for pale pink to red, round and pearly colonies.

DNA extraction

For DNA extraction, volumes of 200 µl were taken i) from the ESwab transport medium of the rectovaginal sample and ii) from the incubated Lim broth tubes. DNA extractions from the sample and from the Lim broth were performed using the NucliSENS easyMAG platform (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, 200 µl of the ESwab transport medium, a modified Amies transport medium, from the rectovaginal ESwab or 200 µl from the

incubated Lim broth was added to 1800 μ l easyMAG lysis buffer and incubated for 10 min at room temperature and stored at -80 °C until extraction. Total nucleic acid extraction was carried out by the off-board protocol and nucleic acids were eluted in 100 μ L of elution buffer.

qPCR assays

GBS nucleic acid detection was based on two different qPCR assays, one targeting the *cfb* gene, which encodes the CAMP factor [12], and the other targeting the *sip* gene, which encodes the surface immunogenic protein Sip [13]. Primers and probes for the *sip* and *cfb* genes were as described previously [12, 13]. For detection of the *sip* gene, one hydrolysis probe (TaqMan Probe) was used for the qPCR assay [13]. The qPCR was performed in a 20 μ L reaction volume on the LightCycler® 1.2 instrument using the LightCycler® Taqman Master kit (Roche Diagnostics, Mannheim, Germany). Each reaction contained reagents to final concentrations of 0.5 μ M of each primer and 0.2 μ M of the hydrolysis probe, and contained 5 μ l of DNA extract. Thermal cycling parameters consisted of 95°C denaturation for 10 min for polymerase activation, followed by 50 amplification cycles of denaturation at 96 °C for 5 sec, annealing at 58 °C for 10 sec and extension at 72 °C for 1 sec.

Detection of the *cfb* gene was done with a pair of fluorescently labeled adjacent hybridization probes, *i.e.* STB-F and STB-C [12]. The qPCR was performed in a 20 μ L reaction volume on the LightCycler® v1.2 instrument using the LightCycler® FastStart DNA Master HybProbe kit (Roche Diagnostics). Each reaction contained reagents to final concentrations of 0.4 μ M of each primer and 0.2 μ M of each hybridization probe and 5 μ l of DNA-extract. Thermal cycling parameters consisted of 95 °C denaturation for 10 min, followed by 45 amplification cycles of denaturation at 95 °C for 10 sec, annealing at 55 °C for 10 sec and extension at 72

°C for 5 sec. The melting curve programme consisted of 45 °C for 30 sec and continuous acquisition of fluorescence data during temperature transition to 80 °C at 0.1 °C /sec.

Using qPCR, the concentration of GBS can be determined as the cycle number whereby the fluorescence signal intensity crosses the detection threshold. This value is expressed as the quantification cycle (Cq). Running along a standard dilution series of GBS DNA (see below) makes it possible to determine the approximate number of bacterial cells initially present. Serial dilutions of a DNA extract of the type strain LMG 14694^T were ran along to produce a standard curve and to establish the lower limit of detection (LOD) of the assays, which was established at 100 copies of GBS genomic DNA per PCR reaction.

Positive controls (highly purified DNA from 5×10^8 GBS cells) and negative controls (HPLC purified water) were included in each run. Samples with discordant results (positive by qPCR and negative by standard culture) were investigated further by DNA sequence analysis of the amplification product.

Statistical methods. The sensitivities of qPCR assays and the culture methods were compared by McNemar's test and the Mann-Whitney U test, with p values of < 0.05 considered as significant.

Results

We compared the sensitivity and specificity of culture on ChromAgar after Lim broth enrichment, with that of two qPCR formats, both with and without Lim broth enrichment, for their ability to detect GBS from rectovaginal swabs from pregnant women at 35-37 weeks of gestation.

Lim broth with subculture on ChromAgar, which we previously showed to be more sensitive than Lim broth and colistin nalidixic agar or Lim broth and Granada agar

[14] was used as gold standard to evaluate the sensitivity of the qPCR formats. In total, 100 pregnant women were tested, of which 66 were negative by both culture and qPCR, 22 were positive by Lim broth enrichment and subculture on ChromAgar, 27 by direct qPCR and 33 by qPCR after Lim broth enrichment. The one sample that was positive by culture and negative by qPCR, was culture positive only after enrichment (Table 1, row: this study).

In summary, the qPCR approach after Lim broth enrichment generated significantly ($p < 0.001$; McNemar's test) more positive results (33%) than did the culture method (22%). qPCR after Lim broth enrichment also detected GBS in six additional women, compared to qPCR directly from the rectovaginal swab (27%) ($p < 0.05$; McNemar's test). No difference was found between both types of qPCR, i.e. hybridization probe technology targeting the *cfb* gene and hydrolysis probe technology targeting the *sip* gene. For the 21 samples that were positive for both culture and qPCR, both the DNA directly from the sample and the DNA extracted after Lim broth enrichment were qPCR positive.

Discussion

Hybridization probes versus hydrolysis probe-based qPCR

To our knowledge, this is the first study that compared two different real-time PCR (qPCR) detection chemistries, thereby targeting two different genes for detection of group B streptococci (GBS). We found complete agreement between both PCR assays, *i.e.* hybridization probe-based qPCR targeting the *cfb* gene and hydrolysis probe-based qPCR targeting the *sip* gene. Both techniques indicated 33% of the women as GBS positive. The identical results with both assays can be taken as indicative for the robustness of the qPCR approach in general.

Lim broth enhanced qPCR versus Lim broth subculture

To prevent GBS disease in neonates, the current recommendation is to screen pregnant women by culture of combined vaginal and rectal mucosae at 35 to 37 weeks' gestation and to treat those with positive cultures. Molecular non-amplification-based methods for identifying GBS have been described almost two decades ago [15]. The Accuprobe system (Gen-Probe, San Diego, Ca.) was shown to be suitable for the identification of GBS from cultures but to be insufficiently sensitive for direct detection from clinical specimens [15]. During the last decade, several studies have demonstrated the utility of real-time PCR (qPCR) for rapidly and reliably detecting GBS colonization in (pregnant) women, as summarized in Table 1.

