Infectious Diseases and Prematurity

Guest Editors: Bryan Larsen, Francesco DeSeta, Joseph Hwang, Mario Merialdi, and José Tirán-Saucedo
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Guest Editors: Paul C. Edwards, Preetha P. Kanjirath, Tarnjit Saini, and Neil S. Norton
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Researchers and clinicians have for several decades focused epidemiologic, basic biomedical and clinical investigation on preterm birth, low birth weight, and numerous sequelae of untimely birth. As the problem has been examined from the expertise of various specialists and from separate but at times overlapping disciplines, different etiologic elements have emerged as important in the genesis of prematurity. Despite continuing discoveries in specific disciplines, preterm birth has not been etiologically tied to one factor such as genetic polymorphism, microbial challenge, or immune dysregulation. In recent years, the idea of “complex disease” has been proposed as a conceptual model for conditions that involve interplay between genetic, environmental, and other factors. This seems an apt description for preterm birth which will explain the breadth of topics in this special issue.

This special issue begins by considering microbial challenges to successful pregnancy. Certainly, the concept of infectious disease as a factor in preterm birth and its sequelae must consider the microorganisms that inhabit the normal host and the way in which host immune system interacts with these microbes. The first five contributions to this issue are focused primarily on microbes that can threaten the pregnancy.

Foremost in understanding the microbial challenge to pregnancy is having the tools to appropriately characterize the maternal microbiome. In the paper by X. Zhou of Forney’s laboratory, we are reminded that only since new, culture-independent, DNA-based methods of analysis have emerged we are able to correctly characterize the lower genital flora and establish that there is no longer one single list of microbes that can be called “normal.” These findings provide important clarity to concepts regarding flora and preterm birth.

The second and third contributions in this series return to the problem represented by Group B Streptococcus (GBS). The concern for early identification and therapeutic intervention for GBS colonization in pregnancy has resulted in protocols that have become widely established. But experience over the past few decades has allowed a retrospective look at protocols again in specific clinical settings.

S. Faro et al. explore the experience of a private American hospital with respect to GBS screening and treatment. In 2 years of the study, they had an 89 percent screening rate with a 29 percent culture-positive rate with an invasive GBS incidence of 0.94 cases per 1000 live births. This is useful in underscoring the continuing need for vigilance for GBS.

The third paper in this issue, also on the topic of GBS, looks at the issue with neonates exposed or presumptively exposed to the organism. B. Buckler and coworkers suggest that some workup of asymptomatic neonates “inadequately treated intrapartum” may be more costly and no better than clinical observation and treatment as warranted. Clearly, clinicians continue to seek refinements to their approaches to individual pathogens, and while the approach to GBS may be seen by some as firmly established, some details of the approach to this organism in pregnancy are open to continued review and assessment.
Bacterial vaginosis is a topic that always emerges in discussions of preterm birth because of its epidemiologic association and the expansive literature that deals with the bacteriology and mechanistic associations with adverse pregnancy outcomes. Interest continues in finding antimicrobial approaches to the condition which in the case of the paper by I.-M. Bohbot and colleagues was aimed at clinical evaluation of secnidazole which they tested head to head with metronidazole.

The fifth paper continues the description of specific organisms of concern in infectious conditions that may threaten pregnancy. The cell-wall deficient bacteria, *Mycoplasma* and *Ureaplasma*, are frequently mentioned as having relationships to adverse pregnancy outcome, but in reality, these organisms are rarely cultured routinely in clinical practice. J. Hwang and B. Larsen have provided an update on the current understanding of these organisms in pregnancy and have reviewed research that reveals the important role of these microbes in eliciting cytokine responses that can be contributors to preterm birth.

The cytokine responses to *Mycoplasma* and *Ureaplasma* provide a nice segue into the sixth paper by J. E. Thaxton and coworkers. A cogent and timely summary of some of the important immune response mediators that can influence premature labor and delivery is provided as the conceptual connection between microorganisms and the immune responses that can lead to preterm birth. More specifically, this report provides a compelling account of the role of toll-like receptor engagement with intrauterine bacteria and the possibility of a cytokine storm involved in prematurity. The possible existence of a second uterine inflammatory pathway is an additional intriguing concept raised by this paper.

As a continuum from term birth to late preterm birth, early preterm birth is one that may consider the most significant result of infectious complications of pregnancy to be the loss of the baby, and pregnancy loss is a problem of international importance with some 4.5 million stillbirths occurring annually worldwide. Thus, it is fitting that this issue includes a paper that recognizes this problem and offers some hopeful information. A cross-sectional study of plasmodial and helminthic infections in Ghana is reported by N. Yatich and coworkers who found a higher risk of stillbirth among pregnant women with parasitic infection and suggested that some pregnancies might be salvaged with early intervention if these conditions can be recognized earlier.

The final paper in this issue by F. Omeaca et al. relates to the issues facing the preterm infant and one of the important threats to its survival and subsequent well-being. The authors explore the immune response of premature infants to hepatitis B vaccine as compared to term babies. In this report, while 6 of 94 premature infants did not respond, there were no significant differences in overall response to immunization due to low birthweight or gestational age suggesting the ability for vaccine application in this population.

The editors of this issue are pleased to offer paper that are not artificially focused but rather through the breadth of topics underscore the concept of complex disease mentioned at the beginning of this editorial. The complexity of genetics, immunity, and microbes acting in a tangled web of interactions reminds the reader why the issue of preterm birth has proven so intractable and why many different disciplines have something to contribute to the ultimate understanding of this clinical problem.

But we are hopeful that as the reader considers these varied contributions, it will be appreciated that despite the clinical descriptions and scientific discoveries, there is an underlying fact that deserves remembering. The clinical and scientific verities involved in adverse pregnancy outcomes are very personally felt by families that lose babies to infection or who have to deal with infants who are severely compromised by their prematurity. These parents undoubtedly are hopeful that the pursuits of scientists and physicians will move ever closer to practical answers to this very prevalent and vexing problem that intrudes on the happiness that is otherwise part of the birth of a child.
Recent Advances in Understanding the Microbiology of the Female Reproductive Tract and the Causes of Premature Birth

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Data derived from molecular microbiological investigations of the human vagina have led to the discovery of resident bacterial communities that exhibit marked differences in terms of species composition. All undergo dynamic changes that are likely due to intrinsic host and behavioral factors. Similar types of bacteria have been found in both amniotic fluid and the vagina, suggesting a potential route of colonization. Given that not all of the species involved in intrauterine infections are readily cultivated, it is important that culture-independent methods of analysis must be used to understand the etiology of these infections. Further research is needed to establish whether an ascending pathway from the vagina to the amniotic cavity enables the development of intrauterine infections.

1. Introduction

Preterm birth is the leading cause of neonatal mortality worldwide [1], yet the underlying etiologies remain largely unknown. Despite the implementation of many public health measures and medical interventions, preterm births continue to increase (Figure 1). A strong body of evidence suggests that intrauterine infection is an important mechanism that might account for 25–40% of preterm births [2, 3]. However, this is probably a conservative estimate because many infections are likely to be subclinical, and the pathogens responsible for these infections may be difficult to detect with conventional culture techniques [4, 5]. Furthermore, our current understanding of normal vaginal microbiota, bacterial vaginosis, and the relationship to intrauterine infection and preterm birth is limited and incomplete.

Efforts to understand the microbiology of the human vagina have been hampered in the past by the use of cultivation-based approaches that have significant limitations, and because longitudinal studies on the dynamics of vaginal bacterial communities have been lacking. Current efforts to understand the human microbiome and its role in preventing infections have entered the metagenomic era in which high throughput DNA sequencing technologies are used to characterize the diversity and function of microbial communities. Not only does this dramatically change the types of data routinely obtained from clinical samples [6], but it provides greater insight to microbial community structure, function, dynamics, and the interspecies interactions that are central to explaining how the human microbiota functions to maintain host health or predispose individuals to diseases [7–9]. We argue that in addition to the technical advances that these methodologies offer we need conceptual advances in the way these data are analyzed, interpreted, and translated into clinical practice. Through these advances our understanding of infectious processes and strategies to prevent and treat infections can be improved.

2. The Value of 16S rRNA Gene Sequence Analyses in Studies of the Human Microbiome

Our understanding of the composition of microbial communities associated with humans has been largely derived
from studies that required the cultivation of microbial populations. Hence, our current understanding of microbe-host interactions is limited because the majority of microbial species resist cultivation in the laboratory [10]. Unquestionably, the cultivation of microorganisms is essential to fully understand the physiology and phenotypic properties of organisms, and thus invaluable to clinical microbiology. However, expansive studies done to assess inter- and intraindividual variation in microbial community composition or to explore ecological relationships and answer epidemiological questions require methods that provide detailed, in-depth information about microbial diversity while being cost-effective and amenable to high throughput sample analysis. In recent years, investigators have begun to rely on culture-independent methods based on analysis of 16S rRNA gene sequences to expand our knowledge of microbial diversity. These methods circumvent the need to cultivate organisms by analyzing nucleic acids directly extracted from samples. Typically the 16S rRNA genes in samples are amplified using primers that anneal to highly conserved regions of the gene and then amplicons are sequenced. Phylogenetic analysis of the sequences obtained allows for classification of the phylotypes (e.g., species) present and is a means to identify the numerically dominant species in communities, changes in community composition that occur in responses to treatments, the influences of habits and practices, and so on. As a result, this has become the favored approach to characterizing microbial populations and communities that reside in or on the human body [11–15].

It has been estimated that one must sample 80% of the species in the community to adequately assess microbial community diversity [17]. Thus, to adequately catalog the majority of microbial taxa in diverse communities, a large number of clones must be sequenced, which is both time-consuming and costly. This high “per sample” cost has historically limited the number of samples that can be analyzed, which in turn placed severe constraints on experimental designs. To alleviate this problem, Sogin et al. [18] pioneered the use of massively parallel DNA sequencing of short, hypervariable regions of 16S rRNA genes to produce detailed surveys of communities that include low abundance taxa. Using technology originally developed by 454 Life Sciences [19, 20] and now manufactured and distributed by Roche Applied Science over 1 million DNA sequence reads can be generated in a single pyrosequencing run, and sequence read lengths are approximately 400–500 bp. To simultaneously analyze multiple samples, each sample is amplified using primers that include a unique 6-bp sequence that provides a way to bin sequences during postsequencing data analysis. Upwards of 250 samples can be analyzed in a single sequencing run, yielding 4000 sequences per sample. By simply reducing the number of samples per run, the depth of coverage can be increased. This technology circumvents traditional approaches that require cloning, which allows users to avoid biases associated with library construction. Moreover, the novel sequencing chemistry permits the sequencing of regions of DNA that have secondary structure or unusual base composition. The lower cost and higher throughput of such technology when applied to 16S rRNA gene sequence analysis affords a way to sample microbial communities at depths that are orders of magnitude greater than is possible by traditional Sanger sequencing of cloned 16S rRNA amplicons [21].

3. Profiling Bacterial Diversity in the Human Vagina Based on the Analysis of 16S rRNA Gene Sequences

3.1. Bacterial Communities in the Human Vagina. In the past 100 years since the first microbiological study of the human vagina [22], lactobacilli have been thought to be the predominant members of normal postpubertal vaginal microflora [23]. Studies reliant on the cultivation of organisms have shown that a diverse array of other bacteria such as Staphylococcus, Ureaplasma, Corynebacterium, Streptococcus, Peptostreptococcus, Gardnerella, Bacteroides, Mycoplasma, Enterococcus, Escherichia, Veillonella, and Bifidobacterium, as well as the yeast Candida [24–26] can be present but typically in much lower numbers. The species of lactobacilli that have been cultivated from vaginal samples of healthy women and identified based on phenetic characters include Lactobacillus jensenii, L. acidophilus, L. casei, L. gasseri, L. crispatus, L. plantarum, L. fermentum, L. cellobiosus, L. brevis, L. minute, and L. salivarius [27–29]. Few studies have been done to assess temporal variation in vaginal community composition within individuals, but those completed suggest that these communities are not subject to dramatic changes in healthy women, even during menses [30–32].

With advances in DNA sequencing technologies and decreased costs, our knowledge of human vaginal microbiota has greatly increased in recent years. Several studies have used culture-independent methods to characterize the vaginal microbial communities of reproductive-age, apparently healthy and asymptomatic women [33–38]. The analytical methodologies used and study designs have varied somewhat in terms of sampling different regions of the vagina, differences in the ethnic backgrounds of women sampled, the geographical location of populations, sampling times in relation to the menstrual cycle, and so on. Nonetheless, these studies are concordant in demonstrating that vaginal bacterial community composition differs both within and
between individuals and several different kinds of communities are known to exist. Thus, a more complicated picture of vaginal microbiota in healthy, asymptomatic women has been painted. For example, in a previous study we analyzed 144 vaginal samples from White and Black women, a subset of those previously collected from more than 3,000 healthy women across North America [39]. The results showed that in 80% of the women microorganisms phylogenetically related to Lactobacillus iners, L. crispatus, L. jensenii, or L. gasseri dominated sampled vaginal communities. Overall, L. iners was the most common species of Lactobacillus in women of both ethnic groups having been recovered in 66% of the women sampled. L. iners is an underappreciated member of the normal vaginal biota, as it does not grow on Rogosa agar that is typically used to isolate lactobacilli. The remainder of communities had low numbers of lactobacilli, exhibited greater species evenness, and included high numbers of clones most closely related to Atopobium and genera of the order Clostridiales, including Megasphaera, Dialister, Anaerococcus, Finegoldia, Peptostreptococcus, and Eubacterium. In addition, 20–30% of the clones from these communities were from novel clades in the phylum Firmicutes. Comparable results were obtained in a recent study of healthy, reproductive-age Japanese women [40]. The findings of these studies indicate there are a limited number of different kinds of vaginal microbial communities in asymptomatic, apparently healthy women. Moreover, from studies of adolescent women (13–15 y) [41], it appears that these communities are established in puberty and may reside in women until menopause.

Recently, we completed a more detailed and expansive study to characterize vaginal microbiota using high-throughput methods based on pyrosequencing of barcoded 16S rRNA genes [42]. The subjects were a cohort of 396 North America asymptomatic women equally representing four ethnic backgrounds (Asian, White, Black, and Hispanic). Women were recruited at three clinical sites: two in Baltimore at the University of Maryland School of Medicine and one in Atlanta, at Emory University. The participants self-identified their race. All women enrolled in the study were not pregnant, of reproductive age ranging from 12 to 45 years (mean 30.6 ± 7.32 years), had regular menstrual cycles (25–35-day menstrual cycles), with a history of sexual activity, and had not taken any antibiotic or antifungal compounds in the past 30 days. Women were asked to refrain from sexual activity in the 48 h before the visit. The vaginal swabs were self-collected by women who were not menstruating or using contraceptive devices, such as NuvaRing [42]. In total there were 282 phylotypes identified in these women. The communities clustered into five groups; four of which were dominated by Lactobacillus iners, L. crispatus, L. gasseri, or L. jensenii, while the fifth had lower proportions of lactic acid bacteria and higher proportions of strict and facultative anaerobes. This low-Lactobacillus group accounted for about 25% of the women sampled. Aside from the different Lactobacillus species, the most abundant taxa identified in the human vagina were Prevotella, Megasphaera, Sneathia, Atopobium, Streptococcus, Dialister, Lachnospira, Anaerococcus, Peptoniphilus, Eggerthella, Finegoldia, Rhodobaca, Anaerotruncus, Ureaplasma, Mycoplasma, Aerococcus, Parvimonas, Staphylococcus, Corynebacterium, Veillonella, Gardnerella, Genella, and Mobiluncus. The most commonly observed taxa in each community group are shown in Table 1. The results further showed that high bacterial species diversity was observed in all vaginal communities, even those where the phylotype abundance distribution was highly skewed and dominated by one or a very few phylotypes.

The study cohort consisted of roughly equal numbers of four ethnicities (white, Asian, black, and Hispanic), and this offered the opportunity to assess the relationship of ethnic background on vaginal bacterial community composition. The proportions of each community group varied among the four ethnic groups (Figure 2), and these differences were statistically significant \[\chi^2(10) = 36.8, P < .0001\]. No statistically significant associations were observed between age and community types within or across ethnic groups. Vaginal bacterial communities dominated by species of Lactobacillus (groups I, II, III, and V) were found in 80.2% and 89.7% of Asian and white women, respectively, but in only 59.6% and 61.9% of Hispanic and black women, respectively. We found that community group IV was overrepresented in Hispanic (34.3%) and black (38.9%) women as compared with Asian (17.6%) and white (9.3%) women. From these data we conclude that vaginal bacterial communities not dominated by species of Lactobacillus are common and appear frequently in black and Hispanic women. The data from this study are in accordance with the results of Zhou et al. [37, 39, 40], who studied the vaginal bacterial communities of white, black, and Japanese women.

3.2. Temporal Dynamics of Vaginal Bacterial Communities

Most studies of vaginal microbiology have employed a cross-sectional study design in which individuals are sampled at one discrete time point or used an interval-censored study design such that participant samples are obtained every few weeks or months [30–32, 43]. As a result little is known about the temporal dynamics of vaginal bacterial communities, and many have the mistaken impression that the composition of these communities is comparatively invariant over time, except perhaps during menstruation and following other deliberate disturbances such as sexual activity or vaginal douching. However, as our understanding of the human microbiome improves, it is becoming increasingly apparent that the bacterial communities of some habitats can markedly change over time and in response to environmental changes. For example, differences or changes in diet can have profound effects on the composition of bacterial communities of the gastrointestinal tract [44, 45]. To understand how microbial communities in the human body fluctuate in response to either defined events or stochastic processes, dynamic community profiling studies are needed [6].

Recently, we completed the sequencing of archived specimens from a longitudinal study of 33 reproductive age women who self-collected vaginal swabs every 3 days over a 16-week time period (see [46], Gajer et al. unpublished). The vaginal bacterial communities of nearly all women were dynamic and exhibited marked changes in the relative
Table 1: Most commonly observed taxa in the vaginal bacterial community groups defined by Ravel et al. [42].

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Additional notes:

- Total number of taxa observed in all samples within a group: 174, 125, 169, 232, 105.
4. The Enigma of Bacterial Vaginosis

The risk of preterm birth and low birth weight infants is markedly increased in women with bacterial vaginosis [47, 48], yet the etiology of bacterial vaginosis remains an enigma [49]. In simple terms, bacterial vaginosis is said to reflect a disturbed vaginal ecosystem in which Lactobacillus species are reduced in number and the community is “overgrown” by strictly anaerobic organisms [50, 51]. Clinically, the diagnosis of bacterial vaginosis requires three of the following four symptoms or signs [52]: (a) homogeneous, thin, white discharge that smoothly coats the vaginal walls, (b) presence of clue cells on microscopic examination, (c) pH of vaginal fluid >4.5, and (d) a fishy odor of vaginal discharge before or after addition of 10% KOH. Alternatively, bacterial vaginosis is diagnosed based on the assessment of bacterial cellular morphologies observed in samples using criteria first introduced by Spiegel et al. [53] and then modified by Nugent et al. [54]. The diagnosis of bacterial vaginosis using the Nugent criteria is based on a numerical scoring system (0–10). The score reflects the relative abundances of three kinds of bacterial cell morphotypes in Gram-stained vaginal smears, namely, large gram-positive rods (Lactobacillus), small gram-variable rods (G. vaginalis/Bacteroides spp.), and curved gram-variable rods (Mobiluncus).

The diagnostic criteria used are a critical issue in studies on the etiology of bacterial vaginosis. While numerous studies have shown that women with high numbers of Lactobacillus species generally do not have bacterial vaginosis, it is a logical fallacy to conclude that women whose vaginal communities have few or no Lactobacillus species have bacterial vaginosis. Unfortunately, this fallacy is the premise of the Nugent criteria wherein the degree of “healthiness” is largely influenced by scoring the relative abundance of Lactobacillus species with typical cell morphology. We assert that while “normal and healthy” can be equated with high numbers of lactobacilli, the converse—that “unhealthy” can be equated with low numbers of or no lactobacilli—is not necessarily true [55]. We postulate that, because of this logical fallacy, bacterial vaginosis is often over-diagnosed by Gram’s staining. This could partly account for the reported high incidence of so-called asymptomatic bacterial vaginosis in reproductive-age women [56, 57] and could also explain a proportion of bacterial vaginosis treatment failures and apparent recurrences of bacterial vaginosis in women [58, 59].

This does not deny the fact that vaginal communities of women with symptoms of bacterial vaginosis have high numbers of strictly anaerobic bacteria, many of which are various taxa that belong to the order Clostridiales. Several studies have reported this to be the case [60, 61]. We postulate that the presence of these organisms in high number is necessary but not sufficient to elicit the symptoms associated with bacterial vaginosis, and that differences in the complex of symptoms that become manifest are likely dictated by differences in the immune response of a host. This seems sensible given that disease results not only from the ill effects of microbial activities and products, but also from the nature and severity of the host immune response to the organism(s). This is apparent if one considers that the clinical diagnosis of infection depends upon the identification of the four signs of inflammation: dolor (pain), rubor (redness), calor (heat), and tumor (swelling) all of which reflect host inflammatory responses (http://www.aboutinflammation.com/fourclassicsymptoms ofinflammation.html). Thus, it is logical to suggest that when examining the vaginal habitat, clinicians might also focus on the microbe-host immune system interaction [62]. Yet, the current diagnosis of asymptomatic bacterial vaginosis relies only on the microbial component of this equation and ignores the host component. Thus it seems unreasonable that the diagnosis of bacterial vaginosis should be based solely on the absence of certain taxa (lactobacilli) and presence of others (strict anaerobes). A similar dilemma occurs in clinical medicine when asymptomatic patients present with bacteria in their urine. For women, a diagnostic...
criterion for asymptomatic bacteriuria is two consecutive midstream clean-catch urine specimens with isolation of the same species in quantitative counts of at least 100,000 CFUs per mL of urine [63]. And in the case of asymptomatic bacteriuria, there are only a few clinical circumstances in which antibiotic treatment has been shown to benefit the patient.

It is important to note in the context of asymptomatic bacterial vaginosis that studies suggest that women with low-Lactobacillus dominated microbiota (many of which would be classified as asymptomatic bacterial vaginosis) are at greater risk for adverse outcomes including STD/HIV infection upon exposure and poor obstetric outcomes [64, 65].

4.1. Normal Vaginal Microbiota. The results of studies done using cultivation-independent methods require that we revise our perceptions of the bacterial species found in the vaginas of normal and healthy women. As mentioned above, recent work by Ravel et al. [42] showed that vaginal bacterial communities could be clustered into five groups demonstrating that there is no single core microbiome. These groups can be readily distinguished on the basis of two criteria: (a) whether the constituent communities are dominated by Lactobacillus or not and (b) the particular species of Lactobacillus present. In the past it has been claimed that the vaginal bacterial communities of healthy women are dominated by species of Lactobacillus that produce hydrogen peroxide [49, 51, 66]. This appears to be true for some but not all women. The most common communities are dominated by L. iners, a species that can be characterized by the inability to produce hydrogen peroxide [23]. Moreover, not only does this organism resist cultivation on commonly used media (which probably accounts for its absence from most surveys done in the past that relied on cultivation), but the cell morphology is atypical by being about 50% smaller (length and width) than the other species of Lactobacillus common to the human vagina (Yuan and Forney, unpublished). This could confound the diagnosis of bacterial vaginosis based on Nugent criteria. The fifth group of vaginal communities found in asymptomatic women is heterogeneous in terms of species composition and typified by a dearth of lactobacilli and higher proportions of strictly anaerobic bacteria including Prevotella, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Sneathia, Eggerthella, Aerococcus, Finegoldia, and Mobiluncus. A large proportion (27%) of White, Black, Hispanic, and Asian women in North America have vaginal communities that cluster within this group, and they are particularly frequent in Hispanic and Black women (38 and 40%, resp.; Figure 2). The fact that these communities are not dominated by species of Lactobacillus has led some to presume that these women have bacterial vaginosis [50, 56]. We postulate that these asymptomatic women with vaginal communities lacking appreciable numbers of lactobacilli may be misdiagnosed as having bacterial vaginosis if Nugent criteria are used. The fact that these communities appear to reflect a natural state and not disease might account for the high recurrence rates and spontaneous cure rate for asymptomatic bacterial vaginosis that have been observed [58, 59, 67, 68]. If this is the case, then vaginal bacterial communities that lack lactobacilli may simply represent another difference found among individuals and highlight the importance of personalized medicine wherein differences among individuals are respected.

There is a widespread discussion over whether asymptomatic gynecologic patients with bacterial vaginosis should be treated [69] since the risk of therapy must be weighed against the benefit to patients, and there is increasing awareness of the need to restrict antibiotic use so as to avoid selection for antibiotic resistance [70, 71]. The arguments presented above suggest that the use of antibiotics for the treatment of asymptomatic bacterial vaginosis might not be sensible since disturbance of a natural state is the “cure” one would be attempting to affect. It should be noted that these communities may tend to revert to their original state once antibiotic therapy has been completed, and this could well account for a portion of the so-called treatment failures that are observed in trials done to assess the efficacy of antibiotic therapy for curing bacterial vaginosis [58, 72].

4.2. Treatment of Asymptomatic Bacterial Vaginosis in Pregnant Women. Controversy surrounds whether pregnant women should be screened for the occurrence of asymptomatic bacterial vaginosis and treated with antimicrobial agents to prevent preterm birth. The basic rationale for screening and treatment is that bacterial vaginosis is associated with intra-amniotic infection and therefore is considered a risk factor for preterm delivery. However, evidence compiled in the Cochrane Reviews does not support the concept of widespread screening for bacterial vaginosis and treatment to prevent premature delivery [73–75]. It is important to note that a history of a prior preterm birth is the most significant clinical factor in identifying women with a propensity for preterm labor and delivery [76, 77]. The Centers for the Disease Control, as well as prominent leaders in the field of infectious disease in obstetrics and gynecology, have recommended that only patients at high risk for preterm delivery—specifically only those with a previous history of a spontaneous preterm birth—should be treated with antibiotics if they are found to have bacterial vaginosis [62].

5. Intrauterine Infection and Preterm Birth

Intrauterine infections are a frequent and important mechanism leading to preterm birth. Intrauterine infection begins in the decidua (uterine lining), extends to the space between the amnion and chorion, and finally reaches the amniotic cavity and fetus [2]. Bacteria have been cultured from the chorioamnion in 15% of nonlaboring women with intact membranes who are undergoing caesarean delivery [2]. Likewise, half of all placentas delivered before the end of the second trimester have been shown to harbor bacteria in the chorion as detected by culture [78]. The prevalence of infection is found to be even higher when molecular
methods are used to detect bacteria. When fluorescence in-situ hybridization is done using a DNA probe specific for a conserved region of the bacterial 16S rRNA gene, then bacteria are found in the membranes of up to 70% of women undergoing elective caesarean section at term [79]. Since these were not cases of preterm birth, these findings suggest that the presence of bacteria in the chorioamnion alone is not always sufficient to cause an inflammatory response that leads to preterm labor and preterm birth. In contrast, an inflammatory response is observed in the amniotic fluid of more than 80% women in early preterm labor with intact membranes. Based on these data, it seems there are two conditions essential for intrauterine infections to cause preterm birth. First, the infectious organisms must enter the amniotic cavity and be recognized as foreign by the host immune system. Second, the bacterial numbers must breach some threshold to trigger an intra-amniotic inflammatory response, which in turn induces preterm labor [80].

5.1. Bacterial Species Found in Amniotic Fluid. *Ureaplasma urealyticum, Fusobacterium* sp., and *Mycoplasma hominis* are the bacterial species most commonly isolated from the amniotic cavity of women with preterm labor and intact membranes [81, 82]. Other microorganisms found in the amniotic fluid include *Streptococcus agalactiae, Peptostreptococcus* sp., *Staphylococcus aureus, Gárdenerella vaginalis, Streptococcus viridans,* and *Bacteroides* sp. Occasionally, *Lactobacillus* sp., *Escherichia coli,* *Enterococcus faecalis,* *Neisseria gonorrhoeae,* and *Peptococcus* sp., while *Haemophilus influenzae, Capnocytophaga* sp., *Stomatococcus* sp., and *Clostridium* sp. are rarely recovered [83–85]. More than one microorganism is isolated from 50% of patients in which the amniotic cavity is infected [79].

In a recent, very comprehensive analysis, DiGiulio and colleagues characterized bacterial 16S rRNA gene sequences in amniotic fluid from women in preterm labor [5]. They found 18 taxa in the amniotic fluids using molecular methods of analysis while only 11 taxa were recovered using culturing methods. In addition, 9 samples were positive only by PCR amplification of 16S rRNA genes, indicating that false negative results can be obtained by using only cultivation methods. In this study, *Mycoplasma* sp., *Ureaplasma* sp., *Streptococcus* sp., *Lactobacillus* sp., *Prevotella* sp., *Delftia* sp., *Neisseria* sp., *Fusobacterium* sp., *Sneathia* sp., and *Leptotrichia* sp. were found in amniotic fluids. In another study by Han et al. [86], twice the numbers of bacterial taxa were isolated in the amniotic fluids of preterm delivery patients using cultivation-independent methods as compared to cultivation-dependent methods. Most taxa were similar to those of DiGiulio’s study, but in addition *Shigella* sp., *Bacteroides* sp., *Bergeyella* sp., and *Peptostreptococcus* sp. were observed. While some bacteria in amniotic fluid have been associated with skin, fecal, and gut microbiota, most are related to those found in the human vagina. This suggests a potential connection between the bacterial species in amniotic fluid with those in the vagina, with the latter being a potential source of infecting organisms [16] (see Figure 3).

Figure 3: Potential routes of intrauterine infection (Source: Goldenberg et al. [16])

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**References**


Research Article

Screening for Group B Streptococcus: A Private Hospital’s Experience

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Objective. To assess the effect of universal screening and administration of intrapartum antibiotic prophylaxis to prevent early-onset neonatal GBS sepsis at a private tertiary care hospital since issuance of the 2002 CDC guidelines for preventing perinatal GBS disease.

Methods. Retrospective analysis of women delivering between January 1, 2003 and December 31, 2004 at a private tertiary care hospital in Houston, Texas. The percentage of women screened, GBS positive women receiving intrapartum antibiotic prophylaxis, and infants developing early-onset GBS sepsis were determined.

Results. 2,108 women delivered 2,135 infants with 1,874 (89%) screened for GBS. Of those screened, 1,322 (71%) tested negative and 552 (29%) tested positive for GBS. In this analysis of 2,135 infants, 3 (0.94 cases/1,000 live births) were diagnosed with invasive GBS sepsis.

Conclusion. High rates of screening of pregnant women for GBS colonization and use of intrapartum antibiotic prophylaxis for GBS carriers can be achieved in a private tertiary care hospital setting. “Synopsis: High screening rates for group B streptococcus in a private tertiary care hospital reduce the incidence of maternal and early onset neonatal GBS infection.”

1. Introduction

Streptococcus agalactiae (group B streptococcus (GBS)) is one of the most common bacterial causes of life-threatening infection in newborns [1, 2]. GBS was first reported as a human pathogen in 1938, but it was not until the 1970s that GBS was described as a major pathogen responsible for neonatal sepsis, pneumonia, and meningitis [3]. GBS neonatal infection is divided into two categories: early-onset disease, which occurs within the first week of life, and late-onset infection, which occurs between one week to 3 months of age [4]. GBS vaginal colonization occurs in 4% to 40% of pregnant and nonpregnant women and appears to be dependent upon geographical location [5–9]. The GBS bacterium also may lead to chorioamnionitis, myonecrosis of the uterus, neonatal pneumonia, premature delivery, premature labor, premature rupture of amniotic membranes, postpartum endometritis, and septic abortion [10–14]. Furthermore, both mother and newborn infant may experience bacteremia, which can cause both septic shock and death.

Prior to extensive prevention efforts in the 1990s, the incidence of invasive neonatal GBS infection ranged from 2 to 3 cases per 1,000 live births [12]. In 1996, the Centers for Disease Control and Prevention (CDC) issued guidelines recommending the use of intrapartum antibiotic prophylaxis and by 1999, the incidence of early-onset GBS infection was reduced to 0.5 cases per 1,000 live births [12].

In 2002, in order to further decrease the incidence of GBS sepsis, the CDC issued revised guidelines that recommended universal screening of pregnant women between 35 and 37 weeks of gestation [12]. However, GBS infection continues to be a considerable problem, causing significant morbidity and mortality in mothers and their newborn infants. The CDC reported for the period 2000–2002, the average early-onset
disease incidence was 0.49 cases per 1,000 live births [14]. Following the revised CDC recommendations in 2002, average early-onset disease incidence decreased to 0.33 cases per 1,000 live births [14].

The purpose of this retrospective study was to determine the screening rate for GBS, the incidence of maternal GBS colonization and early-onset neonatal GBS sepsis. In addition, we determined compliance with the CDC recommendation for the use of intrapartum antibiotic prophylaxis for GBS positive women in a private tertiary care hospital after the 2002 CDC recommendation for universal screening of pregnant women for GBS colonization.

2. Materials and Methods

The study was conducted at The Woman’s Hospital of Texas (WHT), Kelsey-Seybold Clinic (KSC), and the Kelsey Research Foundation (KRF). The WHT is a private tertiary care hospital with private physicians, a level III nursery that accepts maternal-fetal transfers, and the only specialty hospital in Houston, Texas focused on the care of women and infants. Approximately 22% of all WHT deliveries each year are high risk. KSC is a large, multispecialty medical clinic with 300 physicians that serves an ethnically diverse population of over 400,000 patients at 18 locations in Houston, Texas. The KRF is a 501 (c) (3) nonprofit that collaborates with healthcare and research institutions in the Texas Medical Center to conduct health services research, provide patient education, and develop quality improvement initiatives.

Since 1995, the KRF has maintained a database of clinical information from both WHT and KSC about the pregnancy experience of over 20,000 women at KSC. The database contains information describing the pregnancy experience and outcomes of mothers and infants who received care at WHT and KSC. Data for all (9) obstetricians at KSC who routinely performed deliveries at WHT are included in this study.

The database was queried to identify the number of deliveries performed during the two-year period (January 1, 2003 through December 31, 2004). Outcome variables included the number and percentage of pregnant mothers screened for GBS, the GBS status of those women screened, the rate of intrapartum antibiotic usage, and the incidence of early-onset GBS sepsis. Sepsis is defined as a positive blood or spinal culture, or both, in addition to the clinical signs and symptoms of infection. Demographic variables, including age, ethnicity, and insurance status, also were collected from the administrative database. Data was analyzed using Microsoft EXCEL and ACCESS (2003, version 11.6355.6360 SP1) software. Because this is a retrospective analysis, waiver of consent was approved by the Institutional Review Board of WHT.

At 35 to 37 weeks gestation, a standard cotton aerobic bacterial culture swab was gently inserted into the lower third of the vagina and then in a single motion, the perineum was swabbed and the external anal sphincter was swabbed. The swab was then placed in Stuart’s transport medium and sent to the laboratory at room temperature. All specimens were sent to a commercial laboratory as directed by patients’ insurance providers. A screening culture was completed on arrival in labor and delivery if pregnancies were <35 weeks. If GBS bacteriuria was diagnosed during a pregnancy, the patient was considered colonized and treated during labor as per the CDC guidelines [12]. All women who had GBS positive cultures were given antibiotic prophylaxis in labor as recommended by the CDC guidelines [12]. Mothers allergic to penicillin were treated according to 2002 CDC guidelines [12].

3. Results

For the two-year study period, 2,108 women delivered 2,135 infants. Of these 2,108 deliveries, 8% were less than 37 weeks gestation. The mean gestational age at delivery was 38 ± 2.31 weeks. There were 39 sets of twins (1.8%), 20 infant deaths (0.9%), and 171 (8%) premature deliveries. (Prematurity is defined as infants born at a gestational age less than 37 weeks.)

The population was 40% African-American, 25% Caucasian, 28% Hispanic, 5% Asian, and 2% other. Approximately 3% of the women in the sample were less than 18 years of age, 56% were 18–30 years of age, 39% were 31–40 years of age, and 2% were 41 or older. The majority (71%) of women were insured through an HMO, while 27% had coverage with a PPO, 2% with Medicaid, and fewer than 1% were self-insured. The demographics of mothers who were GBS positive, GBS negative or those receiving intrapartum antibiotic prophylaxis were comparable to the demographics of the study sample. Chi-square tests were performed and there were no statistical differences in the subgroups.

Among the 2,108 mothers, 1,874 (89%) were screened for GBS and, of these, 1,322 (71%) tested negative and 552 (29%) tested positive for GBS. There were 231 (11%) of the 2,108 mothers with an unknown GBS status.

Thirty-two (1.5%) of the 2,135 infants in the sample were evaluated for sepsis during their hospitalizations. The sepsis workup included a CBC, blood culture, and lumbar puncture for spinal fluid analysis and culture. Gestational age for 21 (66%) of these infants with sepsis was <30 weeks, for 8 (25%), gestational age was 30 to 36.7 weeks, and for 3 (9%), gestational age was ≥ 37 weeks. These infants were cultured for sepsis at birth and received antibiotics until results were available. For the majority (30) of these infants, culture results were negative and antibiotics were discontinued. For the two culture positive infants, one had positive blood cultures for GBS and the other had positive blood cultures for coagulase negative staphylococci, and none had meningitis. Thirty-one of the 32 (97%) infants were admitted to the NICU and had admissions of more than 30 days. Of these infants, one developed GBS sepsis.

Of the 1,322 mothers who tested negative for GBS, 346 (26%) received antibiotic prophylaxis. Of the 346 that received antibiotics, 314 (91%) received antibiotics for surgical prophylaxis (C-section) and 32 (9%) received antibiotics for acute infection (chorioamnionitis, pylephritis, and
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Flow diagram

Women in the study
($n = 2,108$)

- Known GBS result
  ($n = 1,874$)
  - GBS negative
    ($n = 1,322$)
    Antimicrobial
    - Prophylactic ($n = 314$)
    - Infection ($n = 32$)
  - GBS result unknown
    ($n = 234$)
    - Antibiotic ($n = 165$)
    - No antibiotic ($n = 69$)

- GBS positive
  ($n = 552$)
  - Antibiotic
    ($n = 523$)
  - No antibiotic
    ($n = 29$)

- GBS infants
  ($n = 3$)

Figure 1

Respiratory infection). Among the 314 women who received antimicrobials for surgical prophylaxis, 43 (14%) delivered vaginally. Interestingly, these 43 women, although initially GBS negative by culture at 35–37 weeks, met the CDC risk-based criteria for GBS prophylaxis when admitted to labor and delivery.