Prevalence established by culture and by qPCR

Although the prevalence of colonization in our study on the basis of Lim broth enrichment and subculture onto ChromAgar was 22%, which is in agreement with that reported in the literature, on the basis of culture-based prevalence studies [16-18], we found a significantly higher number of women positive, when direct qPCR was applied (27%). This is in agreement with other studies that reported increases of 12% to 21% GBS positives using direct qPCR [19-21]. In our study, the one sample positive by culture and negative by qPCR, was culture positive only after enrichment, as has also been observed previously [13]. The lower sensitivity of culture can be possibly explained by the presence of antagonistic microorganisms, such as enterococci, which inhibit the growth of GBS or overgrow the GBS [8]. Also antibiotics and feminine hygiene products have been shown to inhibit the growth of GBS [22]. Furthermore, inappropriate storage, collection and transport conditions of the samples from obstetrical clinics to the laboratory could also result

in false negative culture results, especially in cases of light colonization [23]. Besides higher sensitivity and less-demanding sample storage and transport, as offered by PCR, qPCR brings the additional advantage of quantification. It is indeed important to quantify the bacterial load because heavy colonization with GBS has been shown to correlate with a higher risk of neonatal sepsis [24]. In our study, we found that the Cq-values were indicative for the bacterial load, because the samples with the highest Cq-values, which can be assumed to be the samples with the lowest bacterial load, were also the ones that were culture negative, which is also indicative of a lower bacterial load, *i.e.* the mean Cq value for the 21 culture/ PCR positive samples was 21.5 (CI: 18.7-24.3), whereas that for the 12 culture negative, PCR positive samples it was 26.5 (CI: 22.3-30.8), strongly indicative for a lower bacterial load in the latter ($p < 0.05$). For the 12 samples that were positive only by qPCR, the mean Cq value for the six samples, that were positive also when the DNA was extracted directly from the samples, was 22.6 (CI: 15.4-29.8), whereas the other six samples, that were only positive when DNA was extracted from the Lim broth after enrichment, the Cq-value was 30.3 (CI: 27.7-32.9), strongly indicative for a lower bacterial load in the latter ($p < 0.05$). Fig. 1 presents the Cq-values observed for these three categories of samples.

Direct qPCR vs Lim broth-enhanced qPCR

Quantitative PCR may also offer the advantage of reduced time-to-result, making it even useful to be used as an intrapartum screening method [19, 25, 26]. However, our results indicate that combining qPCR with prior Lim broth enrichment significantly increases the sensitivity, compared to qPCR directly on the sample. GBS colonization was determined to be 33% on the basis of qPCR of DNA extracted from Lim broth after enrichment incubation, which is significantly higher than the

colonization rate established by Lim broth with subculture. The increased sensitivity of qPCR after Lim broth enrichment compared to subculture after Lim broth enrichment is in correspondence with most other studies. Goodrich *et al.* [27] established GBS colonization on the basis of rectovaginal swabs for a total of 200 women, after enrichment during 4 h in Lim broth followed by culture, Strep B Analyte Specific Reagent (LightCycler qPCR) and BD-StrepB test, and found colonization rates of respectively 26.5, 29.5 and 30.0%. Correspondingly, Rallu *et al.* [30] reported that Lim broth-enhanced *scpB* PCR identified 37% of a total 605 rectovaginal specimens as GBS positive, whereas subculture identified only 16% as positive. Also, Block *et al.* [28] reported that the rate of *S. agalactiae* detection by carrot broth-enhanced qPCR (33.0%) trended higher than the rates generated by carrot broth culture and subculture (29.6%). However, Scicchitano *et al.* [29] found that both culture and the BD GeneOhm StrepB assay, both after Lim broth enrichment, detected 95.3% of the total number of GBS positives.

Also our finding that qPCR after Lim broth enrichment is more sensitive than qPCR directly on the swabs, is in agreement with other studies. Maloney *et al.* [30] reported that BD GeneOhm StrepB detected more GBS positive women after Lim broth enrichment (no detailed data available). Block *et al.* [28] also concluded that broth-enhanced qPCR was more sensitive than direct swab PCR, although precise data were not provided. Hutchens and Schreckenberger [31] reported that the IDIStrepB Cepheid Strep B SmartCycler assay had a sensitivity of 85.7% when it was performed directly on swab specimens compared to the sensitivity after enrichment culture.

In conclusion, when detecting GBS directly from rectovaginal swabs, the application of qPCR, irrespective of the qPCR assay (hybridization probes qPCR

targeting the *sip* gene or hydrolysis probe qPCR targeting the *cfb* gene), increased the number of GBS positive women compared to culture, and sensitivity was further increase by combining Lim broth enrichment with qPCR.

Because the introduction of overnight incubated Lim broth enrichment prior to qPCR, in order to optimize the sensitivity of GBS detection, reduces the speed – as achieved when direct qPCR is carried out – again, future studies should indicate which of both, i.e. intrapartum screening with a rapid technique or antepartum screening with the currently most sensitive approach, is clinically most relevant.

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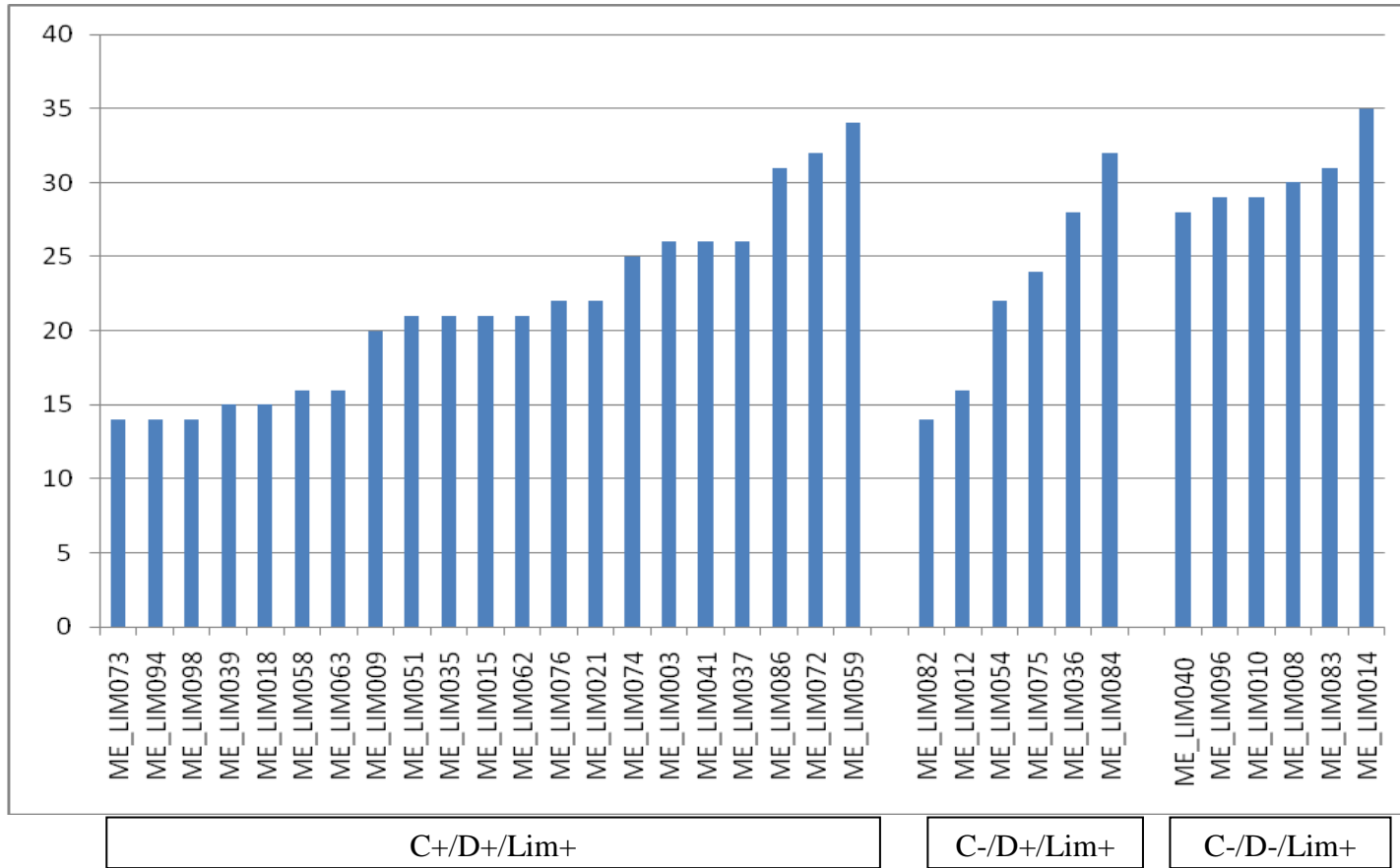
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Table 1. Results of different studies on qPCR tests for detection of GBS from vaginal, rectal and rectovaginal samples