A total of 523 (95%) of the 552 mothers who were GBS positive received intrapartum antimicrobial prophylaxis. The three GBS positive infants were born to GBS positive mothers and all three had a gestational age <36 weeks (33.5, 32.5, and 25.2 weeks). The mother who delivered her baby at 33.5 weeks had severe chorioamnionitis with artificial rupture of membranes and delivered vaginally on the day she was admitted. She received prophylactic ampicillin and subsequently tested positive for GBS. The second mother delivered twins at 32.5 weeks by repeat C-section on the day she was admitted. The mother had premature rupture of membranes with GBS status unknown at time of delivery and subsequently tested positive for GBS. One twin was GBS positive and had intrauterine growth retardation (IUGR). The third GBS positive mother delivered her baby at 25.2 weeks by primary C-section. The mother was admitted eight days prior to delivery and diagnosed with preterm premature rupture of membranes, and chorioamnionitis. She received ampicillin/sulbactam, Amoxicillin, and Erythromycin antepartum, and Cefazolin at the time the umbilical cord was clamped. The infant, in addition to being GBS positive with bacteremia, subsequently was diagnosed with Klebsiella pneumonia and expired 14 days after delivery.

A total of 165 (71%) of the 231 mothers with unknown GBS status received antibiotic prophylaxis for GBS based on weak risk factor assessment. Of these 165 mothers, 106 (64%) delivered babies at <37 weeks gestational age, 1 (0.9%) had rupture of membrane with labor >18 hours, there were no mothers in this group with a temperature >38°C, and 105 (99%) had no recognized risk factors as outlined in the CDC guidelines [12] except for unknown GBS status. The flow diagram in Figure 1 shows the status of the women in the study.

4. Discussion

Our study demonstrates that in a private tertiary care hospital setting, both a high rate of screening for GBS and administration of intrapartum antimicrobial prophylaxis can be achieved. No infants ≥37 weeks gestation developed invasive GBS disease. The three infants with early-onset GBS sepsis born to GBS positive mothers were less than 34 weeks gestation, and they would have been treated based on risk factors alone. The results for this study population are reflective of the overall population delivering at WHT.
A major strength of this study is our ability to capture data on GBS screening and usage of intrapartum antibiotic prophylaxis from our obstetrical database. Similar surveillance studies conducted by the CDC have been limited due to their inability to measure health care provider compliance with screening guidelines [14]. Despite our high rate of GBS screening, 11% of mothers had unknown GBS status at delivery. However, more than two-thirds of these women received prophylaxis for GBS based on risk factors alone. An additional strength is that the results reflect the management of GBS in a private tertiary care nonteaching obstetrical unit, with 80 practicing obstetricians, versus an academic hospital. The widely diverse nonhomogeneous patient population speaks to the nondiscriminatory nature of GBS colonization.

The retrospective nature of the study may be a weakness because the study sample was not controlled and was limited to a small proportion of the total deliveries at WHT. However, the study sample did represent 42% of all KSC deliveries during the two-year study period.

Another weakness is that there were 11% (231) of mothers that were either not screened or data were not available. If a GBS positive rate of 29% is used, then this would contribute an additional 70 women to the GBS positive group.

A confounding finding was that there were three newborns who developed early-onset GBS sepsis born to GBS positive mothers that were administered appropriate intrapartum antibiotic prophylaxis. It is not known whether or not GBS amnionitis was present at the time of admission to labor and delivery. None of these women had clinical signs or symptoms of infection. This emphasizes the fact that universal screening and intrapartum antibiotic prophylaxis cannot eliminate the occurrence of neonatal early-onset GBS sepsis.

Based on our experience reviewing GBS in mothers, we have undertaken an extensive review of neonatal outcomes in newborns of mothers receiving antibiotic prophylaxis within 4 hours of delivery. We also are developing a study to determine whether rapid screening using the PCR method in untested and GBS negative mothers will further reduce GBS infection in newborns.

5. Conclusion

This study found that adherence to the 2002 CDC guidelines for screening and prophylaxis for GBS could readily be accepted, and that in the first year of implementation, 89% of women delivering in a private tertiary care hospital with a large number of practicing obstetricians were screened. This retrospective study demonstrated that universal screening for GBS is effective and can be achieved in a private tertiary care hospital.

Acknowledgments

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References

Research Article

Unnecessary Workup of Asymptomatic Neonates in the Era of Group B Streptococcus Prophylaxis

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Asymptomatic term neonates born to mothers who are Group B Streptococcus (GBS) unknown or GBS positive but “inadequately” treated prior to delivery do not require invasive laboratory evaluation. We conducted a retrospective cohort study of mother/baby dyads born from January 1, 2005 until September 30, 2007 at the Medical College of Georgia. Their current protocol is to obtain a Complete Blood Count with Differential (CBC with D), Blood Culture (BC), and C-reactive protein (CRP) after birth.

Mother/baby dyads (n = 242) that met inclusion criteria were reviewed. Of these 242 babies 25 (10%) were started on antibiotics after the initial lab values were known. None of the blood cultures were positive and the CRP’s were normal. The 2002 GBS guidelines call for laboratory evaluation of “at-risk” neonates, but the workup of these babies is not only costly, it does not provide any advantage over old fashioned clinical observation for the evaluation and treatment of early onset GBS sepsis.

1. Introduction

Group B Streptococcus (GBS) is a gram-positive cocci that may be found in the gastrointestinal tract, respiratory tract, urinary tract, and genital tract [1]. It is one of the most common causes of early onset neonatal sepsis, which occurs within the first 6 days of life. Approximately 30% of women have asymptomatic GBS colonization at some point during their pregnancy, and about 20% remain colonized at the time of birth [1]. GBS has nine serotypes that have been identified, which are differentiated by the polysaccharide capsule of the organism. Types I, II, and III are most commonly associated with neonatal sepsis, with type III being highly associated with central nervous system involvement [2].

Asymptomatic term neonates born to mothers who are GBS unknown or GBS positive but “inadequately” treated prior to delivery do not require invasive laboratory evaluation [1, 3]. This evaluation may include a Complete Blood Count with Differential (CBC with D), a Blood Culture (BC), and a C-reactive protein (CRP). The Center for Disease Control (CDC) 2002 GBS guidelines call for a laboratory evaluation of “at-risk” neonates, but they do not provide data to the usefulness of this practice [4].

Laboratory evaluation of these babies is low yield, costly, and disrupts mother baby bonding soon after birth. These babies can be appropriately managed with careful clinical observation for signs and symptoms of sepsis. Our primary outcome was to determine the number of cases of sepsis that were diagnosed based on these screening practices.

2. Materials and Methods

We conducted a retrospective cohort study of mother/baby dyads born and cared for in the newborn nursery at the Medical College of Georgia (MCG) in Augusta, Georgia. Our study was from January 1, 2005 until September 30, 2007. During this time period 5,342 babies were born. Inclusion criteria consisted of babies born at term (>37 weeks completed gestation), mother’s GBS status unknown or positive
at the time of delivery, no antibiotics or antibiotics less than 4 hours prior to delivery, and a laboratory evaluation upon admission to the newborn nursery. There were no exclusion criteria.

The current protocol in the newborn nursery is to obtain a Complete Blood Count with a Differential (CBC with D), a Blood Culture (BC), and a C-reactive protein (CRP) immediately after birth. Then based on the results of these lab tests and the discretion of the attending the decision about whether to start antibiotics is made.

The study was approved by the Institution Review Board at the hospital.

3. Results

During the study period there were 242 mother/baby dyads that met the inclusion criteria. Of these 242 babies only 25 (10%) were started on antibiotics. The decision to start antibiotics was made by an attending general pediatrician based on the initial lab values. The antibiotic regimen was Amoxicillin 100 mg/kg/dose every 12 hours and Gentamycin 4 mg/kg/dose every 24 hours. Antibiotic therapy was discontinued after 48 hours on 23 out of the 25 babies that were started on antibiotics, and the other 2 babies received 7 days of antibiotics for signs of clinical sepsis. Both of these babies were in the GBS positive No antibiotics group, and both were discharged in good health after completing their antibiotic course. None of the 242 babies had a positive blood culture and the C-reactive protein levels were normal.

4. Discussion

In this era of intrapartum prophylaxis for early onset GBS disease, we have seen a decline in the rate of early onset GBS disease from 0.6 per 1000 in live births in 2000 to 0.39 per 1000 live births in 2006 [5, 6]. The 2002 CDC guidelines have been effective at reducing the incidence of early onset GBS sepsis. However, the guidelines do not address or give support to the “limited evaluation” of asymptomatic babies. Safier et al. estimated that based on the current rate of GBS disease it would take about 10,000 blood cultures to identify 1 case of GBS sepsis [7]. Ottolini et al. found similar findings, as they did not have a positive blood culture in 1665 “at risk” term newborns [3]. In our institution, if a baby is delivered and the mother’s GBS status is unknown or a maternal transfer without her complete medical record, then the obstetricians risk stratify the mother per the 2002 CDC guidelines [4]. If she does not meet the criteria for intrapartum prophylaxis, then she does not receive antibiotics. The problem arises in that the pediatricians treat an unknown GBS status as potential positive, and even though the mother appropriately does not receive antibiotics, the baby is subjected to a laboratory evaluation. The disconnect between the obstetricians and the pediatrician causes serious questions for these mothers who have just given birth. Many times the pediatricians tell the mother that since the GBS status was not known and she did not receive antibiotics that her baby will require an invasive laboratory evaluation. The mother then questions her obstetrician as to why she was not given antibiotics. This has led to obstetricians to give antibiotics to mothers who do not meet criteria based on the 2002 CDC guidelines in order to keep the pediatricians from subjecting asymptomatic neonates from this invasive evaluation. Another factor is the 4-hour window required for appropriate treatment of intrapartum antibiotics from the time the first does of antibiotics is given until the delivery [4]. More than half of our babies were evaluated solely based on the fact that the delivery occurred prior to this 4-hour cutoff. There have been several studies that have questioned this 4-hour window [8, 9]. Bloom et al. showed that bactericidal concentrations of ampicillin could be achieved within 5 minutes after infusion in both maternal and umbilical cord sera [10]. The New Zealand GBS Consensus Working Party that “well appearing babies born >35 weeks gestation to women with GBS risk factors who have received either no or inadequate (<4 hours) chemoprophylaxis should be observed closely in hospital for at least 24 hours” [11].

5. Conclusions

In conclusion, we feel that clinical observation, rather than an in-depth laboratory workup is sufficient for the evaluation of asymptomatic, GBS “at-risk” neonates. The results of our study support the conclusion that subjecting asymptomatic neonates to multiple blood draws and invasive laboratory procedures is low yield. Furthermore, we feel it is costly and disrupts maternal/child bonding. We recommend, rather, that the clinician should implement serial examinations for the observation of sepsis. Laboratory exams should be initially withheld, though implemented if clinical suspicion warrants. We feel this approach is more cost effective and

Table 1: Number of babies treated based on mother’s GBS status and doses of antibiotics given prior to delivery.

<table>
<thead>
<tr>
<th>GBS Status</th>
<th>No. of Babies</th>
<th>No. Babies treated with antibiotics</th>
<th>Positive cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBS unknown no antibiotics</td>
<td>36</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>GBS unknown 1 dose antibiotics</td>
<td>25</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>GBS positive No antibiotics</td>
<td>51</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>GBS positive 1 dose Antibiotics</td>
<td>130</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>242</strong></td>
<td><strong>25</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>

Table 2: Lab results for babies not treated with antibiotics after delivery and babies treated with antibiotics after delivery.

<table>
<thead>
<tr>
<th>Test</th>
<th>No antibiotics</th>
<th>Yes antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell count</td>
<td>19.2</td>
<td>20.9</td>
</tr>
<tr>
<td>I: T ratio</td>
<td>0.09</td>
<td>0.30</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>0.28</td>
<td>0.68</td>
</tr>
</tbody>
</table>
less invasive while providing the same level of care. Further studies are needed to provide data to further stratify the need for invasive examination of asymptomatic Group-B Strep “at-risk” neonates.

References


Clinical Study

Treatment of Bacterial Vaginosis: A Multicenter, Double-Blind, Double-Dummy, Randomised Phase III Study Comparing Secnidazole and Metronidazole

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Objective. Multiple-dose metronidazole oral therapy is currently the reference treatment for bacterial vaginosis (BV). This double-blind, double-dummy, noninferiority study compared the efficacy of secnidazole, another nitroimidazole with pharmacokinetics allowing a single dose regimen, to this standard treatment.

Methods. A total of 577 patients were randomized to receive metronidazole (500 mg, b.i.d for seven days) or secnidazole (2 g, once). Therapeutic cure at D28 was defined as the resolution of vaginal discharge, positive KOH whiff test, vaginal pH > 4.5 and Nugent score > 7 on Gram-stained vaginal fluid.

Results. According to this primary endpoint, the single-dose secnidazole regimen was shown to be at least as effective as the multiple-dose metronidazole regimen (60.1% cured women vs 59.5%, 95% confidence interval with a noninferiority margin of 10%: [−0.082; 0.0094]). Safety profiles were comparable in both groups. Conclusion. The secnidazole regimen studied represents an effective, convenient therapeutic alternative that clinicians should consider in routine practice.

1. Introduction

Bacterial vaginosis (BV) is a common cause of vaginal discharge, occurring in up to 30% of women [1]. It is associated with a complex change in vaginal flora including a decrease in normal Lactobacillus spp., and an increase in Gardnerella vaginalis and anaerobes [1, 2].

Essentially characterized by a fishy-smelling, thin, greyish vaginal discharge [2], BV is clinically diagnosed using the Amsel criteria, including four parameters: presence of the typical discharge, a vaginal pH > 4.5, a positive whiff test (amine odour after adding 10% potassium hydroxide to vaginal fluid) and the presence of “clue cells” (epithelial cells with adhering bacteria) on microscopic examination. At least three out of these four criteria have to be present [2]. The diagnosis may be confirmed by a Nugent score equal or higher than seven on bacteriological analysis of vaginal samples.

Besides being unpleasant for patients when symptoms of discharge and odour occur, BV is associated with an increased risk of several pathological gynaecological conditions as well as major adverse outcomes during pregnancy. It is estimated that BV is associated with a twofold increased risk of preterm birth (odds ratio: 2.4; 95% CI: 1.2–4.8) and a sixfold increased risk of miscarriage (odds ratio: 6.6; 95% CI: 2.1–20.9) [3].

Although the pathogenesis of BV is still not clearly understood and the aetiological role played by the organisms replacing the normal aerobic vaginal flora remains uncertain, antibiotics with good activity against anaerobes but that do not affect Lactobacillus spp. represent the mainstay of BV therapy [2]. Metronidazole, administered either orally or topically according to multiple-dose regimens, has long been established as a standard treatment of BV [4–6]. However, a drawback of these regimens is the necessity to administer them for several days, potentially diminishing compliance...
with a risk of incomplete cure and recurrence of BV [7].

Secnidazole is a new second-generation 5-nitroimidazole product with a broad spectrum of activity against anaerobic bacteria and a longer half-life than metronidazole, making it suitable for single-dose therapy, and therefore potentially offers an advantage over multiple-dose metronidazole regimens. Several studies have consistently suggested its clinical benefits for the treatment of BV [8–10]. However, their methodology was somewhat questionable and there was a need to investigate the efficacy of secnidazole in a well-designed study satisfying the most recent guidelines like those issued by the Food and Drug Administration (FDA).

2. Materials and Methods

This was a national, multicentre, prospective, randomised, comparative, double-blind, double-dummy, Phase III, non-inferiority study comparing the efficacy of secnidazole versus metronidazole in patients with bacterial vaginosis. Nonpregnant women aged 18–65 years with clinical signs of bacterial vaginosis, and from whom a vaginal sample had been collected at the preinclusion visit, were eligible for enrolment. The clinical diagnosis of BV was established on the basis of the following three Amsel criteria: a homogeneous, thin, greyish-white vaginal discharge, positive potassium hydroxide whiff test results, and a vaginal pH above 4.5 [11]. The diagnosis of BV was later confirmed by a Nugent score above seven on bacteriological analysis of the preinclusion vaginal samples. Patients were excluded if they had received antibiotic or antifungal drugs within the past 14 days. The study was approved by the Ethics Committee of Kremlin Bicêtre Hospital (France) and all women gave written informed consent before starting the study.

2.1. Study Design. After baseline screening, patients were randomised to metronidazole (reference treatment) or secnidazole (study treatment) in a 1:1 ratio. Randomisation was stratified by clinical centre; the randomisation list was computer-generated (SAS software) with block sizes of four (two secnidazole, two metronidazole). Investigators and patients were blind to study treatments. After randomisation, patients received either a single 2 g dose of secnidazole or the reference treatment, that is, a seven-day course of 500 mg metronidazole twice daily. In view of the difference in pharmaceutical form and administration regimen between metronidazole (capsules, multiple-dose) and secnidazole (sachet, single-dose), the trial was designed as a double-dummy study. According to the randomisation schedule, patients received either the study treatment and the placebo of the reference treatment or the reference treatment and the placebo of the study medication (one sachet on Day 1, and two capsules per day from Day 1 to Day 7). The patients attended two follow-up visits, at day 14 (D14) ± 2 days and at day 28 (D28) ± 2 days. At each visit, a clinical examination was performed and vaginal samples were taken.

2.2. Endpoints. In accordance with the current FDA guidelines, the primary efficacy endpoint was the therapeutic success, that is, a composite of clinical and bacteriological cure, at D28 [12]. Clinical cure was defined as the normalisation of the three Amsel criteria and bacteriological cure was defined as a Nugent score lower or equal than three. The secondary efficacy criteria were therapeutic success at D14, clinical cure at D14 and D28, bacteriological cure at D14 and D28, mean time to symptom disappearance, and safety. Patients had to complete a daily questionnaire from D1 to D14, recording the intensity of any vaginal discharge (severe, moderate or absent) and the existence of an unpleasant odour (yes/no). Safety was assessed on the basis of adverse events reported.

2.3. Populations. The primary efficacy endpoint was analysed on the intention-to-treat (ITT) population, including all

![Table 1: Major deviations leading to the exclusion of patients from the per protocol population.](image)

<table>
<thead>
<tr>
<th>Description</th>
<th>Metronidazole n = 287</th>
<th>Secnidazole n = 290</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonrespect of scheduled visits</td>
<td>4.9 (14)</td>
<td>3.8 (11)</td>
</tr>
<tr>
<td>Nonevaluability of primary efficacy endpoint</td>
<td>4.5 (13)</td>
<td>1.7 (5)</td>
</tr>
<tr>
<td>Concomitant antibiotic treatment</td>
<td>3.5 (10)</td>
<td>2.1 (6)</td>
</tr>
<tr>
<td>Nonrespect of compliance</td>
<td>2.4 (7)</td>
<td>1.4 (4)</td>
</tr>
<tr>
<td>of which missing data on compliance</td>
<td>1.4 (4)</td>
<td>1.4 (4)</td>
</tr>
<tr>
<td>Error in randomisation</td>
<td>1.4 (4)</td>
<td>1.4 (4)</td>
</tr>
<tr>
<td>Treatment initiated more than 3 days after randomisation</td>
<td>1.0 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nonrespect of inclusion criteria</td>
<td>0.3 (1)</td>
<td>0.7 (2)</td>
</tr>
<tr>
<td>Vaginal treatment</td>
<td>0 (0)</td>
<td>0.7 (2)</td>
</tr>
<tr>
<td>Nonrespect of exclusion criteria</td>
<td>0 (0)</td>
<td>0.3 (1)</td>
</tr>
</tbody>
</table>

Table 2: Overall therapeutic success at D28 (primary efficacy endpoint).

<table>
<thead>
<tr>
<th>Population</th>
<th>ITT</th>
<th>mITT</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secnidazole</td>
<td>58.3%</td>
<td>60.1%</td>
<td>63.4%</td>
</tr>
<tr>
<td>(169/290)</td>
<td>(146/243)</td>
<td>(137/216)</td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>57.8%</td>
<td>59.5%</td>
<td>62.9%</td>
</tr>
<tr>
<td>(166/287)</td>
<td>(141/237)</td>
<td>(127/202)</td>
<td></td>
</tr>
<tr>
<td>95% CI Secnidazole</td>
<td>[−0.076;0.085]</td>
<td>[−0.082;0.094]</td>
<td>[−0.087;0.098]</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.085</td>
<td>0.094</td>
<td>0.098</td>
</tr>
</tbody>
</table>

NS: not significant.
randomised patients who received at least one dose of the treatment, on the modified ITT (mITT) population defined as all the patients of the ITT population in whom the diagnosis of BV was confirmed after bacteriological examination, and on the per protocol (PP) population comprising all patients included in the mITT population who completed the study protocol without any major deviation and were evaluable at all study visits. The main population was mITT population, but consistent results between mITT and PP populations were expected due to the noninferiority design of the study. Patients not evaluable at D28 were reported as “therapeutic failures” in the ITT and mITT populations. Safety analyses were performed on the ITT population.

2.4. Sample Size and Statistical Analysis. The study was designed with ≥90% power to test the hypothesis that a single dose of secnidazole is noninferior to a seven-day course of metronidazole. According to published studies, the cure rate with the reference treatment was estimated to be approximately 87% [13]. Assuming this cure rate, a noninferiority margin fixed at 10% and a similar dropout rate in the two groups, a sample size of 432 patients (216 per group) was needed to determine noninferiority with a power of 80% and an alpha error of 2.5% (one-sided test). Postulating that 25% of recruited patients would not be evaluable for the primary criteria, the targeted recruitment was 287 patients per group.

Noninferiority was tested using the confidence interval (CI) approach: the primary and secondary efficacy endpoints were analysed by calculation of the bilateral (two-sided) 95% CI of the difference in rate “Secnidazole group-Metronidazole group”. The lower limit of the CI was compared to the −10% limit of noninferiority. The mean time to symptom disappearance was compared between the two groups using the log-rank test. Safety data were analysed using Chi-square tests (Pearson's or Fisher's).

3. Results and Discussion

3.1. Results. The study was performed between March 2007 and July 2008 at 27 sites in France. A total of 577 women (mean age: 36 years in both groups) were enrolled and randomised to receive secnidazole (ITT, n = 287) or metronidazole (ITT, n = 290) (Figure 1). Approximately 28% of patients (secnidazole: 27.2%; metronidazole: 28.6%) had experienced at least one episode of BV during the two years preceding inclusion. After bacteriological examination, the diagnosis of BV was confirmed in 237 patients (81.7%) in the metronidazole group and 243 patients (84.7%) in the secnidazole group (mITT population). During the study, 16 premature withdrawals (metronidazole, n = 12; secnidazole, n = 4; P = .045) were reported, the main reasons cited being “personal convenience” (metronidazole, n = 4; secnidazole, n = 3) and “lost to followup” (metronidazole, n = 3). At the end of the study, major deviations from the protocol, most frequently nonrespect of scheduled visits, nonevaluableity of the primary efficacy endpoint and concomitant antibiotic treatment, were reported for 35 subjects in the metronidazole group and 27 subjects in the secnidazole group (Table 1). “Nonrespect of compliance” was reported for seven patients in the metronidazole group and four patients in the secnidazole group. Overall, 202 patients in the metronidazole group and 216 patients in the secnidazole group were included in the PP population.

3.1.1. Primary Efficacy Endpoint: Therapeutic Success at D28. In the mITT population, therapeutic success (both clinical and bacteriological cure) at D28 was achieved in similar percentages of patients in both groups: 59.5% (141/237) in the metronidazole group and 60.1% (146/243) in the secnidazole group (Table 2). The lower limit of the 95% confidence interval of the difference “secnidazole-metronidazole” was above −10% ([−0.082; 0.094]), confirming the noninferiority of secnidazole compared to metronidazole. The rates of therapeutic success in the PP and ITT populations confirmed the noninferiority of secnidazole compared to metronidazole (Table 2).

3.1.2. Therapeutic Success at D14. At D14, therapeutic success in the mITT population was observed in 66.2% (157/237) of patients in the metronidazole group versus 65% (158/243) of patients in the secnidazole group (Table 3). The noninferiority of secnidazole was confirmed by the limits of the 95% CI for the difference “secnidazole-metronidazole” (95% CI: [−0.097; 0.073]). In the PP population, the percentages of patients achieving therapeutic success were similar in the two treatment groups (Table 3), corroborating the noninferiority of secnidazole. In the ITT population, the lower limit of the 95% CI was slightly below −10%, but the difference between the groups was not statistically significant.

3.1.3. Clinical and Bacteriological Cures Assessed Separately. At D28, clinical cure was achieved in 77% of patients in the secnidazole group and bacteriological cure in 70.3%, higher percentages than the therapeutic success rate (≈60%),
Table 3: Overall therapeutic success at D14.

<table>
<thead>
<tr>
<th>Population</th>
<th>ITT</th>
<th>Population</th>
<th>mITT</th>
<th>PPD14*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secnidazole</td>
<td>62.4%</td>
<td>65.0%</td>
<td>68.7%</td>
<td></td>
</tr>
<tr>
<td>(191/290)</td>
<td>(158/243)</td>
<td>(147/214)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>65.2%</td>
<td>66.2%</td>
<td>69.0%</td>
<td></td>
</tr>
<tr>
<td>(187/287)</td>
<td>(157/237)</td>
<td>(149/216)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>[-0.106; 0.051]</td>
<td>[-0.097; 0.073]</td>
<td>[-0.09; 0.085]</td>
<td></td>
</tr>
<tr>
<td>Secnidazole-Metronidazole</td>
<td>[-0.106; 0.051]</td>
<td>[-0.097; 0.073]</td>
<td>[-0.09; 0.085]</td>
<td></td>
</tr>
</tbody>
</table>

* PPD14 (per protocol population at day 14): patients who were assessable and presented no major deviation from the protocol at day 14 (214 patients in the metronidazole group and 216 patients in the secnidazole group).

Table 4: Clinical and bacteriological cures at D14 and D28 in the mITT population.

<table>
<thead>
<tr>
<th></th>
<th>Clinical cure (%)</th>
<th>Bacteriological cure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D14</td>
<td>D28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secnidazole</td>
<td>79.7%</td>
<td>77%</td>
</tr>
<tr>
<td>(n = 243)</td>
<td>(n = 193)</td>
<td>(n = 187)</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>77.9%</td>
<td>79.3%</td>
</tr>
<tr>
<td>(n = 237)</td>
<td>(n = 145)</td>
<td>(n = 188)</td>
</tr>
<tr>
<td>95% CI</td>
<td>[-0.056; 0.093]</td>
<td>[-0.098; 0.052]</td>
</tr>
<tr>
<td>Secnidazole-Metronidazole</td>
<td>[-0.056; 0.093]</td>
<td>[-0.098; 0.052]</td>
</tr>
</tbody>
</table>

NS: not significant.

3.1.4. Mean Time to Symptom Disappearance. Among the patients of the mITT population completing the self-assessment diary, more than three-quarters reported the disappearance of BV symptoms, within a mean of 7.12 days in the metronidazole group and 6.83 days in the secnidazole group (Table 6). Similar results were observed in the PP and ITT populations (data not shown).

3.1.5. Safety. Safety was evaluated in all randomised patients who took at least one dose of the study treatment. In the two treatment groups, a similar proportion of patients experienced at least one adverse event (AE): 109 (38%) in the metronidazole group and 113 (39%) in the secnidazole group. No differences were observed in the frequencies of AE classified by Organ System, with the exception of headaches, more frequent, although rare, in the secnidazole group (n = 10 versus n = 4 in the metronidazole group). The difference in the rate of patients reporting an expected AE between the metronidazole group (n = 27, 9.4%) and the secnidazole group (n = 16, 5.5%) was at the limit of statistical significance testing. The percentages of subjects reporting at least one drug-related AE were similar in the two treatment groups: 22.7% (n = 65) in the metronidazole group and 22.4% (n = 65) in the secnidazole group, most of these AE being mild in intensity (66.2% in the metronidazole group and 67.7% in the secnidazole group) and associated with complete recovery by the end of the followup period (28 days) (49.2% of patients in the metronidazole group and 53.9% of patients in the secnidazole group). The most frequent unresolved events (vaginitis and abnormal genital discharge) could be considered as treatment failure.

3.2. Discussion. This large, randomised, double-blind, double-dummy Phase III clinical trial designed according to the most recent FDA guidance [12] confirmed the efficacy and safety of a single-dose regimen of secnidazole compared to the standard multiple-dose metronidazole regimen. In all patient populations (ITT, mITT, and PP), around 60% of patients in both treatment groups achieved both bacteriological and clinical cure at D28. The time to symptom disappearance was also similar in both groups. The observed therapeutic success rate was lower than those reported in previous trials investigating oral metronidazole in this indication. In a systematic review of metronidazole treatment of BV published in 1992, the four studies evaluating metronidazole 500 mg bid for seven days reported an initial cure rate within four weeks ranging between 83% and 97% [13]. The recently published Cochrane Review assessing the effectiveness of antimicrobial agents used to treat BV in nonpregnant women, excluded these four studies, as the methods used for BV diagnosis were deemed doubtful, but selected seven other similar trials, mostly performed in
the late 1980s and early 1990s [2, 14–20]. Once again, the cure rates reported, ranging between 78% and 96%, were substantially higher than that recorded in our study (60%).

Several explanations may be postulated for this discrepancy. Firstly, whereas in our study, diagnosis and cure were defined according to the stringent definitions given in the FDA guidance [12] taking into account both bacteriological and clinical criteria, most previous studies used less rigorous endpoints often based on clinical criteria only. Interestingly, the clinical cure rate achieved at D28 (secondary endpoint) in our metronidazole group of patients (77%) is close to the rates reported in these previous studies. Secondly, these older studies, some being open-label, included fewer patients. Finally, therapeutic cure was sometimes assessed after too short a time posttreatment, and this is a crucial limitation since evaluation of BV before one month of treatment is known to be inaccurate [21].

Considering only the two previous randomised, double-blind, controlled studies including at least 100 patients, “clinical cure rates” reported at D28 after treatment with metronidazole were lower [17, 19]. Both these studies compared oral metronidazole (500 mg twice a day for seven days) versus vaginal clindamycin in approximately 400 patients. In one study, cure of BV defined as a pH ≤ 4.5, absence of amine odour after addition of potassium hydroxide, and absence of clue cells, was observed in 54% of patients in the metronidazole group at one month posttherapy [17]. Cure or improvement (requiring two of the criteria defining cure) was achieved by 78% of patients. In the other study, the overall cure rate determined on the basis of the absence of clue cells and of an amine odour reached 76.3% at the second followup visit scheduled between 28 and 42 days after the start of treatment [19]. Interestingly, these rates are of the same order of magnitude as the clinical cure rate achieved at D28 (secondary endpoint) in our metronidazole group: 77%.

Similarly, a recent review recalculating the four-week cure rate for oral metronidazole based on the results of published placebo-controlled studies, quoted an anticipated average cure rate of 66%, which is in accordance with our therapeutic success rate [22].

In the previous studies assessing the efficacy of a single oral 2 g dose of secnidazole for the treatment of BV, the reported cure rates (72% and 93%) were also higher than the therapeutic success rate observed at D28 in our secnidazole-treated patients (≈60%), probably for the same methodological reasons [8, 10].

The secondary endpoint analysis showed that therapeutic success rates at D14 were slightly superior to those observed at D28, both in the secnidazole group (62.4% versus 58.3%) and in the metronidazole group (65.2% versus 57.8%), testifying either relapse or recurrence, probably due to the persistence of the original imbalance in vaginal flora. The difference in success rates at D14 and D28 observed was of the same order within each treatment group. Available data indicate that when BV reappears, it is more likely to represent reactivation rather than a new infection [22]. This result highlights the relevance of assessing treatment efficacy at D28, as recommended in the FDA guidelines.

Secnidazole has been extensively used for the past 20 years to treat various parasitic diseases, including trichomoniasis, and its good safety profile is well established. Neither the preclinical toxicity studies nor the accumulated postmarketing experience in its approved indications gave evidence of a risk of adverse events with secnidazole during pregnancy. This study confirmed that secnidazole is well-tolerated, adverse events recorded being mild in severity and predominantly those known to be associated with imidazole
derives as a whole. The relationship of adverse events to secnidazole was difficult to evaluate in this study, as the occurrence of adverse events was evaluated three weeks after the administration of a single dose of the drug.

4. Conclusion

In conclusion, these results are important to the extent that this randomised, double-blind, double-dummy clinical trial is the first one to assess the efficacy of the reference treatment with oral metronidazole in a large population of patients with BV following a rigorous methodology which conforms to the recent FDA guidance. Secnidazole was at least as effective as metronidazole with a similar favourable safety profile. With its more convenient posology, that is, a single-dose regimen versus a twice-a-day regimen for seven days with the reference drug, secnidazole represents an attractive therapeutic option that should be considered in routine practice, particularly in women whose likely compliance is doubtful or in women who are asymptomatic and question the necessity of a treatment over several days [22].

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References

Review Article

Mycoplasma, Ureaplasma, and Adverse Pregnancy Outcomes: A Fresh Look

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Recent work on the Mollicutes that associate with genital tract tissues focuses on four species that may be of interest in potential maternal, fetal, and neonatal infection and in contributing to adverse pregnancy outcomes. Mycoplasma hominis and Ureaplasma urealyticum have historically been the subject of attention, but Mycoplasma genitalis which causes male urethritis in addition to colonizing the female genital tract and the division of Ureaplasma into two species, urealyticum and parvum, has also added new taxonomic clarity. The role of these genital tract inhabitants in infection during pregnancy and their ability to invade and infect placental and fetal tissue is discussed. In particular, the role of some of these organisms in prematurity may be mechanistically related to their ability to induce inflammatory cytokines, thereby triggering pathways leading to preterm labor. A review of this intensifying exploration of the mycoplasmas in relation to pregnancy yields several questions which will be important to examine in future research.

1. Introduction

Mycoplasma hominis, Ureaplasma urealyticum have had several decades of history among experts in genital tract infectious disease with indications that the former can be part of the normal flora of sexually experienced women and both may play a role in chorioamnionitis, salpingitis, bacterial vaginosis, and postpartum endometritis. Despite an abundance of reports on these organisms, work progressed slowly, mainly due to the fastidiousness and technically challenging culture methods needed to link the organism to clinical conditions. The availability of molecular methods has substantially altered our ability to derive valid information about the pathogenic potential of these bacteria which lack rigid cell walls (Mollicutes, specifically the family Mycoplasmataceae).

More recently, interest in Mycoplasma genitalium has developed among basic scientists; not so much because of it being an organism that can infect the human reproductive tract—though it can, but because it is a self-replicating microorganism with a minimally sized genome and has been sequenced, showing how little DNA is actually needed to permit microbial life. The genome of this organism is 580,000 base pairs and contains 482 genes. By comparison the genome of Neisseria gonorrhoea is about 2.2 million base pairs. Despite attention in Mycoplasma genitalium being largely of interest in its genetic organization and the focus of the creation of a synthetic Mycoplasma by the J. Craig Venter Institute, it has also been given substantial attention as a genital tract colonizer or pathogen.

In this paper, we will review contemporary information about Mycoplasma and Ureaplasma with special attention to the manner in which these organisms may be associated with premature birth and related syndromes. As a convenience, at times when both genera are being referred to, the term genital mycoplasmas will be used to denote that the discussion encompasses all the Mycoplasmataceae that may occur in the female genital tract.

2. Common Pathways to Preterm Labor and Adverse Pregnancy Outcome

Inflammatory reactions within the genital tract tissues of the pregnant female represent a common pathway that leads to delivery, not only when labor is initiated prematurely,
but also when it occurs spontaneously at term. While a complete discussion of the details of parturition could run to a book-length work, just a few points require mention as background for the discussion of the very small bacteria considered here that can contribute to preterm labor and delivery.

First, the components of parturition include, uterine activity, cervical effacement, and rupture of the fetal membranes, while mechanical processes at one level are ultimately dependent on mediators that are released prior to these mechanical actions occurring. Several sources of proinflammatory substances can be noted including stress on the mother or fetus, blood borne infection of the placenta (even if occult), short cervix allowing vaginal flora to be in abnormally close proximity to the fetal membranes, overdistention of the uterus, and altered vaginal flora in which elevated concentrations of proinflammatory microorganisms may be present. Recent observations indicate that inflammatory cells invade the chorion and amnion in both premature and term labor, and inflammatory cells may be a source of inflammatory mediators [1]. Uterine contractions are driven by prostaglandin in the form of PGF2-a which is increased in the amniotic fluid in preterm labor both when the inflammation is associated with positive microbial culture and even in the absence of positive culture [2].

As a result, there is concern about microbial stimuli that can lead to inflammatory reactions in the gravid uterus as these could initiate the cascade of events leading to precipitous delivery. The source of such inflammation-inducing insults includes specific bacteria whether acquired exogenously (such as STD pathogens) or endogenously (altered ecology of the normal flora), expanding numbers of certain bacteria which are otherwise normal inhabitants of the healthy host, escape of normal bacteria to otherwise privileged sites to anatomic locations in proximity to the fetus, and specific genetic backgrounds of the pregnant woman that allow modified responses to microbial challenge. Any of these might incite inflammation in the cervix, membranes, amniotic fluid, placenta, or cord. Indeed, even the fetus may become part of generating an inflammatory response that may result in preterm labor.

Thus, attention may be paid to specific organisms, such as the Mycoplasmas and Ureaplasmas, even though they may elicit adverse pregnancy outcomes in ways that are functionally similar to the way other bacteria elicit inflammatory reactions that lead to preterm labor and birth.