Reference	Population	Samples	Culture technique	qPCR technique	Total number	C + / P+	C + / P-	C - / P+	C - / P-	Sensitivity	Specificity	PP+	PP-
Bergh et al. 2004	consecutive	V	Direct & selective culture	sip, LC, Taqman probe	100	24	1	8	67	96	89	75	98.5
Davies et al. 2004	Intrapartum Calgary	VR	selective culture	IDI-Strep B	427	76	1	12	338	99	97	86.4	99.7
	Intrapartum Houston				75	17	2	4	52	89	93	81	96.3
	Intrapartum Milwaukee				48	11	2	0	35	85	100	100	94.6
	Intrapartum Montreal				166	14	1	11	139	93	93	56	99.3
	IntrapartumPittsburgh				87	22	3	0	62	88	100	100	95.4
	Intrapartum Total				803	140	9	27	626	94	95.9	83.8	98.6
Convert et al. 2005	Antepartum(33-37 w)	V	selective culture	cfb, LC, HybProbe	400	75	0	47	278	100	85	61	100
Réglier-Poupet et al. 2005	Antepartum (35-37 w)	V	Direct culture	cfb, LC, HybProbe	269	44	1	8	216	97	96	84.6	99
Bergeron et al. 2006	Antepartum (35-37 w)	R, V, VR	selective culture	cfb, LC, HybProbe	112	32	1	0	79	97.0	100	100	98.8
Bergseng et al. 2007	Intrapartum	R + V	Direct & selective culture	sip, LC, Taqman probe	251	84	2	3	87	97.6	97	96.5	89.5
Gavino et al. 2007	Intrapartum	VR		GenXpert (Cepheid)	55	23	1	11	20	95.8	64.5	67.6	95.2
Goodrich et al. 2007	Antepartum (35-37 w)	VR	selective culture	LC Strep B ASR(ptsI, LC, HybProbe)	200	53	0	6	141	100	95.9	89	100
				BD-StrepB test		49	4	11	136	92.5	92.5	81	97
Chan et al. 2008	Intrapartum	LV	selective culture	cfb, LC, HybProbe	143	9	11	1	122	45	99	90	91.7
Edwards et al. 2008	Antepartum-36 w (366) & intrapartum (418)	VR	selective culture	XPert GBS	784	173	17	24	570	91.1	96.0	87.8	97.1
				IDI-Strep B									
Money et al. 2008	Intrapartum	VR	selective culture	IDI-Strep B	190	49	5	3	121	90.7	93.2	94.2	96
& Bourbeau 2009	Antepartum (35-37 w)	V, VR	Direct & selective culture	BD GeneOhm StrepB assay (previously IDI-Strep B)	498	136	5	5	349	96.6	99.1	96.4	98.5
Wernecke et al. 2009	Antepartum (35-37 w)	V	selective culture	(RiboSEQ, LC, HybProbe)	159	107	4	2	46	96.4	95.8	98.2	92
				BD GeneOhm™ StrepB Assay		105	6	5	43	94.6	89.6	95.5	87.8
El Helani et al. 2009	Intrapartum	V	Direct & selective culture	Xpert GBS; Cepheid	968	135	3	2	723	98.5	99.6	97.8	99.7
Wilson et al. 2010	Antepartum (35-37 w)	G, V, VR	selective culture	Smart GBS assay	206	64	0	0	142	100	100	100	100
This study	Antepartum (35-37 w)	VR	Direct & selective culture	cfb, LC, HybProbe									
				sip, LC, Taqman probe									
				Direct DNA	100	21	1	6	72	95.4	92.3	77	98.6
				Lim broth DNA	100	21	1	12	66	95.4	84.6	63.6	98.5

FIG 1. Cq values of the GBS-qPCR positive rectovaginal samples using the hybridization probe assay (*cfb* gene).



Legend: x-axis: C+: culture positive, D+: qPCR positive, directly from sample, Lim+:qPCR positive only after Lim broth enrichment.

y-axis: Cq-value of qPCR.

Chapter III: General discussion and future perspectives

III.1. Characterization of vaginal microflora of pregnant women

Characterisation of the normal vaginal microflora (VMF) may provide a better understanding of the mechanisms involved in the stability of the lactobacilli-dominated VMF, or conversely, with their failure to maintain homeostasis of the vaginal ecosystem. The bacteria normally present in the human vagina play a key role in preventing successful colonization by 'undesirable' organisms, including those responsible for bacterial vaginosis, yeast infections, sexually transmitted diseases and urinary tract infections (89, 152, 343, 373). Four *Lactobacillus* species are now generally agreed to dominate the VMF, in particular *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners* (128, 377, 380). Verstraelen *et al.* (382) reported that the presence of different *Lactobacillus* species in the normal VMF is a major determinant to the stability of the VMF in pregnancy. *L. crispatus* is associated with a particularly stable vaginal ecosystem. Conversely, microflora comprising *L. jensenii* elicits intermediate stability, while VMF comprising *L. gasseri/L. iners* is the least stable. A fifth species, *L. vaginalis*, may be present more frequently than is now assumed, but may be missed when using anaerobic culture in jars for culture (see below).

Microscopy, culture and different molecular techniques have been used to study the composition of the VMF. The most widely performed method is microscopy in combination with the Gram-stain based scoring system of vaginal smears, developed by Nugent *et al.*, with high intracenter and intercenter, as well as intraobserver and interobserver reproducibility (266). Verhelst *et al.* (380) showed that it is possible to differentiate to some degree between *L. crispatus* (short plump rods), *L. gasseri* (long tender rods) and *L. jensenii* (very long bacilli) on the basis of Gram smear. De Backer *et al.* (75) found that *L. iners* cells surprisingly present as Gram-negative small coccobacilli, instead of as Gram-positive bacilli, which, together with the inability to grow on MRS medium and its formation of small colonies on other media, is probably the reason why this species has been long overlooked and has been confused with *Gardnerella vaginalis*.