Nevertheless, there is a second layer of concern and interest in preterm birth elicited by excursions in microbial populations. If preterm birth occurs secondary to an inflammatory stimulus occurring because of an expanded bacterial population, the infant at the time of birth may be exposed to a qualitatively or quantitatively abnormal bacterial challenge and if premature, this exposure could contribute to the pathologies that are well known among preterm infants. Waites and colleagues noted that the association, though not the actual causality of Ureaplasmas with premature infant bronchopulmonary dysplasia, has well been established [3], and even as more data is collected on etiologic and mechanistic connections, more efficacious and targeted therapies are needed.

3. **Mycoplasma genitalium (MG)**

Among sexual partners in a recent study of Mexican American and African American individuals, there was a 9.5 and 10.6% infection rate with MG in women and men, respectively, and symptoms of urethritis among men, but lack of symptoms in women [4]. Further underscoring the role of this organism in male nongonococcal urethritis, an Australian case control study recently published indicated that MG prevalence was 10% among cases versus 2% among controls, but C. trachomatis among cases was 33.5% [5].

The organism has also been incriminated as a cause of cervicitis. In a study of a cross section of women attending an STD clinic in Baltimore, the rate of MG in women with cervicitis was 28.6%, while C. trachomatis was 15.8% among cervicitis patients, and MG was found in 19.2% of all patients in the study, while *Chlamydia trachomatis* was present in 11.0%. Although coinfections were common, multiple logistic regression revealed that only MG colonization was significantly associated with cervicitis [6]. Among nonpregnant women, MG also has been associated with salpingitis and in a study of Cefoxitin treatment of salpingitis, failure to eliminate symptoms was attributed to eradicate MG [7]. And in a study of Swedish women undergoing elective termination, postabortal salpingitis was associated with MG colonization in 2.8% of women, furnishing an odds ratio above 6-fold compared to noncolonized women [8].

In an animal study, rapid dissemination from vaginal deposition of MG to the upper genital tract and to joints was observed to occur in a mouse model of infection, providing a hint that this mycoplasma may behave through pathogenic pathways similar to other mycoplasmas that have been found in salpingitis [9]. Indeed, a study by Short and coworkers, who studied 22 MG monoinfected women, with pelvic inflammatory disease, comparing them to 172 gonococcal and or chlamydiial infected women found natural history and epidemiologic characteristics of the three infections to be similar [10].

The role of MG in premature birth is less well defined and is complicated because it may be superimposed on the carriage of other organisms also implicated as causes or at least associates of adverse pregnancy outcome. A recent case-control study from analyzed pregnant women tested for several genital tract pathogens and after multivariate analysis found that young maternal age and MG colonization were independent risk factors for preterm birth [11].

One of the early studies conducted in London looked at more than 1200 pregnant women and found that colonization rate by MG was only 0.7% and only one miscarriage, and no evidence of a connection with preterm birth was discovered. In a study from Japan, investigators also failed to find an association of MG with prematurity, although they did associate *Ureaplasma parvum* (and not *U. urealyticum*) with preterm birth and late spontaneous abortion [12]. In Blanchard and co-workers study, they examined 232
amniotic fluids and were unable to find PCR evidence of MG [13]. Thus, the preponderance of evidence suggests that MG, while quite prevalent, is more important in male urethritis and nonpregnant women than in pregnancy. Nevertheless, indications that MG may have a role in adverse pregnancy outcomes were reported in very recent papers, suggesting that as technology improves and diligence in searching for associations with MG in pregnancy increases, new evidence of its significance may yet emerge.

4. *Mycoplasma hominis* and *Ureaplasma urealyticum* (MH and UU)

These two organisms are considered together because much of the literature related to these organisms has developed together. In 1985, a Canadian study by Ebmil and Pereria noted that cervical cultures of MH and UU revealed that the organisms were found simultaneously in women from family planning and prenatal clinics much more frequently than ether was found alone [14].

What was first learned about the importance of these organisms in the female genital tract was based on detection by arduous culture methods and in some cases by antibody studies. The early work on these organisms suggested, MH was a marker for sexual activity, with higher prevalence in cervicovaginal cultures of sexually active women than prior to sexual debut. UU was typically thought of as a more virulent organism. These organisms have been associated with bacterial vaginosis and salpingitis, but their role in gynecologic infections has often been a matter of dispute. In a recent survey of women from a sexual health clinic in Australia, the rates of colonization with UU and MH were 6.1 and 13.7%, respectively, however, another *Ureaplasma, U. parvum*, was found in 57% of women [15].

The term “genital mycoplasmas” is taxonomically imprecise way of referring jointly to MH and UU, and because of its use in the literature, it will also be used in the following paragraphs. Current literature related to the genital mycoplasmas reports on observations that are made both by culture-based detections and by molecular diagnostic methods. Relatively few reports rely on antibodies to these organisms for detection or diagnosis. There is little doubt that molecular methods have revolutionized our understanding of these microorganisms, because when culture and molecular detection are used simultaneously, methods such as PCR seem to offer great sensitivity. As noted by Oh et al. [16], cultivation missed most *Ureaplasma* present in genital tract tissues in women with placental insufficiency. Such individuals would be expected to have a high rate of genital mycoplasma migration into the amniotic fluid or fetal membranes, but by culture, 91% of women with PCR evidence of *Ureaplasma* had negative cultures for the organism [17].

Despite limitations in methods, the preponderance of reports implicates UU more frequently in relationship to prematurity-linked conditions. Thus, for preterm premature rupture of the fetal membranes, preterm labor, intra-amniotic infection, chorioamnionitis, funisitis, and placental invasion, the presence of genital mycoplasmas is often interpreted as these organisms having a role in pathogenesis. This may be an overinterpretation, but cautious investigators describe these epidemiologic associations as influencing the risk of adverse pregnancy outcomes. Several reports mentioned below will emphasize the apparent greater importance of UU compared to MH.

It is appropriate to note that in a Czech study of 225 women with pPROM, 68% had cervical colonization by UU compared to 17% among control patients, and 28% of pPROM patients were colonized by MH compared to 15% among control patients [18]. Kasper et al. [19] made an extensive analysis of microbial flora with several categories of vaginal conditions including BV, partial BV, altered vaginal flora, aerobic vaginosis, and genital *Mycoplasma* colonization and reported that after 24 weeks gestation, MH was a risk factor for preterm birth (as were partial BV and abnormal vaginal flora characterized by a diminution of *Lactobacillus*). In contrast, another study [20] of 977 pregnancies in which Nugent scoring was done found that 14% of individuals had a high (8 or greater) Nugent score and this was associated with preterm birth, but genital mycoplasma colonization was not. Interestingly UU colonization was much higher than high Nugent score (UU was found in 88% and MH was present in only 3%).

A study of nearly 2000 women in Brussels found a preterm birth rate of 4.9%, and 53.6% of those who delivered prematurely showed UU colonization. In this study, the description of abnormal bacterial flora often accompanied colonization by UU. Although logistic regression showed a significant risk associated with UU, it did not show a commensurate risk associated with what the authors referred to as abnormal flora [21]. Another recent study of 150 women with pPROM reported that UU was present in 96% but was only found in 32% of women who did not experience membrane rupture [22]. Ureaplasma will certainly not be the only threat during pregnancy as noted by a study of bacterial invasion of the amniotic fluid, but it is striking that in 15 women for whom cervical insufficiency was the predisposing cause to amniotic invasion, 7 women had intra-amniotic bacteria (one or more species) and 5 of these 7 had UU [23]. Finally, a recent study of placental cultures from Japan found that among 151 placentas from pregnancies that ended with spontaneous preterm birth before 32 completed weeks, 63 were culture positive for Ureaplasma and 83% of these showed histologic chorioamnionitis, whereas only 30% of Ureaplasma culture negative placentas showed signs of chorioamnionitis [24].

The preceding paragraphs support the idea that both species of genital mycoplasmas may infect the products of conception, but predominantly, UU seems the more frequent and by inference the more virulent of the two opportunistic organisms. This raises further questions as to what constitutes virulence among the genital mycoplasmas and whether virulence can be measured. Further, and probably related, is how the genital mycoplasmas are able to elicit the adverse pregnancy outcomes, and in particular, how may they be related to premature birth?
5. Ureaplasma parvum (UP)

The taxonomic designation of UP as phylogenetically distinct from other mycoplasmas is a relatively recent occurrence and makes backward looks at the literature challenging. It is possible that earlier papers subsumed this organism under UU, but it is also possible that its presence was missed. Therefore, emphasis will be placed in the next paragraphs on the literature that has recognized the separate status of UP.

A modern innovation in microbiology is using the genetic material rather than phenotypic information as a point from which to understand the organism. A groundbreaking paper by Perevre and colleagues [25] compared genomes of MH, MG, and UP and identified 247 coding sequences that were common to the three organisms and for UP there were 280 coding sequences unique to that organism. In addition, analysis of the genomes revealed the energy metabolism, and growth substrates were distinct for the three species. This is notable because it implies that despite living in a common environment, they each derive their energy from the host milieu in different ways.

Given the availability of reagents that can detect UP, it may be expected that a large number of reports will be seen in the future that articulate the ecology of this organism in the human genital tract. For example, a recent report found that in healthy nonpregnant women UP was identified in 57% of human genital tract. For example, a recent report found that the future that articulate the ecology of this organism in the may be expected that a large number of reports will be seen in ways.

Several reports have focused on the frequency of mycoplasmas including UP in the male genital tract with potential relationships to urethritis, male infertility, and sexual transmission. These topics are not immediately germane, but the literature suggests a role for male partners in female infections.

The arrival of a new taxonomic classification is usually met with the question of what specific role does this newly named organism play in clinical infections? For UP there is emerging evidence that it may play a role in infections of pregnancy or in eliciting conditions associated with prematurity. A recent study indicated that there is a dose-related intra-amniotic inflammatory response to UP and that this is related not only to pPROM, preterm labor, and chorioamnionitis, but also to early onset sepsis in the baby and bronchopulmonary dysplasia [27]. Kataoka’s study from 2006 indicated a high prevalence of UP and a statistical association with late abortion and early preterm birth [12].

6. Clinical Features of Host-Mycoplasma Interaction and Mechanisms of Adverse Outcomes

The pathogenicity of mycoplasmas in the female genital tract was previously confirmed by the presence of antimycoplasma antibodies among women with intra-amniotic infection and postpartum fevers [28], but currently the details of immunologic networks are better known and it is possible to make more direct links to clinical outcomes.

Even before specific immunity in the form of antibody is engaged, the host employs mechanisms for recognizing molecular motifs that lead to intracellular signaling and upregulation of host defense factors. A system of recognition factors includes the toll-like receptors or TLRs which have been identified in the genital tract [29]. Activation of TLRs results in the expression of cytokines that can elicit inflammation and phagocytosis leading to antigen presentation and ultimately specific immunity. If we are able to conduct cell culture experiments that indicate that specific molecular motifs known as PAMPs or pathogen-associated molecular patterns exist in genital mycoplasmas, it could explain how these organisms elicit the inflammatory reactions that can lead to labor.

It is appropriate to explore the question of whether genital mycoplasmas have the ability to ligand TLRs with the result of that inflammatory mediators are elaborated. MG is known to upregulate the key signaling molecule NFκB through the Toll 2 and 6 receptors on epithelial cells [16]. This research also indicated vaginal epithelial cells were less responsive than cervical epithelial cells. In a study of detergent-extractable macrophage stimulating activity from UU, activation of Toll 2 and 4 showed activation of a monocyte cell line [30]. Trophoblast cells from term placentas are also activated (producing elevated NFκB and p38 MAP kinase and ERK 1/2 in response to mycoplasma lipoprotein [31]). These trophoblastic cells contain TLRs 2, 4, and 6 and the stimulation through exposure to lipopeptide ultimately elicits production of COX2 and PGE2. Thus, cervical and trophoblast tissue, are able to respond to common elements of mycoplasma, namely the lipopeptide portion of the cell membrane and evidence points to the TLR2 being a key receptor in this process. Cytokines are part of a highly regulated network and include proinflammatory factors as well as anti-inflammatory factors. Proinflammatory cytokines have been associated with ammonia and placental infections. Interleukins 1β, 6, and 8 as well as TNF-α [16, 32], are typically elevated in amniotic fluid, cord blood, and expression in tissues that simultaneously contain bacterial DNA [33]. The cytokines, prostaglandin synthetic pathway (cyclo-oxygenase), and prostaglandins provide a mechanistic connection between the inflammatory stimulus and the ultimate initiation of labor.

7. Mycoplasma Virulence

Certainly the substances that elicit an inflammatory response may be considered among the most important virulence factors present in the genital mycoplasmas. However, additional...
factors may be important in the pathogenic potential of these normally opportunistic organisms. The bulk of the existing literature on this topic relates to the hundreds of mycoplasmas that infect animals, where long standing interest in veterinary medicine has existed. There is evidence to suggest that membrane active substances with hemolytic activity are found in all the arginine using mycoplasmas [34] such as MH. Adherence factors may be predicted as are typical for epithelial microorganisms and like other mucosal pathogens, an IgA protease has been reported for UU [35]. While the difficulty in culturing and working with mycoplasmas in the same way that more conventional organisms are studied has probably limited the pursuit of virulence factors, the annotated genomes of these organisms will allow the prediction of the presence of factors that may be analogous to virulence factors in other organisms and will provide a fertile area of research for theoretical biologists.

8. Remaining Issues

The story of mycoplasmas that are found in pregnant women and specifically in the reproductive tract and in occasional association with adverse pregnancy outcome is an incomplete and sometimes confusing story. One complicating factor is the fact that the mycoplasmas can reside in the normal flora and when pregnancy complications that could have a microbial etiology arise, it is often difficult or even illogical to incriminate one microorganism. The difficulty in understanding the mycoplasmas in relation to prematurity is much like the difficulty in connecting preterm birth in women with bacterial vaginosis (BV) to the BV. While the condition has a statistical association with preterm birth, BV itself involves organisms that are part of the normal flora. While the numbers and relationships among the normal vaginal flora organisms are altered in BV, there is a natural tendency among schooled clinicians singling out an individual organism as an object of therapeutic drug treatment, to think that an organism such as Gardnerella vaginalis may be the important target when in reality a complex bacterial milieu seems to be important and some bacterial species that may be a part of the process have only been identified relatively recently (Atopobium vaginae, for one).

Realizing that mycoplasmas are also part of the bacterial milieu of BV, we again face the dilemma of whether we can incriminate the mycoplasmas alone for adverse pregnancy outcomes or must consider only the entirety of the flora as responsible. Increasingly, multivariable analysis is being used to tease individual factors out of complex collections of epidemiologic, statistical, and clinical data. In this way, certain organisms that are part of the normal flora can be associated as independent risk factors for clinical conditions. Moreover, it will be important for those who follow this field to pay close attention to the questions being asked and specific associations being hypothesized because there is a difference in whether an association is being made between mycoplasmas and prematurity, and/or low birthweight, and/or amnionitis, and/or amniotic fluid infection, and/or preterm rupture of the membranes. Likewise, the clinical question could be whether one or more of these outcomes is related to genital tract colonization by a particular organism or whether a specific tissue (e.g., amnion, placenta, amniotic fluid) must be infected to result in clinical symptoms.

Future research will undoubtedly continue to dissect details about the role of mycoplasmas in adverse pregnancy outcome through statistical means and more specific questions. But a limitation of these kinds of investigation will be dependent on the success with which mycoplasmas are identified among cohorts of patients to be studied. Mycoplasmas have been included as a subject of investigation for many years, their presence was first based on culture, and because the culture techniques are challenging, differences in technical expertise between laboratories may have slowed the process of discovery and certainty about the clinical role of these organisms.

Currently, we have the advantage of molecular detection methods. Polymerase chain reaction is now commonly used to detect the presence of these organisms with great specificity, and when used in a quantitative mode has the ability to make inferences about dose response relationships in clinical situations. The ability to exploit molecular methods to examine microbe interactions with their receptors and transduction of cellular signals and upregulation of cytokines and other effector molecules from susceptible tissues puts us on the edge of a clear understanding of the interaction of the genital mycoplasmas and host cells at the most fundamental level. New opportunities for therapeutic interventions should follow understanding infectious mechanisms in extreme detail.

One last point to be made in the ever increasing emphasis on medicine delivered by health care teams, we should be reminded that the issues of infectious disease leading to preterm birth and other complications of pregnancy do not end with the delivery. The fact is that the infant may be born with infection that threatens survival, but the microorganisms that may help precipitate labor may also participate in other significant problems of the premature infant. The growing literature on this topic is beyond the scope of this paper, but associations of mycoplasmas with bronchopulmonary dysplasia, fetal respiratory distress syndrome [36], and intraventricular hemorrhage are beginning to appear in the literature and should be watched carefully over the coming years. These significant advances in understanding of the consequences of infection should heighten the determination of obstetricians and neonatal intensivists to focus on good communication for the benefit of both mother and her offspring.

References


Review Article

TLR-Mediated Preterm Birth in Response to Pathogenic Agents

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The incidence of preterm birth in developed countries has risen in the past decades. Underlying causes for this enigmatic pregnancy complication are numerous, yet infectious agents that induce dysregualtion of immunity at the maternal-fetal interface pose one of the most probable causes of preterm birth. This paper highlights two factors regarding maternal infections that trigger unscheduled inflammatory sequences that are deleterious to the maternal-fetal balance necessary to maintain pregnancy. Firstly, we discuss the role of Toll-like receptors (TLRs) as sentinels of uterine immunity in the context of response to pathogens. We highlight the idea that particular TLR activations lead to differential immune cascades that induce preterm birth. Secondly, two alternative routes of pathogenic entry may prove to be critical for inducing preterm birth via a cytokine storm or a secondary and currently unknown cell-mediated mechanism of uterine inflammation. This paper summarizes pathways that underlie activation of adverse and diverse immune responses to foreign agents that may result in preterm birth.

1. Introduction

Healthy pregnancy is the result of a tightly regulated system of crosstalk between maternal and fetal structures. Implantation and parturition are specifically characterized by states of inflammation [1, 2]. Proinflammatory cytokines, matrix degrading proteins, altered transcriptional factors, rapid hormonal changes, and immune cell activity are paramount for uterine activation and the onset of labor [3]. In contrast, the gestation period, comprised of decidualization, placentation, and fetal development, requires uterine quiescence guided by high levels of progesterone and the production of anti-inflammatory cytokines from both maternal and fetal cells [4, 5]. Due to the immediacy with which the onset of labor takes place and the resultant necessary shift from anti- to proinflammatory signal cascades, it is not surprising that unscheduled parturition is the most high risk state of pregnancy for adverse outcomes.

Preterm birth can result from a range of causes such as exposure to environmental triggers, maternal stress, fetal or maternal genetic abnormalities, or hormonal imbalance. However, infection is one of the most heralded causes of preterm birth due to the drastic link between underlying infectious agents and their ability to promote inflammatory responses [6–8]. It is well documented that the vast majority, upwards of 90% of preterm births that occur before gestational week 28 can be correlated to the presence of infectious agents and severe inflammation [9]. Furthermore, a significant number of placentas obtained from preterm deliveries show pathological signs of chorioamnionitis which can result from a number of differential pathogenic agents [10]. Thus, while the evidence for infection-mediated preterm birth is substantial, the underlying mechanisms that induce early birth in response to pathogenic presence remain vague.

Investigation into the mechanisms that lead to preterm birth in response to pathogenic agents should take into account several key factors. Firstly, the route of entry that a given foreign agent takes determines where the agent will ultimately subsist and what pathways will be activated. Recent evidence demonstrates that the same pathogen, delivered through alternative routes, can lead to differential inflammatory responses ([11–13], our unpublished results). Therefore, the mechanisms that underlie initiation of preterm birth may be dependent on the organ or tissue where a pathogen enters. Secondly, different pathogens may elicit disparate inflammatory responses. Data from humans
and animal models show that alternative sets of cytokines coupled with activation of maternal or fetal cells are triggered in response to different pathogenic agents, yet all result in unscheduled inflammation [14, 15]. A strong explanation for initiation of distinct immune pathways is probably the activation of toll-like receptors (TLRs). TLRs are a diverse set of innate immune sentinel receptors highly conserved throughout evolution. Each TLR1–10 is specific for a different pathogen associated molecular pattern (PAMP) [16]. Importantly, TLRs are highly expressed at the maternal-fetal interface on trophoblasts and uterine immune cells [17]. It is likely that differential uterine immune responses occur due to the diversity of pathogens that ensues activation of any one of the TLRs, ultimately leading to deleterious inflammation and preterm birth.

This paper aims to summarize specific viral and bacterial pathogens that may program preterm birth outcomes. Furthermore, we expound upon possible routes of transmission and areas of replication that different foreign organisms may home to. Finally, after a brief discussion of the necessary steps toward an inflammatory environment that programs term labor, we elaborate on pathogenic triggers of this process that may activate TLRs to induce preterm birth.

2. Keys to Parturition

In order to delineate the aberrant induction of inflammation by infectious agents, the role of inflammation in healthy pregnancy outcomes must be understood. The act of birth is characterized by the onset of uterine contractions that lead to expulsion of the fetus from the uterine cavity. The act of giving birth is the ultimate step in a proinflammatory signaling cascade that is orchestrated by an intrauterine milieu coupled to hormonal cues.

Temporal increase in inflammatory signals initiates labor. Inflammatory cytokines and chemokines such as TNF-α, IL-1β, and IL-8 increase in the placental microenvironment, including amniotic fluid and fetal membranes. This induces signals for innate immune cells to become activated [18, 19]. Upon initiation of a proinflammatory cascade including NF-κB activation, uterine immune cells produce inflammatory chemokines and cytokines. Increased uterine activation of transcription by NF-κB leads directly to high levels of COX-2, PGE₂, the gap junction protein connexin 43, and upregulation of the oxytocin receptor [20]. The increase of the aforementioned proteins and receptors leads to active uterine stretching and thus to the induction of birth.

The pregnant uterus is replete with specialized immune cells primed to play roles in implantation, placentation, and parturition. The major cell types studied thus far are uterine NK cells (uNK), dendritic cells, T regulatory cells (T reg), and macrophages. uNK cells are the most thoroughly studied uterine immune cells due to lack of their natural cytotoxicity, an alternative receptor repertoire to peripheral blood counterparts, and the ability to produce vascular growth factors, cytokines, and chemokines during early stages of pregnancy [21, 22]. In humans, uNK cells are reduced after the second trimester. This trend is also followed in mice. However, uNK cells can be amplified in mice in response to inflammatory triggers at later stages of pregnancy. It is thus tempting to speculate that such a phenomenon may also occur in humans. Research has expanded to investigate the ability of uNK cells to create a balanced milieu at the maternal-fetal interface due to their ability to produce IL-10, IP10, and IL-8 during cross-talk with trophoblasts and dendritic cells [23–35]. uNK cells are found interwoven throughout decidual areas where spiral arteries are bountiful. This positioning is demonstrative of their role in growth and decidualization of the placental organ [22]. uNK cells peak in humans in first trimester and their numbers start to decrease at the end of second trimester. Mouse models deficient in uNK cells show that proper birth takes place in the absence of these cells; however, the placenta that occurs is shallow and less developed than models where uNK cells are available [26]. While uNK cells are not thought to play a direct role in term birth, several murine studies have shown that these cells can become cytotoxic in nature in the presence of certain pathogens and ultimately lead to preterm birth through production of TNF-α [13, 14]. Thus, it stands to reason that the disappearance of uNK cells in mid-third trimester is a fail-safe mechanism for the course of term birth that allows the uterus to minimize unscheduled inflammatory events.

While uNK cells do not play a documented role in term birth, macrophages may hold the key to conduction of proper parturition. Uterine macrophages are found in proportions upwards of 20% of the total uterine lymphocyte population. Over the course of pregnancy, uterine macrophages are present as innate immune sentinels due to their expression of TLRs. However, macrophages are immunomodulatory over the course of gestation as they simultaneously produce TNF-α, IL-10, and TGF-β throughout placentation [27]. Along similar lines, matrix metalloproteinases (MMPs) are secreted in early and late gestation for tissue degradation which in turn induces MIP-1α and MCP-1 to signal the further invasion of macrophages for phagocytosis [28–30]. Reports show that late stage stimulation of uterine macrophages induces high levels of IL-8 production, a neutrophil and macrophage chemoattractant. In line with these findings, the act of parturition itself is associated with high levels of macrophage and neutrophil infiltration as these two cell types can quickly engulf, remove, and remodel tissue [31].

The function and phenotype of dendritic cells in the uterus is beginning to be expounded upon. Recent studies have highlighted their role in crosstalk with uNK cells and trophoblasts in order to orchestrate production of cytokines such as TNF-α, IL-12, and IL-10 [32]. While the role of DCs in parturition remains unknown, it is fair to assume that these cells add to the balanced cytokine and signaling milieu required throughout gestation. Furthermore, recent characterization in mice of a uterine-specific subset of DCs that is skewed toward production of IL-15 and CCL6 adds to evidence that their actions are dichotomous [33]. Thus, while these cells contribute to an immunosuppressive atmosphere over the course of gestation, they possess the capacity to contribute to proinflammatory responses upon pathogenic activation.
While uNK cells, macrophages, and dendritic cells aid to orchestrate the balance between pro- and anti-inflammatory milieu over the course of gestation, T regulatory cells Tregs in the uterus are thought to be mainly immunosuppressive. Studies demonstrate the importance of these cells as adoptive transfer of Tregs into abortion prone mice leads to pregnancy rescue [34]. T-cell-deficient Rag1−/− mice given the TLR4 agonist LPS deliver preterm. Infusion of Tregs into these mice rescues pregnancy to term, indicating an advantageous role for these cells in TLR-pathogen mediated response to adverse pregnancy outcomes [35]. The function and phenotype of Tregs found in the uterus remains unknown, but these cells are presenting as a possible therapeutic cell to aid in rescue of pregnancy loss.

While the aforementioned cellular activities are highly orchestrated throughout pregnancy and particularly during the onset of labor, these actions are correlated to specific hormonal cues that allow for these changes. For the majority of pregnancies one of the main mechanisms that allows for uterine quiescence and lack of uterine muscle movement is the increased presence of the hormone progesterone [36, 37]. Female sex hormones have been shown to decrease the T-cell stimulatory capacity of DCs during pregnancy [38]. In rodents, direct treatment of DCs with progesterone has been shown to increase the inhibitory capacity of DCs and these changes are associated with progesterone receptor (PR) regulation. Due to the fact that human and murine uNK cells do not possess PRs, the capacity of DCs to produce IL-15 for uNK development may work through indirect mediation of DCs by progesterone [39, 40]. Importantly, macrophages possess PR and are directly regulated by progesterone as their migration and nitric oxide production is severely impaired when progesterone levels are high. In support of these findings, PR+ mice show high levels of macrophage and neutrophil infiltration in the pregnant uterus [3]. Taken together, the functional withdrawal of progesterone is critical to the initiation of parturition as its decreased action allows for control of immune cells needed to initiate inflammatory cascades to be relinquished.

3. Preterm Birth: Activated Uterine Immunity

How does the presence of viral or bacterial organisms lead to preterm birth? It is rarely the foreign organism itself that causes preterm birth. Rather, it is the immune response of the host evoked by the pathogen that leads to aberrant pregnancy outcomes. With the knowledge of parturition as a process mediated by inflammation, we can begin to understand that pathogenic insults can trigger unscheduled uterine immunity. Evidence demonstrates that the activated TLR pathways and the route of pathogenic entry may determine the immunological cascade of aberrant cellular and cytokine activity that lead to preterm birth ([11–15], our unpublished data). The majority of these pathways commonly lead to increased NF-κB activity that allows for production of inflammatory cytokines and mediation of progesterone withdrawal through modulation of the participation of different PRs [20].

Briefly, the immune system at the maternal-fetal interface thus far has been discussed due to its virtue as specialized toward growth and decidualization of the placental organ and delicately regulated by sex hormones. However, new observations have deciphered that innate immune defenses, characteristic of innate immunity on peripheral blood cells, are present at the placental level as well. The TLRs are expressed at the placental level on trophoblasts, uNK cells, DCs, and macrophages [17, 21]. Activation of any of the TLRs leads to a downstream cascade of proinflammatory cytokine production, and most notably, to activation of the NF-κB transcription factor. It stands to reason that TLR activation at the maternal-fetal interface may tip the tightly regulated cytokine and hormonal anti-inflammatory milieu necessary for successful term pregnancy.

Two forms of pathogenic entry are studied in rodent models due to their correlations with human infection. Intrauterine ascension through the vaginal tract and systemic infiltration are mimicked by intrauterine infusion and intraperitoneal injections, respectively (Figure 1). Bacteria show stronger correlations throughout the literature with increased incidence of preterm birth than viruses. This may be due to the differential sites of infection that bacteria versus viruses target. Generally, bacteria are found in mucosal membranes that surround the amniotic sac or those lining the intrauterine canal [8]. On the other hand, reports demonstrate that viruses, in need of host cell machinery for replication, tend to infect trophoblast cells of the placenta as these cells possess specific receptors needed for viral particle entry [41–43]. Importantly, bacteria are easily cultured from amniotic fluid or from swab samples taken from the uterine tracts of pregnant women [7, 44]. Due to the nature of viruses, these pathogens are more difficult to pinpoint, though some studies have been able to isolate HIV and adenovirus from amniotic fluid samples [8, 45].

4. Bacterial Entry

Bacterial infections manifest in the pregnant uterus generally between maternal and fetal membranes or directly within amnion or chorion specific fetal membranes. Infection of the amniotic cavity is denoted amnionitis, direct infection of fetal membranes is denoted chorioamninitis; whereas funisitis is infection of the umbilical cord. Interestingly, several reports note that bacterial agents are rarely found at the placental level, in contrast to viral pathogens [7, 8, 45]. Due to the fact that bacteria can subsist extracellularly within membranes, it is reasonable to postulate that intrauterine bacteria or fetal DNA due to necrosis of fetal cells are visualized by TLRs that are extracellular such as TLR2, TLR4, TLR5, and TLR6 [46, 47].

Effective methods of probing for the presence of bacterial agents are immunostaining of membranes of the chorion or amnion from term versus preterm deliveries. Membranes from preterm delivered placentas show higher levels of chemotactic proteins MCP-1 and CXCL6 (Granulocyte chemotactic protein-2), inflammatory cytokines IL-1β, TNF-α, and COX-2 by immunohistochemical staining [48–50].

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The general route of entry in placental viral infection and the resultant inflammatory events can lead to preterm birth. Though data is relatively scarce, evidence suggests that viral entry into trophoblast cells induces trophoblast apoptosis. 

5. Viral Entry

Though data is relatively scarce, evidence suggests that viral entry into trophoblast cells induces trophoblast apoptosis and the resultant inflammatory events can lead to preterm birth [52]. The general route of entry in placental viral infections is cell-specific as different viruses enter trophoblasts that express their viral receptor [42, 52, 53]. Once viral uncoating or shedding occurs within a cell, intracellular TLRs are available as sentinels within endosomal pockets where viruses may replicate. Intracellular TLRs, TLR3, TLR7/8, and TLR9 are specific for viral genomic motifs and, not surprisingly, are associated with several viruses that prove correlation with adverse pregnancy outcomes.

6. TLRs: Sentinels of Uterine Immunity

Here, we summarize known pathogens, viral or bacterial, associated with induction of preterm birth. We highlight immunological pathways that demonstrate specific PAMPs that activate TLRs to induce preterm birth. Work in mouse models illustrates differential TLR-mediated cascades of inflammatory-based uterine immunity that can lead to preterm birth. Importantly, clinical correlates where amniotic fluid is tested or maternal/fetal membranes are probed begin to support these findings.

6.1. TLR2. TLR2 is one of the more promiscuous TLRs in that upon activation it can concomitantly signal with TLR1 and TLR6. In line with these findings, TLR2 is not only specific for gram positive bacteria due to its specificity for peptidoglycan, but also signals upon recognition of meningococcal porins, fungal, parasitic, and viral pathogen-associated molecular patterns [12]. Studies show that women who deliver preterm with the condition of chorioamnionitis show significant upregulation of the TLR2 receptor as compared to controls, implicating a role for the receptor in preterm birth and infection [54]. Murine studies demonstrate preterm birth occurs when TLR2 is activated either through systemic or intrauterine delivery ([11], our unpublished data). Systemic activation induces activation of the NF-κB transcription factor, and first trimester trophoblasts produce significant amounts of IL-8 and IL-6 ultimately undergoing apoptosis [12]. In contrast, intrauterine delivery of TLR2 agonists did not induce increased TNF-α production or NF-κB activation [11] (Table 1).

TLR2-specific human pathogens that correlate with increased incidence of preterm birth are ureaplasma ureaticulum, group B streptococcus, and cytomegalovirus (CMV) [55, 56]. Ureaplasma ureaticulum is one of three bacteria that are grouped together and termed bacterial vaginosis. While common genital infections such as Chlamydia trachomatis and neisseria gonorrhoea are rarely associated with aberrant pregnancy outcomes, high loads of bacterial vaginosis in pregnant women prove nine times more likely to lead to prematurity [47]. Thus, the activity of TLR2 in the presence of ureaplasma ureaticulum is an important correlation in the literature to follow at the clinical level. CMV shows the greatest correlation of human infection and adverse pregnancy outcomes as this herpes virus is present in 80% of the general population, but becomes active under conditions of immune suppression. Data demonstrate that human CMV may infect syncitiotrophoblasts or cytotrophoblasts and induce proinflammatory cascades such as IL-8 production [57, 58].

6.2. TLR3. While TLR2 generally “sees” pathogens that are extracellular, TLR3 possesses specificity for double stranded RNA viral motifs and is found in endosomal pockets.
TNF-α demonstrate that preterm birth in WT mice is induced by INF-α, IL-12, and MCP-1 [11, 60]. Our unpublished results demonstrated marked serum and placental production of IL-6, IL-12, and MCP-1 [42]. Studies in human and mouse trophoblasts show that cells were treated with synthetic TLR3 ligand poly(I:C), and the NF-κB pathway was activated leading to downstream cascades of inflammatory signals. Studies have utilized poly(I:C) as well to assess the role of preterm birth through TLR3 activation in mouse models. Where poly(I:C) was injected intraperitoneally to give preterm birth in WT mice is induced by a TNF-α-mediated axis. In contrast, in a model of intrauterine infusion, INF-β and CCL5 were the major contributors to preterm birth outcomes in response to poly(I:C) and TNF-α production. Similar unpublished data from our lab agree with this data as pregnancy where cellular turnover is abundant [59]. Evidence suggests that TLR3 activation in trophoblasts may be a key mechanism to preterm birth outcome induced by infectious agents [60].

Trophoblasts express the coxsackievirus and adenovirus receptor (CAR). Upon adenoviral infection, trophoblasts underwent apoptosis and this led to recruitment of a receptor (CAR). Upon adenoviral infection, trophoblasts show that cells were treated with synthetic TLR3 ligand poly(I:C), and the NF-κB pathway was activated leading to downstream cascades of inflammatory signals. Studies have utilized poly(I:C) as well to assess the role of preterm birth through TLR3 activation in mouse models. Where poly(I:C) was injected intraperitoneally to give preterm birth. Furthermore, the seropositive women cause increased uNK cell cytotoxicity and TNF-α production, an NF-κB dependent mechanism and demonstrated marked serum and placental production of IL-6, IL-12, and MCP-1 [11, 60]. Our unpublished results demonstrate that preterm birth is induced by a TNF-α-mediated axis. In contrast, in a model of intrauterine infusion, INF-β and CCL5 were the major contributors to preterm birth outcomes in response to poly(I:C) and TNF-α production. Similar unpublished data from our lab agree with this data as pregnancy where cellular turnover is abundant [59]. Evidence suggests that TLR3 activation in trophoblasts may be a key mechanism to preterm birth outcome induced by infectious agents [60].

6.3. TLR4. It is well established that TLR4 recognizes lipopolysaccharide motifs found on the majority of gram negative bacteria. Two of the three bacterial strains associated with preterm birth outcomes, mycoplasma hominis and trachtonomas vaginalis, are gram negative and thus have the capacity to signal via TLR4 [46]. Activation of TLR4 at the placental level has been studied in order to assess the possible immune activation that ensues when TLR4 specific bacterial loads overwhelm the uterine cavity and trigger preterm birth [61]. Our lab has demonstrated that LPS and E. coli cause increased uNK cell cytotoxicity and TNF-α production, ultimately leading to preterm birth. Neutralization of TNF-α or abolition of uNK cells rescued pregnancy to term. In contrast, our unpublished model of E. coli intrauterine infusion demonstrates that preterm birth does occur, but not in a TNF-α-dependent manner ([13], our unpublished data). Similar unpublished data from our lab agree with this data as the NF-κB pathway was not activated in a model of preterm birth mediated by LPS intrauterine infusion (Table 1). Again, these results present strong evidence for the hypothesis that alternative routes of pathogenic entry, for both viral and bacterial pathogens, can cause markedly different immune responses, both ultimately leading to preterm birth.

6.4. TLR7/8. TLR7/8 are specific for single stranded RNA motifs, thus retroviruses are strong candidates to activate these receptors. Though HIV is not yet correlated to a specific TLR for activation, evidence suggests that TLR7 activation mimics HIV related pathologies [62]. Importantly, strong associations exist between HIV seroprevalence and incidence of preterm birth. A cohort study that followed 600 women, approximately half seropositive for HIV and half seronegative, showed that HIV+ women were significantly more likely to give preterm birth. Furthermore, the seropositive women who gave preterm birth showed significantly diminished levels of CD4+ T cells [63]. Taken together, these data demonstrate that either HIV or the immune-compromised state of mothers may lead to increased risk of preterm birth. Studies in nonpregnant patients aim to activate TLRs in order to discourage HIV activity due to the proinflammatory activity of antiretroviral therapy [64]. Taken together, the immunosuppressive state of pregnancy needs to be thoroughly considered when therapy is employed in pregnant women, but lessons may be learned from how the virus travels and replicates during this state.