Both culture and molecular techniques have their advantages and limitations when studying the composition of the complex VMF. The human vagina harbors a complex microflora, including many fastidious bacteria. Hence, culture-dependent analysis is likely to overestimate the role of cultivable bacteria while uncultivable species are overlooked (295). During this doctoral research, it was possible to identify the vaginal *Lactobacillus* species rapidly and with high discriminatory power by means of tDNA-PCR (10, 375, 393), whereas lactobacilli remain difficult to identify with biochemical means. Other molecular techniques than tDNA-PCR remain laborious. Using tDNA-PCR, we found that a total of 121 (91.6%) of 132 vaginal samples and 51 (38.6%) of 132 rectal samples were positive for lactobacilli (100). *L. crispatus* was the most frequently isolated *Lactobacillus* species from the vagina (40% of the subjects were positive), followed by *L. jensenii* (32%), *L. gasseri* (30%) and *L. iners* (11%). *L. gasseri* was the most frequently isolated *Lactobacillus* species from the rectum (15%), followed by *L. jensenii* (12%), *L. crispatus* (11%) and *L. iners* (2%) (100). We also observed that incubation in anaerobic gas containers (GasPak, Becton Dickinson, Erembodegem, Belgium) yields much lower numbers of *L. vaginalis*, compared to culture in an anaerobic cabinet (BugBoxPlus, LED Techno, Heusden-Zolder, Belgium), as was used for our studies. *L. vaginalis* was not found by Marrazzo *et al.* (238) and virtually absent in the study of Antonio *et al.* (6) and Verhelst *et al.* (379), all using jars, whereas we found a vaginal carriage rate of approximately 10%.

In our study, a total of 46 (35%) of 132 pregnant women carried two or more *Lactobacillus* species vaginally and/or rectally. Marrazzo *et al.* (238) reported 72% of 237 participants to be colonized overall by lactobacilli and 24% to be colonized with more than one *Lactobacillus* species. Antonio *et al.* (5) reported that 8% carried more than one *Lactobacillus* species in the vagina, with 3.5% colonized by both *L. crispatus* and *L. jensenii*.

A total of 18 women (12%) in our study were colonized by both *L. crispatus* and *L. jensenii* (17 vaginally and one rectally). Vaginal colonization of women with *L. crispatus* and *L. jensenii* has been suggested to be advantageous in the maintenance of a normal microflora and the prevention of sexually transmitted diseases (5, 6).

II.2. Vaginal/rectal colonization

Several bacterial species are known to colonize both the gastrointestinal and the reproductive tract, and it is largely agreed that the rectum plays an important role as a source or reservoir for organisms that colonize the vagina. For example, group B *Streptococcus* (GBS) colonizes both the vagina and the rectum of many women of reproductive age (256), as was shown in this doctoral study as well (99). Also, *Escherichia coli*, known as an intestinal commensal, is the most important cause of urinary tract infection in women. Colonization of the vagina by intestinal microflora is also clear from studies with orally administered probiotic lactobacilli that were able to colonize the vagina (293). Of 100 pregnant women in our study, 17 harbored *E. coli* in both rectum and vagina, 7 only in the vagina and 76 exclusively in the rectum. Also for *Candida albicans*, there is a close correlation between the vagina and rectum, which has been considered as the reservoir whence vaginal reinfection occurs (225). We found 12 women harboring *Candida albicans* in both rectum and vagina, five only in the vagina and two exclusively in the rectum.

Also organisms associated with bacterial vaginosis, i.e. *Mobiluncus* spp. *Mycoplasma hominis* and *G. vaginalis*, have been claimed not to be transmitted sexually but to colonize the vagina from an endogenous intestinal tract site (155, 178).

Recently, Song *et al.* (345) and Antonio *et al.* (6) studied lactobacilli from rectal and vaginal origin by DNA-based identification of cultured isolates. Antonio *et al.* (6) found that co-colonization of the vagina and rectum by H₂O₂-producing lactobacilli was associated with the lowest prevalence of bacterial vaginosis (5%), whereas females colonized only vaginally, only rectally, or at neither site had an increased risk of bacterial vaginosis. Because of the close proximity of the rectum to the vagina, the isolation of H₂O₂-producing vaginal *Lactobacillus* species from the rectum suggests that it may play a role as a reservoir for these microorganisms and that the presence of the right *Lactobacillus* species in the rectum may contribute to the maintenance of undisturbed VMF (6).

In this doctoral study, we not only compared the prevalence of the same species in both vagina and rectum, but also genotyped the obtained isolates, to assess whether not only the species but also the genotypes of the isolates were identical. To our knowledge, this is the first study comparing vaginal and rectal isolates at the strain level. RAPD-analysis was used in our study to assess genotypic similarity of vaginal and rectal isolates of the same bacterial species, for those species that were found to be present simultaneously at both sites (100). Several of the total of 63 species identified, were found only vaginally (9, i.e. 14.3%) or only rectally (26, i.e. 41.3%), but 29 species (44.4%) were isolated from both sites, indicating that many species can colonize the vagina from the rectum or migrate to the rectum from the vagina. For the 8 species for which isolates were present simultaneously in the same subject in vagina and rectum, we found that the genotype of the vaginal isolate was identical to that of the rectal isolate for 70% of the 50 vaginal/rectal species pairs that had been obtained. These data show that the simultaneous presence of the same species in rectum and vagina is not a random event, but that indeed it is the same strain, which has colonized both sites (100).

In addition, we found genotypic similarity between the vagina and rectum when studying group B streptococci (GBS). For five out of the 19 women from which both vaginal and rectal GBS isolates were obtained, all the rectal and vaginal isolates that were genotyped were identical. For another 13 of these 19, at least one of the vaginal isolates had a similar genotype as one of the rectal isolates (99). Strong genotypic and serotypic heterogeneity was observed between women and for individual women, *e.g.*, we found a total of 66 genotypes among 118 isolates (from 32 women), of which 4 women had up to 4 different genotypes in total (rectal + vaginal) and 14 women had up to 2 different genotypes vaginally (99). The high level of heterogeneity established in our study, compared to other studies, may be due to the different culture media that were used and to the use of separate swabs for sampling vagina and rectum for each individual woman. The high overall genotypic intraspecific diversity indicates that our findings of genotypic identity between rectal and vaginal isolates of the same subject is genuine and not due to a bias as might be caused *e.g.* by low discriminatory power of the

genotyping method used, or overall low genotypic diversity within the species *S. agalactiae*.

Using qPCR, it was also possible to assess whether the vaginal and rectal bacterial loads corresponded (96). We found indeed significant positive correlations between vaginal and rectal colonization, i.e. there was high degree of correspondence between vaginal bacterial load and rectal bacterial load for five of the six species, i.e. *A. vaginae*, *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, indicating that not only the same species and strains can be present simultaneously in both niches, but also that cell densities per bacterial species do correlate between both niches, confirming that both niches are in close correspondence.

This study (96) also confirmed previous findings of our group (75), as we found positive correlation between the bacterial load for *G. vaginalis* and *A. vaginae*, i.e. the bacterial load of both species was low in normal VMF (grades Ia and Iab), and high in disturbed VMF (grades II and III). Negative correlations were found between *L. crispatus* and *G. vaginalis* and also between *L. crispatus* and *A. vaginae*. In our study, *A. vaginae* was found less frequently than *G. vaginalis* in non-grade III samples, but was present in high concentrations in 10 of the 12 grade III samples, confirming that the presence of *A. vaginae* seems to be a diagnostically more valuable marker for BV than the presence of *G. vaginalis*, as suggested by Verhelst *et al.* (379) and confirmed by several other studies (38, 44, 75, 117).

In accordance, Swidsinski *et al.* (358) concluded that bacterial vaginosis does not occur as a result of simple growth per continuum of perianal bacterial biofilm migrating to the vagina, but that some species originating from the intestinal tract, e.g. *G. vaginalis*, do display pronounced vaginotropism, whereas many other species do not.

III.3. Comparison of culture and qPCR to characterize the vaginal and rectal microflora

Both culture and molecular methods have their advantages and drawbacks. Cultivation of bacteria enables to obtain critical insights about the microbial phenotypic

characteristics, that are difficult to derive from molecular studies. Furthermore, cultivated microbes allow for the experimental manipulation of these organisms in the laboratory and the testing of hypotheses about pathogenesis and virulence factors. Accordingly, cultivation studies remain an important area of investigation in vaginal microbiology research (85).

Besides for basic research, the VMF is also studied for diagnostic reasons. Because the microflora of the human vagina can affect the health of women, their fetuses, and newborns and because conventional cultivation methods fail to detect some fastidious vaginal bacteria, leading to an incomplete census, the development of highly sensitive molecular methods for detection of (pathogenic) bacteria has been a central topic of research in gynaecological and obstetric microbiology during the last decades.

The major limitation of culture-based methods has been described as 'the great plate count anomaly', because only a small fraction of microorganisms that are present in a population can be cultured (170). Cultivation-based characterization of the VMF is further biased by the type of culture medium and microbial density present in the sample. For example, *L. jensenii* forms larger colonies on Schaedler than on Columbia agar, which results in an easier isolation of *L. jensenii* from the former medium, but on the other hand this renders it more difficult to isolate other species on Schaedler agar from a sample mainly colonized with *L. jensenii*. *L. iners* does not grow on MRS agar, routinely used for detection of lactobacilli in vaginal samples, and has therefore been overlooked in many studies.

Recent advances in molecular biology have facilitated the detection and identification of bacteria without cultivation. The most commonly employed target for molecular identification of bacteria is the small ribosomal subunit or 16S rRNA gene. The 16S rRNA gene is useful, because it is present in all bacteria and has regions of sequence conservation that can be targeted with broad range PCR primers and areas of sequence heterogeneity that can be used to identify bacteria or infer phylogenetic relationships (15, 317, 394). Molecular studies of the VMF help to augment, but do not replace, the census data generated using cultivation-based approaches. They have made it possible to discover many non-cultivable or fastidious bacterial species, such as *A. vaginae* (379),

BV-associated bacteria (BVAB) - belonging to the *Clostridiales*, and bacteria related to *Megasphaera*, *Leptotrichia* and *Dialister* (349).

We established significant differences between the detection of six bacterial species of the vaginal and rectal microflora using culture and qPCR. The carriage rate was 3-fold (for *L. crispatus*) to 17-fold (for *A. vaginae*) higher according to qPCR than according to culture. Women were positive for the six species in 4.0 to 32.3% of cases according to culture, compared to 68.5 to 91.5% of cases according to qPCR. Using qPCR, we could also detect *L. iners* in high numbers vaginally and rectally when compared to other studies of the rectal microflora (5, 238), which may be of additional importance because several recent molecular studies (119, 186) indicate that the possible role of this peculiar lactobacillar species in the transition between balanced and imbalanced VMF needs further elucidation.

Using two different qPCR assays in GBS detection from pregnant women(97), we found that qPCR approach after Lim broth enrichment generated significantly ($p < 0.001$; McNemar's test) more positive results (33%) than did the culture method (22%). qPCR after Lim broth enrichment also detected GBS in six additional women, compared to qPCR directly from the rectovaginal swab (27%) ($p < 0.05$; McNemar's test). No difference was found between both types of qPCR, i.e. hybridization probe technology targeting the *cfb* gene and hydrolysis probe technology targeting the *sip* gene.

While molecular methods have many advantages over cultivation approaches for characterizing microbial diversity, there are also limitations (18, 123). Use of universal primers targeting conserved regions of the 16S rRNA gene may not detect all bacteria present in a sample due to the presence of polymorphic nucleotides at conserved positions. This was pointed out by Verhelst *et al.* (379), who found that the 'universal' 16S rRNA gene primers, used by many research groups, failed to amplify this gene from *G. vaginalis*, because of the presence of 3 mismatches in the forward primer.

Another problem is primer specificity, *e.g.*, when using the primers of *L. jensenii* for qPCR, we recently discovered (personal communication Piet Cools) an aspecific - false positive - reaction in some (African) vaginal samples. We could show that this was due to the amplification of an undefined fragment present in the *L. iners* genome and thus

occurred only for samples where *L. iners* was present. The use of a hydrolysis probe, so that only specific amplification is reported, could solve this problem.

Heterogeneity of the 16S rRNA gene can also hamper analysis (313), because the conserved primer regions may contain some mismatches for some species anyway. Lowering the annealing temperature during PCR to enable amplification of those species with some primer mismatches, may also allow nonspecific amplification of DNA from human tissues (349).

The DNA extraction step is also vital to obtain a representative pool of DNA, which will then be used for PCR amplification. Species bias for different extraction methods has been described (240, 351), and can originate from differences in the kits used for DNA extraction, or from the fact that the DNA of Gram negative bacteria is much easier to isolate than that of Gram positives (131), due to the thick peptidoglycan layer of the latter.

Presence of inhibitors in the clinical samples from blood, mucus, or vaginal products can lead to failed or reduced amplification. Amplification controls are useful in tracking DNA quality whereby the efficiency of amplification of human target genes such as beta-globin (130) or the 18S rRNA gene (199) can be assessed. Use of internal amplification controls by adding an exogenous template at known concentrations to the clinical samples can help in detection of PCR inhibitors (239), particularly when performing quantitative PCR analysis.

The opposite problem is formed by false positives, which can occur especially when broad range bacterial primers are used. Low levels of bacterial DNA may be present in the laboratory (carry over of previously amplified DNA), in DNA extraction kits or in PCR reagents, such as recombinant *Taq* polymerase (32, 318). A way to monitor this problem is to include negative (no template) controls in every run of PCR, which allow for detection of this type of contaminants.

The PCR amplification step itself can introduce biases such as skewed representation of a sample based on the different guanosine plus cytosine (G+C) contents of the bacterial species (93), because bacteria with a higher G+C content of the target may be more difficult to amplify when compared with bacteria with lower G+C.