6.5. TLR9. TLR9 recognizes unmethylated CpG motifs. Though reports identify this intracellular receptor as specific for double stranded DNA viruses or fetal DNA, these types of CpG motifs constitute over 80% of bacterial genomes as well. Studies that activate TLR9 with CpG in pregnant mice thus far have demonstrated that a lack of IL-10 causes high susceptibility to CpG-mediated pathogenic mimics and inflammatory responses composed of severe placental macrophage and neutrophil influx coupled to TNF-α production and preterm birth [15] (Table 1).

<table>
<thead>
<tr>
<th>TLR</th>
<th>(PAMP)</th>
<th>Route of Entry</th>
<th>NF-κB</th>
<th>TNF-α</th>
<th>Pregnancy Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>peptidoglycan</td>
<td>IU</td>
<td></td>
<td></td>
<td>Preterm Birth&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>TLR3</td>
<td>poly(I:C)</td>
<td>IU</td>
<td></td>
<td></td>
<td>Preterm Birth&lt;sup&gt;11, up&lt;/sup&gt;</td>
</tr>
<tr>
<td>TLR3</td>
<td>poly(I:C)</td>
<td>Systemic</td>
<td>+</td>
<td>+</td>
<td>Preterm Birth&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS/E. coli</td>
<td>IU</td>
<td>N/A</td>
<td></td>
<td>Preterm Birth&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS/E. coli</td>
<td>Systemic</td>
<td>N/A</td>
<td>++</td>
<td>Preterm Birth/IUFD&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG</td>
<td>IU</td>
<td>N/A</td>
<td></td>
<td>Preterm Birth/IUFD&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG</td>
<td>Systemic</td>
<td>N/A</td>
<td>++</td>
<td>Preterm Birth/IUFD&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>– up</sup> is our unpublished data.
Importantly, since herpes viruses are latent or chronic, if the virus is inactive during the term of pregnancy there are no significant findings that viral carriers have adverse pregnancy outcomes [65]. While cytomegalovirus belongs to the herpes virus family, it has been associated with activation of TLR2 in humans, while murine CMV is specific for TLR9. Mouse models demonstrate that CpG motifs specific for TLR9 activation do lead to adverse pregnancy outcomes in IL-10−/− mice [15]. In wild type mice, the adverse effects were on nonlive born pups that experienced cranial-facial and distal limb malformation [66]. Interestingly, infants born to CMV-infected mothers have shown similar malformations [67].

7. Conclusion

Taken together, the aforementioned studies and resultant data demonstrate that pathogenic route of entry as well as the specific TLR activated may be two important determinants of the immune responses that induce preterm birth. Murine studies have begun to delve into the role of NF-κB and its direct role in TNF-α production. Importantly, an amassment of current literature seems to suggest that systemic inflammatory responses may induce TNF-α through the NF-κB pathway to activate events leading to preterm birth. In contrast, in the intrauterine setting the mode of action is most likely TNF-α-independent. Therefore, more research needs to occur to better understand the underlying inflammatory or hormonal imbalances that are induced by intrauterine infection that lead to preterm birth outcomes.

In regards to activation of specific TLRs, systemic injection of different PAMPs demonstrates that alternative immune cell subsets respond based on the pathogen at play. Bacterial pathogens signaling through TLR2 or TLR4 show strong staining within human chorion and amnion membranes. Murine studies emphasize the role of uNK cell amplification and TNF-α production in response to TLR4 activation. TLR3 activation data from our lab demonstrates, in WT mice, that uNK cells amplify and produce TNF-α in response to poly(I:C), a viral mimic. Importantly, other reports show that systemic injection of poly(I:C) induces trophoblast apoptosis after a vigorous cytokine storm leading to a preterm birth outcome. Taken together, these two reports are not disparate. Finally, in the context of TLR9 activation it has been shown that macrophages and neutrophils infiltrate the placental zone and lead to preterm birth or intrauterine fetal death outcomes.

As a whole, the reports discussed in this paper lend strong evidence to the postulate that the pathway to induction of preterm birth is dependent on the TLR activated—a product of the type of pathogenic infection. Secondly, the route of entry, intrauterine ascension versus systemic infection, plays a role in determining the cytokine profile activated and the ensuing mechanism of fetal rejection. From a clinical standpoint, it may be paramount to account for not only the type of infectious agent at play within the maternal-fetal system, but also the route through which the pregnancy was compromised.

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Research Article

Malaria, Intestinal Helminths and Other Risk Factors for Stillbirth in Ghana

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Objective. The objective of the study was to assess Plasmodium/intestinal helminth infection in pregnancy and other risk factors for stillbirth in Ghana. Methods. A cross-sectional study of women presenting for delivery in two hospitals was conducted during November-December 2006. Data collected included sociodemographic information, medical and obstetric histories, and anthropometric measures. Laboratory investigations for the presence of \textit{Plasmodium falciparum} and intestinal helminths, and tests for hemoglobin levels were also performed. Results. The stillbirth rate was relatively high in this population (5%). Most of the stillbirths were fresh and 24% were macerated. When compared to women with no malaria, women with malaria had increased risk of stillbirth (OR = 1.9, 95% CI = 1.2–9.3). Other factors associated with stillbirth were severe anemia, low serum folate concentration, past induced abortion, and history of stillbirth. Conclusion. The fact that most of the stillbirths were fresh suggests that higher quality intrapartum care could reduce stillbirth rates.

1. Introduction

Of the 130 million babies born worldwide every year, approximately 4 million are stillborn [1], more than 98% of these occur in developing countries [2]. Stillbirth accounts for more than half of perinatal mortality in developing countries [3]. In Sub-Saharan Africa, stillbirths account for more than 3% of deliveries each year [2]. While countries in South-East Asia and South America report the highest overall numbers of stillbirth, countries in Africa report the highest incidence rates per 1000 live births [4]. The average stillbirth rate in developing countries has been reported to be 26 per 1000 live births, about five times higher than in developed countries (5 per 1000) [4]. One fourth to one third of all stillbirths is estimated to take place during delivery [5, 6]. Stillbirths occurring in the intrapartum period generally have a normal appearance and are often called “fresh” stillbirths [5]. The skin not being intact implies death more than 24 hours before delivery (antepartum), often called “macerated” stillbirths [5].
Stillbirths have not been widely studied, have been under-reported, and rarely have been considered in attempts to improve birth outcomes in developing countries [5, 6]. There are many factors associated with stillbirth including inadequate access to obstetric care, inadequate care [7], malaria, hypertensive disease, poor nutritional status, history of stillbirth, congenital anomalies, sickle cell disease, and high burden of infectious comorbidities [5, 8–10].

Conceptually, infection may result in fetal death through several pathways [11]. First, maternal infection may cause severe illness, leading to fetal death [12, 13]. Also, an infection in the uterus or anywhere else in the mother’s body may precipitate preterm labor [14]. Last, the placenta may be directly infected, leading to reduced blood flow to the fetus, a likely cause of stillbirth associated with malaria infection [15]. When malaria parasites infect the placenta, placental insufficiency results because of lymphocyte and macrophage accumulation, and increased expression of pro-inflammatory cytokines; these impede maternal blood flow through the placenta [16, 17]. Intestinal helminths, including hookworms and *Trichuris trichura*, have been associated with anemia [18, 19]. A study in Tanzania showed that 63% of stillbirths were attributable to maternal anemia [20]. It has been suggested that low hemoglobin concentrations can cause a state of chronic hypoxia, which is presumably exacerbated in pregnancy when oxygen demands are particularly high because of the metabolism of the mother and the fetus, and that oxygen transfer to the fetus is probably reduced in anemic women [21]. Folate deficiency causes megaloblastic anemia [22]. Circulating folate concentrations decline in pregnant women, hence the need for supplementation [22]. A strong association has been observed between maternal plasma, cord plasma, and placental folate concentrations, suggesting that transplacental folate delivery depends on maternal plasma folate concentrations [22].

According to the World Health Organization’s Opportunities for Africa’s Newborns 2006 report, the stillbirth rate for Ghana is 24 per 1000 deliveries. Even though stillbirths represent a large proportion of perinatal deaths, causes of stillbirths are poorly understood in Ghana. To our knowledge, the association between malaria and intestinal helminth coinfection in pregnancy and stillbirth has not been studied. Few studies have studied the association between malaria and helminths in pregnancy, with conflicting results. This study provides baseline data in this area. Given that 98% of stillbirths occur in developing countries, especially sub-Saharan Africa [2], which also has a high burden of malaria and intestinal helminth [23] infections, it is important to investigate the role of these infections in contributing to stillbirth.

2. Methods

The study was conducted in Kumasi, the capital of the Ashanti region of Ghana. Kumasi is the second largest city in Ghana with a population of 1.2 million [24]. The climate in Kumasi is humid and tropical, with two rainy seasons, April to June and September to October. Helminth infection is endemic in the Ashanti region [25], which also has an intense perennial malaria transmission, the predominant parasite being *Plasmodium falciparum* [24].

The Institutional Review Board of the University of Alabama at Birmingham and the Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi reviewed and approved the study protocol before its implementation.

A cross-sectional study of women presenting for delivery at two hospitals in Kumasi, the Komfo Anokye Teaching Hospital (KATH), and the Manhyia Polyclinic was conducted in November and December 2006. All women with a singleton, uncomplicated pregnancy were asked to participate. After informed consent was obtained, a questionnaire was administered to collect sociodemographic information, and medical and obstetric histories. Body weight and mid upper arm circumference (MUAC) were measured for each woman. Obstetric information was also obtained from the mothers’ antenatal care (ANC) charts. ANC charts provided information on number of antenatal care visits, gestational age as assessed by palpation at first ANC visit or ultrasound at first ANC, tetanus shots, malaria prophylaxis, antihelminth medications, hemoglobin level, and illnesses and treatments during pregnancy. Blood was drawn by venipuncture for determination of hemoglobin levels, serum folate level, and malaria antigen tests. Stool samples were obtained for determination of intestinal helminths.

At delivery, state of the newborn (alive or stillbirth), sex, weight, and length were obtained as recorded by the midwives.

Determination of malaria antigen in plasma was done using the Malaria Antigen Celisa (Cellabs, Brookvale, Australia). The malaria Antigen Celisa kit is a monoclonal antibody-based assay specific for *P. falciparum* malaria. The assay detects a merozoite antigen that circulates in the blood for up to 14 days postinfection. Determination of Hookworms, *Ascaris lumbricoides*, and *Trichuris trichura* was done using the Kato-Katz thick smear technique (WHO, 1991). Stool samples were processed within 12 hours of collection and examined microscopically within one hour of preparation to avoid missing hookworm ova. For *Strongyloides stercoralis*, samples were processed using the Baermann method [26]. Serum folate was measured by radioimmunoassay. Hemoglobin level was measured in an automatic cell counter (Sysmex M-2000; Digitana AG, Hamburg, Germany) about 30 minutes after blood sampling.

Variables were defined as follows—uncomplicated pregnancy: absence of hypertension, pre-eclampsia, history of a previous caesarean section and hemorrhage, and a normal presentation of the fetus [27]. *Malaria infection*: presence of malaria antigen in the mother’s peripheral blood at the time of delivery. *Intestinal helminth infection*: presence of helminth eggs or larvae in stool collected at the time of delivery. *Coinfected*: positive for both malaria and intestinal helminths at delivery. *Anemia*: hemoglobin levels <11 g/dL, and severe anemia: hemoglobin level <8 g/dL [28]. *Low serum folate*: serum folate concentration <6.8 nmol/L [29]. *Stillbirth*: an intrauterine death of a fetus weighing...
at least 500 grams after 20 completed weeks of gestation occurring before the complete expulsion or extraction from its mother [30]. An intrauterine death of a fetus during labor or delivery was considered a fresh stillbirth, and an intrauterine death of a fetus sometime before the onset of labor, where the fetus showed degenerative changes was considered a macerated stillbirth [30]. *Induced Abortion:* the purposeful interruption of an intrauterine pregnancy with the intention other than to produce a live-born infant, and which does not result in a live birth [31].

Sample size was calculated using unpublished reports on stillbirth from the two study hospitals, which estimated that at least 1%–1.5% of 1000 births would be stillbirths. We made the assumption that if we obtained 10 stillbirths, and that 10–25% of women with normal births had both malaria and intestinal helminth infections, at a 5% significance level, we would have 80% power to detect an odds ratio of 7.5–9.0; assuming 15 stillbirths, we would be able to detect an odds ratio of 6.0–7.5.

### 3. Data Analysis

Data analysis was performed using SAS software version 9.1 (SAS Institute, Cary, NC). Differences in socio-demographic and obstetric characteristics by stillbirth were assessed by chi-square or t-test. Correlation analyses were performed to identify potential multicollinearity between independent variables. To determine factors associated with stillbirth, we used multiple logistic regression. Variables that were significant (*P* < .05) on bivariate analysis and those that are known to be associated with stillbirth based on previous studies were entered into a regression model [32]. Through this procedure, we calculated odds ratios (OR) and 95% confidence intervals (CI).

### 4. Results

Seven hundred and eighty five (785) women were recruited into the study before delivery in the two hospitals in Kumasi. We obtained both malaria and intestinal helminth results from 746 women, and data analysis was limited to these women. None of the women smoked and only 14 (1.8%) consumed alcohol. Overall, the mean age of the women was 26.8 years (range: 15 to 48 years); 21.1% were single, 30.2% were primigravidae, 30.6% were anemic, 29.5% had a prior induced abortion, and 5.2% had a history of stillbirth (Table 1).

There were 37 cases of stillbirths (4.9% of all deliveries). Of these, 9 (24.3%) were macerated. A higher proportion of women who were single did not receive SP during pregnancy, had fewer than 5 ANC visits, had low folate levels, were anemic, had had a prior induced abortion or a prior stillbirth and delivered a stillborn infant compared to their counterparts (Table 1).

Of the 746 women, 407 (54.6%) had neither infection, 147 (19.7%) were infected with *P. falciparum* only, 68 (9.1%) were infected with helminths only, while 124 (16.6%) were coinfect ed. A higher proportion of women with either organism presented with stillbirth than women with neither infection. Women who were coinfect ed had a modestly higher rate of stillbirth than women with a single infection (Table 2).

Low serum folate, severe anemia, prior induced abortion and prior stillbirth were each strongly, independently associated with stillbirth, with increased odds ranging from over 3-fold to a 6-fold increase (Table 3). Women with malaria irrespective of whether or not they had intestinal helminths had a 90% increased odds of stillbirth. Although intestinal helmint infection had a stronger association, it was not statistically significant (Table 3).

### 5. Discussion

This study demonstrated that the study population had a relatively high rate of stillbirth (5% of all deliveries). Factors associated with stillbirth were malaria, severe anemia, low serum folate concentration, past induced abortion, and history of stillbirth.

Many stillbirths were fresh (75.7%), an indication that a proportion of these cases could likely have been prevented [5].

It has been suggested that stillbirths occurring in the peripartum period could be prevented through appropriate cesarean section, improved obstetric care, and improved emergency response to obstetric complications [5]. In this study, women who had fewer antenatal care visits had an increased risk of stillbirth, suggesting that stillbirths are closely linked to use and quality of maternal services [33].

Malaria is endemic in many African countries, and is thought to play a role in contributing to stillbirth [9]. We observed an association between malaria and stillbirth. A similar finding has been observed in sub-Saharan Africa [34]. Intestinal helmint infections, especially Hookworms and *Trichuris* can cause anemia [18, 19], which in turn leads to adverse birth outcomes including stillbirth [20]. We did not observe an association between intestinal helmint infections and stillbirth, a finding that has been previously reported [35]. However, our observation could be the result of small numbers, that is, malaria was more common than intestinal helmintes. Coinfection with malaria and intestinal helmintes did not increase the risk for stillbirth but as in the case of intestinal helmintes, this could be a matter of numbers. A study in Tanzania found that 63% of stillbirths were attributable to anemia [20]. Malaria contributes to anemia by hemolysis or destruction of parasitized cells and causes shortened red cell survival [36, 37], while hookworms and *Trichuris* cause anemia through direct blood loss [19, 38]. Since the mechanisms by which malaria and intestinal helmint infections cause anemia differ, it is possible that their impact on anemia are additive [39] and could exacerbate adverse birth outcomes. Anemia was a risk factor for stillbirth in this study. The association between anemia and stillbirth has been demonstrated previously [20]. Low serum folate was associated with stillbirth. Folate deficiency causes megaloblastic anemia [22]. Circulating folate concentrations decline in pregnant women, hence the need for folate supplementation [22].
Table 1: Demographic and obstetric characteristics of Ghanaian women by stillbirth status, 2006 (N = 746).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ALL</th>
<th>Stillbirth</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Age:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>102</td>
<td>13.7</td>
<td>96</td>
<td>13.5</td>
<td>6</td>
</tr>
<tr>
<td>20–24</td>
<td>188</td>
<td>25.2</td>
<td>178</td>
<td>25.1</td>
<td>10</td>
</tr>
<tr>
<td>25–29</td>
<td>215</td>
<td>28.8</td>
<td>205</td>
<td>28.9</td>
<td>10</td>
</tr>
<tr>
<td>≥30</td>
<td>241</td>
<td>32.3</td>
<td>230</td>
<td>32.4</td>
<td>11</td>
</tr>
<tr>
<td>Formal education</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>164</td>
<td>22.1</td>
<td>157</td>
<td>22.2</td>
<td>7</td>
</tr>
<tr>
<td>Primary</td>
<td>98</td>
<td>13.2</td>
<td>89</td>
<td>12.6</td>
<td>9</td>
</tr>
<tr>
<td>Middle or Junior Secondary</td>
<td>363</td>
<td>48.9</td>
<td>348</td>
<td>49.2</td>
<td>15</td>
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<tr>
<td>≥Senior Secondary</td>
<td>117</td>
<td>15.8</td>
<td>113</td>
<td>16.0</td>
<td>4</td>
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<tr>
<td>Weekly income</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100,000</td>
<td>175</td>
<td>23.7</td>
<td>162</td>
<td>23.0</td>
<td>13</td>
</tr>
<tr>
<td>100,000–199,000</td>
<td>49</td>
<td>6.6</td>
<td>48</td>
<td>6.8</td>
<td>1</td>
</tr>
<tr>
<td>200,000–354,000</td>
<td>295</td>
<td>39.9</td>
<td>284</td>
<td>40.3</td>
<td>11</td>
</tr>
<tr>
<td>≥355,000</td>
<td>220</td>
<td>29.8</td>
<td>211</td>
<td>29.9</td>
<td>9</td>
</tr>
<tr>
<td>Marital Status</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>156</td>
<td>21.1</td>
<td>143</td>
<td>20.3</td>
<td>13</td>
</tr>
<tr>
<td>Living in union</td>
<td>140</td>
<td>18.9</td>
<td>134</td>
<td>19.0</td>
<td>6</td>
</tr>
<tr>
<td>Married</td>
<td>445</td>
<td>60.1</td>
<td>429</td>
<td>60.8</td>
<td>16</td>
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<tr>
<td>Gravidity</td>
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<td></td>
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<tr>
<td>One</td>
<td>225</td>
<td>30.2</td>
<td>216</td>
<td>30.5</td>
<td>9</td>
</tr>
<tr>
<td>Two</td>
<td>141</td>
<td>18.9</td>
<td>137</td>
<td>19.3</td>
<td>4</td>
</tr>
<tr>
<td>≥Three</td>
<td>380</td>
<td>50.9</td>
<td>356</td>
<td>50.2</td>
<td>24</td>
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<tr>
<td>Trimester at first ANC visit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>389</td>
<td>52.8</td>
<td>370</td>
<td>52.5</td>
<td>19</td>
</tr>
<tr>
<td>Second</td>
<td>325</td>
<td>43.9</td>
<td>311</td>
<td>44.1</td>
<td>14</td>
</tr>
<tr>
<td>Third/none</td>
<td>23</td>
<td>3.5</td>
<td>24</td>
<td>3.4</td>
<td>2</td>
</tr>
<tr>
<td>Less than 5 ANC visits</td>
<td>318</td>
<td>43.2</td>
<td>296</td>
<td>42.2</td>
<td>22</td>
</tr>
<tr>
<td>Sulfadoxine pyrimethamine doses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>197</td>
<td>26.4</td>
<td>177</td>
<td>25.0</td>
<td>20</td>
</tr>
<tr>
<td>One</td>
<td>196</td>
<td>26.7</td>
<td>188</td>
<td>26.5</td>
<td>8</td>
</tr>
<tr>
<td>Two</td>
<td>99</td>
<td>13.3</td>
<td>94</td>
<td>13.3</td>
<td>5</td>
</tr>
<tr>
<td>Three</td>
<td>254</td>
<td>34.1</td>
<td>250</td>
<td>35.3</td>
<td>4</td>
</tr>
<tr>
<td>No deworming</td>
<td>719</td>
<td>96.9</td>
<td>685</td>
<td>96.9</td>
<td>34</td>
</tr>
<tr>
<td>Folate level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>290</td>
<td>55.6</td>
<td>262</td>
<td>53.5</td>
<td>28</td>
</tr>
<tr>
<td>Normal</td>
<td>232</td>
<td>44.4</td>
<td>228</td>
<td>46.5</td>
<td>4</td>
</tr>
<tr>
<td>Hemoglobin level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (≥11 g/dL)</td>
<td>512</td>
<td>69.5</td>
<td>496</td>
<td>70.8</td>
<td>16</td>
</tr>
<tr>
<td>Moderate anemia (8–10.9 g/dL)</td>
<td>192</td>
<td>26.1</td>
<td>179</td>
<td>25.5</td>
<td>13</td>
</tr>
<tr>
<td>Severe anemia (&lt;8 g/dL)</td>
<td>33</td>
<td>4.5</td>
<td>26</td>
<td>3.7</td>
<td>7</td>
</tr>
<tr>
<td>Previous induced abortion</td>
<td>217</td>
<td>29.5</td>
<td>192</td>
<td>27.5</td>
<td>25</td>
</tr>
<tr>
<td>History of stillbirth</td>
<td>27</td>
<td>5.2</td>
<td>16</td>
<td>3.3</td>
<td>11</td>
</tr>
</tbody>
</table>