III.4. Prenatal screening of group B streptococci

In 2002, the Center for Disease Control and Prevention (CDC) published revised guidelines for the prevention of perinatal GBS disease, and recommended routine culture for all pregnant women between 35 and 37 weeks of gestation (320). These recommendations were prompted by the recognition that a culture-based screening strategy is at least 50% more effective in preventing neonatal infection than the previously recommended risk-based screening (322).

Different culture-based approaches have been developed. Because GBS are components of the complex normal microflora in the human vagina and rectum, GBS-selective broth media supplemented with antibiotics that inhibit growth of non-GBS organisms have been developed to improve GBS recovery from clinical samples (190).

In 2010, the Center for Disease Control and Prevention (CDC) published new guidelines for the prevention of perinatal GBS disease (58), and recommended the use of selective broth that incorporates chromogenic pigments for GBS detection and as optionally, direct broth testing using DNA probe, latex agglutination or nucleic acid amplification techniques.

In our first study (99), which included 150 pregnant women, we compared the number of GBS positive women when assessed Granada agar, only after Lim broth enrichment, and Columbia CNA agar, with or without enrichment. Lim broth combined with Granada agar enabled detection of all the women that were found GBS positive in this study (n = 36), whereas direct inoculation onto Columbia CNA agar achieved a sensitivity of 40% and subculture of the Lim broth onto Columbia CNA agar detected 81% of the carriers (99).

In our second study (98), which included an additional 100 pregnant women, we compared Granada agar and Chromagar, with and without prior Lim broth enrichment, and found the same sensitivity for both media. In addition, we found that Lim broth enabled the detection of only one additional sample, leading to 22 GBS (22%) positive cultures. This is in agreement with some studies that found comparable sensitivity

between Granada agar plating directly from the sample and after Lim Broth enrichment (78, 140, 386), whereas other studies found direct plating significantly less sensitive (151, 270). Although the published materials and methods do not enable to fully support the following statement, this difference might possibly be explained by different brands of the plates used.

Our data suggest that Granada agar and Chromagar are not only faster and easier to use than the CDC recommended method, but is also at least as sensitive for the detection of GBS. This is in agreement with other studies (277, 282, 362). The cost of using direct Granada agar or Chromagar may be comparable to Lim Broth enrichment and subculture on blood agar. Finally, direct plating onto Chromagar, besides being equally sensitive and rapid as direct plating onto Granada agar, in addition did not require anaerobic incubation as is the case for Granada agar. The specificity problems associated with the use of Chromagar, *i.e.* three false positive colonies, which were shown to be *Streptococcus anginosus*, can be resolved by confirmation of Chromagar positive isolates with the CAMP test.

The only other research groups studying chromogenic agars so far did so only for vaginal samples (277, 362), whereas we also included the CDC recommended vaginorectal sampling method. We concluded that vaginorectal swabs were the best samples to detect colonization by *S. agalactiae* in pregnant women, in correspondence with previous reports (2, 57, 83).

Rapid detection methods for GBS may be advantageous for women who present in labor without any information on their GBS colonization status. Ke *et al.* (196) developed a qPCR method based on amplification of a *cfb* gene fragment that is present in virtually every strain of GBS. Also Bergh *et al.* (22) established a qPCR targeting the *sip* gene universally present across all serotypes of GBS. We compared both approaches and found both of them had the same sensitivity and specificity (97). qPCR methods that offer the ability to detect GBS colonization within 1 h of sample receipt, have also been developed (72). The BD GeneOhm StrepB test (BD GeneOhm Sciences, San Diego, CA; formerly IDI-StrepB), the Cepheid GeneXpert GBS and Smart GBS (Cepheid, Sunnyvale,

CA) offer detection of GBS directly from vaginorectal swabs for antepartum or intrapartum detection of GBS colonization.

We also compared the sensitivity of the most sensitive culture method, *i.e.* Lim broth enrichment combined with Chromagar (97), with the two qPCR methods (22, 196). Our study found that 33% of pregnant women were colonized with GBS according to the qPCR assays, whereas only 22 women were found positive using Lim broth and subculture on Chromagar. This was in agreement with other studies that report increases of GBS positive women of 12% to 21% using PCR (68, 72, 288).

Interestingly, in our study, GBS was detected with qPCR from six additional women, only from DNA extracted from the Lim broth and not from the DNA extracted directly from the sample: In total, 33 GBS positive women were detected by Lim broth enrichment, followed by qPCR, whereas only 27 were detected by direct qPCR. Few other studies compared direct molecular methods with molecular methods after broth enrichment. In a conference abstract, Maloney *et al.* (236) reported that the sensitivity of the BD GeneOhm StrepB was higher for specimens pre-incubated in Lim broth than for the same specimens with direct testing, but no data were provided. Also in correspondence to our findings, Rallu *et al.* (30) reported that, out of a total of 605 vaginorectal specimens, Lim broth-enhanced *scpB* PCR identified 226 samples (37%) as GBS positive, whereas culture identified only 96 samples (16%) as positive.

It may be of importance to note that the prevalence of GBS positive women, which was established as 33% according to our findings on the basis of qPCR, exceeds largely the estimated prevalence of 13-25%, as estimated by the Belgian reference laboratories in collaboration with the section of epidemiology of the Scientific Institute for Public Health (ISP-WIV, Brussels) (150). The data of this National Institute are of course based on culture, and our culture based prevalence estimation (22%) indeed corresponds with these data.

The advantages of molecular applications, increased sensitivity and decreased time of result relative to culture, are clear, also from our study. One disadvantage of the molecular detection of GBS, directly from the samples, is that there is no isolate readily available for susceptibility testing as needed for penicillin-allergic patients, but our most

sensitive method, Lim broth enrichment combined with qPCR, circumvents this problem, at the expense of increased time-to-result.

In conclusion, the combined data from our studies and from most other studies indicate that direct culture on selective media like Granada agar and Chromagar is equally sensitive as Lim broth enriched culture. Because we found Chromagar slightly more sensitive and especially because it can be incubated aerobically, the latter may be the preferable method. Furthermore, when comparing two different qPCR formats targeting different genes, we found both approaches to be equally sensitive, but more importantly, to be more sensitive and rapid than culture. The most sensitive approach turned out to be Lim broth enrichment followed by qPCR (recommended as an option by the most recent CDC guidelines of 2010 (58), which reduces speed again and therefore intrapartum applicability.

III.5. Further perspectives

In our study we reported high species- and strain similarity between vaginal and rectal microflora (96, 99, 100), strongly suggestive for the important role of the rectum as a source of vaginal colonization not only at the level of the normal VMF but also of species like *G. vaginalis* and *A. vaginae*, typical for disturbed microflora. Although it is difficult to establish studies which clearly indicate that it is the rectum that serves as a source for vaginal colonization and not vice versa, the former is more likely, also because studies with orally administered probiotic lactobacilli showed that these were able to colonize the vagina (293). Studies whereby the dynamics of the vaginal and rectal microflora are detailed, by collecting consecutive samples from the same subject during *e.g.* two menstrual cycles (ongoing PhD Research Guido Lopes dos Santos Santiago, LBR), might shed more light on the primary source of the vaginal microflora. In addition, metagenomic studies, as possible by next generation sequencing (229, 286), may enhance largely our capacities to document the microbial overlapping and exchange between both niches, although it should be kept in mind that the biases, introduced by

nucleic acid extraction and amplification, as discussed above, should be taken into account.

Furthermore, because of the likeliness that the rectum serves as the source for vaginal (re-)colonisation, the capacity of vaginally introduced lactobacilli to colonize the rectum may also be an important criterion that should be considered in the choice of a probiotic strain for the reconstitution of vaginal lactobacilli and the normal vaginal microflora.

Given the epidemiological differences between *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners* (380, 381), confirmed by one of our studies (96), detailed characterization of metabolic pathways and adherence properties of these species through pan-genomic studies (see I.5.2) might pinpoint why *L. crispatus* apparently confers colonization resistance while the other *Lactobacillus* species do not or to a lesser extent. The question is which characteristics of *L. crispatus* inhibit growth of other bacteria and why the presence of *L. crispatus* in pregnant women decreases the likelihood of other vaginal bacteria are found later in pregnancy (382). For *A. vaginae* and *G. vaginalis*, two species predominantly present in disturbed VMF, pan-genomic studies can help to determine whether the presence of these species is merely a sign of disturbance rather than that they possess special enzymatic activity which confers to the disturbance of the normal VMF.

Regarding our studies on *Streptococcus agalactiae* or group B streptococci (GBS), it is clear that also here technological advances will further improve infection prevention and treatment efforts. Our work, although on a limited series of women (97, 99), convincingly showed the value of the selective solid media, such as Granada agar (79, 304) and Chromagar (277, 362), obviating the need for broth enrichment and speeding up the culture-based procedure. Probably, this is the best culture-based solution at the moment and little further improvement on the basis of culture seems possible, except maybe in reducing the already limited number of false positive (Chromagar) and false negative (Granada agar) colonies.

Our study, comparing culture and qPCR (97) showed, that - although culture has been optimized as much as possible (see above) - the estimation by qPCR of the number of women positive for GBS, largely exceeds that of the culture-based estimation. Future

research might consist of renewed screening on a national basis to establish the prevalence of GBS positive women in Belgium, as assessed by molecular techniques.

Advantages of rapid and reliable intrapartum screening, that can be performed in the labor and delivery area, could lead to an additional decrease in the incidence of early-onset neonatal GBS infections and in morbidity and mortality associated with those infections. The availability of such screening methods offers the potential for GBS detection among women without prenatal care, or in women who are admitted in preterm labour. The qPCR technology which makes intrapartum screening possible, is relatively new, and thus far its use does not appear to be widespread among hospitals. At present, only biochemistry and haematology laboratories can deliver results within 1 h. As qPCR tests become more widely available, the possibility of precise microbial identification for selected pathogens in 30–45 min instead of 2–3 days may be generalized, increasing the appropriateness of clinical management of pregnant women at delivery.

The cost of in house qPCR and culture (chromogenic agars and enrichment broths) may be comparable. Commercial qPCR (*e.g.*, 40 Euro for one GeneExpert Assay on the Cepheid Smart Cycler) is at present much more expensive than culture.

Future research should document how relevant the diagnostic value of intrapartum screening really is, compared to prepartum screening. It should be noted that our quantitative molecular data, as possible by means of qPCR, indicate that women who are positive only after Lim broth enriched qPCR are colonized by lower numbers of GBS. Future studies should clarify whether it is of clinical importance to detect also pregnant women colonized by low numbers of GBS. If it turns out that it is clinically important to detect every GBS positive woman, including those with a low colonisation load, there will be a continued need to combine molecular methods with culture-based enrichment to obtain maximum sensitivity. As a consequence, intrapartum screening, although possible because of the extremely rapid and technically non-demanding approaches, such as GeneXpert GBS assay, whereby DNA-extraction, qPCR and interpretation are reduced to a minimum of handling (101, 136), obviating the need of culture, may need to be reconsidered. It will have to be decided whether the advantages of culture-free and

thus rapid intrapartum screening outweigh the fact that a category of the carriers, i.e. the low load GBS carriers, is missed.

As mentioned in the CDC recommendation, chemoprophylaxis for prevention of GBS disease has some limitations, including the emergence of antibiotic-resistant bacteria and the occurrence of side effects associated with the administered antibiotics (59). Possibly, the continued development of strategies aiming at active maternal immunisation might offer a better solution for prevention of GBS disease. Vaccination of women could be a future alternative in preventing both early-onset and late-onset neonatal GBS infection by transplacental transfer of protective IgG antibodies (19, 141). This strategy might be effective in reducing neonatal infection and may as well have an impact on perinatal and maternal infectious morbidity (335).

Trials of protein conjugate vaccines have been performed, as reviewed by Pettersson *et al.* (278). These trials have demonstrated that these vaccines are safe, well tolerated and immunogenic, producing an increase in antibody levels known to prevent disease in 90% of the subjects. Moreover, in these trials the antibody response was observed to persist for more than a year in the mother while the passive protection in the neonate persisted for at least 3 months. The authors concluded that a large randomized trial is needed, confirming the high efficacy in reducing neonatal infection, before a vaccination strategy can be considered as an alternative in preventing GBS infection in newborns.

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Curriculum vitae

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- (1997- 2000): (part time) Teaching assistant in Medical Technology Department, Islamic University, Gaza, Palestine.
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Publications (A1)

- El Aila NA, Tency I, Claeys G, Claeys G, Cool piet, Deschaght, P. Decat, E. , Santiago GL, Verstraelen H, Temmerman M, Vaneechoutte M. Comparison of culture and different qPCR assays for detection rectovaginal carriage of Group B streptococci in pregnant women. Res. Microbiol. Accepted January 2011.
- Lopes das Santos Santiago, G., Deschaght P, El Aila NA, Kiama TN, Verstraelen H, Jefferson KK, Temmerman M, and Vaneechoutte M. 2011. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. Am. J. Obstetr. Gynecol. Accepted December 2010.
- El Aila NA, Tency I, Claeys G, Saerens B, Cools P, Verstraelen H, Temmerman M, Verhelst R, Vaneechoutte M. Comparison of different sampling techniques and of different culture methods for detection of Group B streptococci carriage in pregnant women. BMC Infect Dis 2010, 10:285.
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- El Aila NA, Tency I, Claeys G, Saerens B, De Backer E, Temmerman M, Verhelst R, Vaneechoutte M. Genotyping of *Streptococcus agalactiae* (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy. *BMC Infect Dis* 2009, 9:153.
- Kotłowski R, Shamputa IC, El Aila NA, Sajduda A, Rigouts L, van Deun A, Portaels F. PCR-based genotyping of *Mycobacterium tuberculosis* with new GC-rich repeated sequences and IS6110 inverted repeats used as primers. *J Clin Microbiol* 2004 42:372-7.
- Shamputa IC, Rigouts L, Eyongeta LA, El Aila NA, van Deun A, Salim AH, Willery E, Loch C, Supply P, Portaels F. Genotypic and phenotypic heterogeneity among *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients. *J Clin Microbiol* 2004, 42:5528-36.

Submitted manuscripts

- El Aila NA, Tency I, Claeys G, Saerens B, De Backer E, Verstraelen H, Temmerman M, Verhelst R, Vaneechoutte M. Correlation of the vaginal and rectal occurrence and bacterial load of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae*, as established by culture and qPCR. Submitted to *Research in Microbiology*

Manuscripts in preparation

- Development of real time PCR for *S. pneumoniae*.
- Adhesion to teeth and dental filling materials of potential oral probiotic strains in the prevention of caries.

Conferences and symposia

- Second Scientific Molecular Diagnostics Meeting, December 14th, 2010. **Antwerp, Belgium.**
- *16th Annual Symposium of Belgium Society of Microbiology*, BSM, “Molecular dialogue in host-parasite interaction” November 18 - 19, 2010. **Brussels, Belgium.**
- *20th European Society of Clinical Microbiology and Infectious Diseases*, ECCMID, April 10 - 13, 2010. **Vienna, Austria.**
- Advances in Genomics, Symposium, January 28-29, 2010. **Gent,, Belgium.**
- First Scientific Molecular Diagnostics Meeting, December 15th, 2009. **Ghent, Belgium.**
- *15th Annual Symposium of Belgium Society of Microbiology*, BSM, “Molecular dialogue in host-parasite interaction” December 11th, 2009. **Brussels, Belgium.**
- qPCR Congress, November 16-17, 2009. **London, UK.**
- Benelux qPCR Symposium, Gent, October 6, 2008, **Belgium.**
- Middle East Consortium on Infectious Disease Surveillance (MECIDS): *Salmonella* Identification Workshop, March 7-10, 2005. **Jerusalem, Palestine.**
- Fourth Intercountry Meeting of National Containment Coordinators for Laboratory Containment of Wild Polioviruses and Potential Infectious Materials, 7-8 December, 2004. **Muscat, Oman.**
- Regional Symposium, Improved Diagnosis, Drug Resistance Detection and Control of Tuberculosis in Latin America, 6-8 July, 2002. **Antwerp, Belgium.**
- Workshop on diagnosis of *Brucella* species, University of Crete, June, 1999. **Athens, Greece**
- Workshop on diagnosis of *Mycobacterium tuberculosis*, March, 1997. Institute of Tropical Medicine, **Antwerp. Belgium**

Posters

- El Aila NA, Tency I, Claeys G, Verstraelen H, Deschaght P, Decat E, Santiago GL, Cools P, Temmerman M and Vaneechoutte M. Evaluation of different culture media, swabs and sampling techniques for rapid detection of vaginal and rectal Group B streptococci in pregnant women. *16th Annual Symposium of Belgium Society of Microbiology, BSM*, November 18 - 19, 2010. **Brussels, Belgium.**
- El Aila, NA Tency I, Claeys G, Saerens B, Temmerman M, Verhelst R, and Vaneechoutte M. Evaluation of different culture media, swabs and sampling techniques for rapid detection of vaginal and rectal Group B streptococci in pregnant women. *20th European Society of Clinical Microbiology and Infectious Diseases, ECCMID*, April 10 - 13, 2010. **Vienna, Austria.**
- El Aila NA, Tency I, Verstraelen H Saerens B, De Backer E, Temmerman M, Verhelst R, Vaneechoutte M. Correlation of the vaginal and rectal occurrence and bacterial load of lactobacilli, *Gardnerella vaginalis* and *Atopobium vaginae* as established by qPCR. *Advances in Genomics*, January 28-29, 2010, **Gent, Belgium.**
- El Aila, NA., Tency I, Claeys G, H. Verstraelen, Saerens B, Santiago GL, De Backer E, Cools P, Temmerman M, Verhelst R, and Vaneechoutte M. Identification and genotyping of bacteria from paired vaginal and rectal samples from pregnant women indicates similarity between vaginal and rectal microflora. *Belgium Society of Microbiology meeting 11th December, 2009.* **Brussels, Belgium.**
- El Aila, NA., Tency I, Claeys G, Saerens B, De Backer E, Temmerman M, Verhelst, R and Vaneechoutte M. Genotyping of *Streptococcus agalactiae* (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy. *Belgium Society of Microbiology meeting 11th December, 2009.* **Brussels, Belgium.**
- El Aila NA, Tency I, Verstraelen H Saerens B, De Backer E, Temmerman M, Verhelst R, Vaneechoutte M. Correlation of the vaginal and rectal occurrence and

bacterial load of lactobacilli, *Gardnerella vaginalis* and *Atopobium vaginae* as established by qPCR. qPCR Congress, November 16-17, 2009, **London, UK.**

- Verhelst, R., De Backer E, Verstraelen H, Saerens B, El Aila NA, Santiago GL, Temmerman M, and Vaneechoutte M. 2008. RAPD fingerprinting of *Lactobacillus crispatus* strains in a Belgian population reveals two predominant strains. Poster at the 2nd EUPROBIO Conference. 15-17 October 2008. **Krakow, Poland.**
- Vaneechoutte M, Pirnay JP, De Vos D, T. Pitt L, Cornelis P, Pirson J, El Aila NA, S. Van daele, Schelstraete P, and De Baets F. 2007. Only a subset of *Pseudomonas aeruginosa* strains is able to colonize CF patients. Poster at 30th ECFS, June 13-16. **Belek, Turkey.**

Courses

- qPCR course from 29 May till 1 June, 2010 organized by BioGazelle, Gent University.
- Course in Statistics 'Analysis of Variance' from 24 December 2009 till 15 February, 2010 organized by Institute for Continuing Education in Science, Gent University.
- Course in Statistics 'Introductory Statistics' from 24 November till 15 December, 2009 organized by Institute for Continuing Education in Science, Gent University.
- Advanced Academic English: Conference Skills, from 2nd October till 11th December 2009, organized by the doctoral schools, Gent University.
- Advanced Academic English Writing Skills, from 2nd October till 11th December 2009, organized by the doctoral schools, Gent University.
- Project management (October 2009) organized by the doctoral schools, Gent University.
- Personal effectiveness (August/September 2009) organized by the doctoral schools, Gent University.
- Several Molecular Biology courses organized by Ghent University, 2007, (21 hours), including the following courses:
 - Biotechnological Techniques in Medical Diagnostics
 - Human Molecular Genetics
 - Host bacteria interaction
 - Molecular Medical Immunology
 - Molecular pathogenesis
 - Molecular Neurology

Memberships

- European Society of Microbiology and Infectious Diseases (ESCMID).
- Belgium Society of Microbiology (BSM).