Numbers in each category may be less than the total due to missing values; Bold P < .05; N = number.
Table 2: Malaria and intestinal helminth infection status of 746 Ghanaian women according to whether or not they had stillbirths, 2006.

<table>
<thead>
<tr>
<th>Infection Status</th>
<th>ALL</th>
<th>col %</th>
<th>N</th>
<th>Stillbirth</th>
<th>row %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes*</td>
<td>271</td>
<td>36.3</td>
<td>22</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>475</td>
<td>63.7</td>
<td>15</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>P =</td>
<td></td>
<td></td>
<td></td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>Helminths</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes*</td>
<td>192</td>
<td>25.7</td>
<td>17</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>554</td>
<td>74.3</td>
<td>20</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>P =</td>
<td></td>
<td></td>
<td></td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>407</td>
<td>54.6</td>
<td>10</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Malaria alone</td>
<td>147</td>
<td>19.7</td>
<td>10</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>Helminths alone</td>
<td>68</td>
<td>9.1</td>
<td>5</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>Malaria and helminth coinfect</td>
<td>124</td>
<td>16.6</td>
<td>12</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>P =</td>
<td></td>
<td></td>
<td></td>
<td>&lt;.01</td>
<td></td>
</tr>
</tbody>
</table>

Col = column; N = number; * with or without other infection. Bold P < .05.

Table 3: Risk factors associated with stillbirth in Ghana, 2006.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Crude (a)</th>
<th>(a) Adjusted</th>
<th>(b) Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (per 5 years)</td>
<td>1.4</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Single</td>
<td>2.3</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Primigravidae</td>
<td>0.7</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>No SP doses</td>
<td>2.5</td>
<td>2.7</td>
<td>2.3</td>
</tr>
<tr>
<td>First trimester ANC visit</td>
<td>1.0</td>
<td>2.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Low serum folate</td>
<td>3.6</td>
<td>3.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Anemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate versus normal</td>
<td>2.3</td>
<td>3.3</td>
<td>2.9</td>
</tr>
<tr>
<td>Severe versus normal</td>
<td>4.8</td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Past induced abortion</td>
<td>5.3</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Past stillbirth</td>
<td>5.7</td>
<td>6.4</td>
<td>6.1</td>
</tr>
<tr>
<td>Infection status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria (yes versus no)</td>
<td>2.7</td>
<td>1.9</td>
<td>N/A</td>
</tr>
<tr>
<td>Intestinal helminths (yes versus no)</td>
<td>2.6</td>
<td>2.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Infection status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria only versus uninfected</td>
<td>2.9</td>
<td>N/A</td>
<td>1.7</td>
</tr>
<tr>
<td>Helminths only versus uninfected</td>
<td>3.2</td>
<td>N/A</td>
<td>2.8</td>
</tr>
<tr>
<td>Coinfected versus uninfected</td>
<td>4.3</td>
<td>N/A</td>
<td>1.7</td>
</tr>
</tbody>
</table>

OR-Odds Ratio. CI- Confidence interval. Bold P < .05.

(a) Model includes 2 individual infections with or without the other infection.
(b) Model includes single infections and coinfection.

A strong association has been observed between maternal plasma, cord plasma, and placental folate concentrations, suggesting that transplacental folate delivery depends on maternal plasma folate concentrations [22]. Some studies [40, 41] have reported higher rates of stillbirth in women with megaloblastic anemia than those without.

Another risk factor for stillbirth in our study is history of induced abortion. Abortion is legal in Ghana only for medical reasons, and is not available upon request (Ministry of Health, Ghana). Most women seeking abortion therefore sometimes attempt illegal abortions, and then go to the hospital for treatment of complications [42]. Removal of retained products of conception in the hospital setting is usually performed by cervical dilation and curettage [43]. There has been concern that this may result in cervical insufficiency, hence future adverse birth outcomes [44].

History of stillbirth substantially increased the risk of stillbirth in the study population. The tendency to repeat pregnancy outcomes in successive births is well known and includes risk of stillbirth [45]. Previous studies have
demonstrated that women with a history of stillbirth may have a 6 to 10-fold increased risk of stillbirth [46, 47]. The causal mechanism may involve impaired placental development and function due to compromised vascular support system [48].

A methodological weakness of our study lies in limited power due to the number of stillbirths, therefore our findings should be interpreted with caution. The fact that it was a cross-sectional study also limits our ability to draw causal or temporal associations. The study however has several strengths including new findings, high participation and consistency with other studies in some risk factors of stillbirth, which strengthens confidence in the new findings. Another strength of the study lies in the fact that the hospitals in which the study was conducted were secondary and tertiary hospitals which cater to large numbers of women of all socioeconomic status from Kumasi and surrounding areas. The 2008 Demographic Health Survey for Ghana (DHS, 2008) reported that 82.4% of women in urban areas in Ghana deliver in a health facility. Our results are therefore representative of most pregnant women in the area.

The fact that most of thestillbirths were fresh suggests that higher quality intrapartum care could reduce stillbirth rates. More studies need to be conducted to further assess the association between stillbirth and malaria and intestinal helminth infection. It is important to conduct further studies to investigate risk factors of stillbirth to determine which stillbirths are preventable so that targeted interventions can be developed and tailored for resource-poor settings.

Acknowledgments

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References


Clinical Study

Hepatitis B Response of Premature Infants after Primary and Booster Immunisation with a Diphtheria-Tetanus-Acellular Pertussis-Hepatitis B-Inactivated Poliovirus/Haemophilus Influenzae Type B Vaccine

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A range of schedules are recommended for hepatitis B vaccination of premature infants. This open-label study (217744/083) compared the immune response of premature (N = 94) and full-term infants (N = 92) to hepatitis B antigen following primary administration of hexavalent DTPa-HBV-IPV/Hib vaccine at 2–4–6 months and a booster dose at 18 months. Anti-HBsAg antibodies were determined before and one month after primary and booster doses. There were no significant differences in postprimary seroprotection rates (anti-HBsAg > 10 mIU/mL; preterm 93.4%; full-term 95.2%) or geometric mean concentrations (634 versus 867 mIU/ml), and neither appeared to be related to gestational length or birth weight. Prebooster seroprotection rates were 75 and 80.6%, respectively. Six premature infants did not respond to primary and booster doses. Primary and booster vaccinations with DTPa-HBV-IPV/Hib elicit satisfactory anti-HBsAg responses in preterm infants, which are not influenced by gestational age or birth weight. This schedule and vaccine will greatly facilitate the immunisation of premature infants.

1. Introduction

Infection with hepatitis B virus (HBV) persists as a worldwide public health problem, with vertical transmission of HBV being responsible for approximately one third of all new cases of hepatitis B. Childhood hepatitis B immunisation has significantly reduced the incidence and prevalence of HBV infection [1], and currently more than 160 countries use hepatitis B vaccine in their national immunisation programmes. Exciting new global initiatives have been implemented that allow the poorest countries in the world to afford this vaccine [2].

The number of infants born prematurely has risen in the last 15 years, and recent advances in the care of premature infants have substantially increased their survival rates [3]. It is now thought that prematurity, rather than a specific gestational age or birth weight, is more predictive of immunologic HBV response compared with full-term infants. The latest recommendation is that medically stable preterm or low-birth weight babies (weighing >2000 g) who are born to hepatitis B surface antigen (HBsAg) negative mothers should receive HBV at birth or shortly thereafter [4].

Concern among parents and paediatricians about the number of injections required during each immunisation visit has contributed to the observation that routine paediatric vaccination is often delayed in preterm infants [5, 6]. However, several combined vaccines are now available which reduce the required number of injections and medical visits. The acceptability of multiple immunisations and increasing the coverage of each vaccine can thereby be achieved.

We have previously shown preterm infants less than 37 weeks of gestational age to display satisfactory immune response to all component antigens of a hexavalent
diphtheria-tetanus-acellular pertussis-hepatitis B-inacti-
vated poliovirus-Haemophilus influenzae type B vaccine
(DTPa-HBV-IPV/Hib), with seroprotection/vaccine response
rates generally similar to those seen in full-term infants
following primary vaccination and a booster dose [7–9].
This paper details the results of a further analysis of the
immune response in the preterm cohort to the hepatitis B
component of this hexavalent vaccine, including a follow-up
at 18 months and a specific focus on the nonresponders.

2. Materials and Methods

2.1. Study Design. This was an open-label study with two
parallel groups: preterm subjects (<37 weeks’ gestation) and
a control group of full-term infants. The study protocol was
approved by the Clinical Investigation Research Board of
La Paz Hospital and conducted in accordance with Good
Clinical Practice Guidelines. Written informed consent was
obtained from the parents or guardians of all subjects prior
to enrolment. Trial number: 217744/083 (DTPa-HBV-IPV-
083).

2.2. Subjects. Preterm subjects were stratified according to
clinical characteristics including length of gestation, birth
weight, pre- and postnatal steroid use, need for red blood cell
transfusion, and weight at 6 months. Length of gestation was
determined by date of the last menstrual period and/or early ultrasound scan and subsequently confirmed by neonatal
examination. Exclusion criteria comprised major congenital
defects or serious chronic illnesses, severe neurologic damage
or nontreatable convulsions; known or suspected immune
defects or serious chronic illnesses, severe neurologic damage
ultrasound scan and subsequently confirmed by neonatal
weight, pre- and postnatal steroid use, need for red blood cell

2.3. Vaccines. All subjects were primed with a DTPa-HBV-
IPV/Hib vaccine [Infanrix hexa, GlaxoSmithKline (GSK)
Biologicals, Rixensart, Belgium] at 2, 4, and 6 months and
received a booster dose with the same vaccine at 18 months of
age. The vaccine was administered as a 0.5 mL intramuscular
injection into the right anterolateral thigh.

Each dose of DTPa-HBV-IPV/Hib vaccine contained ≥30
international units (IU) of diphtheria toxoid, ≥40 IU of
tetanus toxoid, 25 µg of adsorbed pertussis toxin, 25 µg of
adsorbed filamentous hemagglutinin, 8 µg of adsorbed per-
tactin, 10 µg of HBsAg, 40, 8, and 32 D-antigen units of polio
virus types 1, 2, and 3, respectively, and 0.7 mg of aluminum
salts. The lyophilized Hib vaccine pellet contained 10 µg
of Haemophilus influenzae type b polysaccharide conjugated
to 20 to 40 µg of tetanus toxoid, 0.12 mg of aluminium
phosphate, and 10 mg of lactose.

For infants not responding to primary and booster doses
(anti-HBs <10 mIU/mL), three further 10 µg doses of hepatic
s recombinant vaccine (Engerix-B; GSK Biologicals) could
be administered in the third year of life. If the anti-HBs titre
was above 100 mIU/mL after the second additional dose (6th
in total), then the third dose was not given. If it was below
100 mIU/mL, then the third dose was administered.

2.4. Immunogenicity Analysis. Blood samples were drawn
before the first dose and one month after the third in primary
course and before and one month after the booster dose.
Serum samples were stored at −20 ºC until analysis at GSK
Biologicals. Antibodies against HBV were determined using
ELISA (enzyme-linked immunosorbent assay), with an assay
cut-off of 10 mIU/mL for HBsAg.

2.5. Statistical Analysis. Statistical analysis of the immuno-
genicity results was performed on the according-to-protocol
(ATP) cohort. Seroprotection rates and antibody geometric
mean concentrations (GMCs) were calculated with exact
95% confidence intervals (CI) at each time point. Differences
in seroprotection rates between the preterm and full-term
groups were compared using Fisher’s exact test.

3. Results

A total of 186 infants were enrolled (94 preterm and 92 full-
term), of whom 93 and 89 infants, respectively complied
with the criteria for inclusion in the ATP cohort for analysis
of immunogenicity of the primary vaccination course. The
demographic and neonatal characteristics of the infants have
been previously described [7]. The mean age at the time of
first immunisation was 8.6 ± 0.63 weeks (range 8–11 weeks)
and 8.2 ± 0.8 (range 6–11 weeks), respectively.

Of the 155 infants who received booster doses, 152
subjects were included in the ATP cohort for analysis of
immunogenicity of the booster dose (84 prematurely born
subjects and 68 full-term subjects). Mean age at the time
of booster vaccination was 18.2 ± 0.47 months (range 18–20
months) and 18.3 ± 0.55 months (range 18–20 months),
respectively.

3.1. Postprimary Immunogenicity Results. The mean
immunogenicity data after primary course and before and after
booster dose are included in Table 1. A total of 93.4% of
the preterm and 95.2% of the full-term infants responded to
primary vaccination (anti-HBs ≥10 mIU/mL). Although the
GMCs after the primary course were numerically lower in the
preterm group, the differences were not significant.

The immunogenicity results by gestational age are shown
in Table 2. All subjects with a gestation below 31 weeks
were seroprotected compared with 83.3% and 92.6% in
the groups with a higher gestational age. However, with
respect to GMCs, a numerically higher response was seen
in the groups aged 34–36 weeks and 28–30 weeks but the
differences were not significantly different. After the booster
dose, the seroprotection rates were lower for the groups with
higher gestational age but GMCs retained a similar pattern to that observed after the primary course.

A nonconsistent response was also seen in the analysis according to birth weight (Table 3), where 92% of subjects with the lowest birth weight were protected compared with all babies with birth weight 1000–1500 g. Only 82.4% of those weighing more than 2000 g at birth were seroprotected. No specific pattern of GMC response was observed. Similarly, there was no significant difference according to babies with (N = 15) or without (N = 76) intrauterine growth restriction (sero protection rates: 86.6 and 94.7%, resp.), or with (N = 67) or without (N = 24) prenatal steroids (seroprotection rates: 91 and 100%, resp.).

Post-primary anti-HBs seroprotection rates and GMCs were numerically lower in the nine subjects who had received postnatal steroids (88.9% and 188.1, resp.) than the 81 subjects who had not received them (93.9% and 724.6, resp.), but the differences were not significant.

Blood transfusion during the neonatal period did not significantly affect seroprotection rates (anti-HBs ≥10 mIU/mL) or GMCs ([transfused infants: 94.7% (95% CI 82.3–99.4) and 486.6 (95% CI 280.0–845.5) mIU/mL, respectively; no transfusion: 92% (95% CI 81.8–97.9) and 766.8 (95% CI 542.8–1298.7) mIU/mL, resp.) in all babies.

Considering weight at 2 and 6 months as a variable independent of gestational age and birth weight, no significant patterns of differences were observed in seroprotection rates (Table 4).

### 3.2 Prebooster Persistence and Booster Response
Before the booster dose, both the seroprotection rates of anti-HBs GMCs were low in both the pre- and full-term groups (Table 1). Seroprotection rose to 91.6 and 98.5%, respectively one month after the booster dose and the GMCs increased to 1771 and 1965 mIU/mL, respectively. There were no significant differences between the responses in the preterm and full-term groups.

### 3.3 Nonresponders
Six preterm infants (6.59%) did not respond to primary immunisation and also failed to respond to the booster dose (anti-HBs ≥10 mIU/mL; Table 5). All of these infants had a gestational age above 31 weeks. Two babies weighed less than 1000 g at birth and had severe intrauterine growth restriction (IUGR; 32 weeks/600 g and 31 weeks/800 g), but the other four babies did not have any risk factors.

One infant responded to the 5th HBV dose, two to the 6th, and one to the 7th. Two babies (2.19%) never responded.

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### Table 1: Anti-HBs seroprotection rates and GMCs in preterm and full-term infants one month after the primary course and before and after the booster dose (ATP cohort for immunogenicity).

<table>
<thead>
<tr>
<th></th>
<th>Seroprotection</th>
<th>GMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n</td>
</tr>
<tr>
<td>Postprimary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm</td>
<td>91</td>
<td>85</td>
</tr>
<tr>
<td>Full-term</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>Prebooster</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm</td>
<td>84</td>
<td>63</td>
</tr>
<tr>
<td>Full-term</td>
<td>67</td>
<td>54</td>
</tr>
<tr>
<td>Postbooster</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm</td>
<td>83</td>
<td>76</td>
</tr>
<tr>
<td>Full-term</td>
<td>68</td>
<td>67</td>
</tr>
</tbody>
</table>

### Table 2: Anti-HBs seroprotection rates (SR) and geometric mean concentrations (GMC) by subgroup of preterm infants after primary course and booster dose (ATP cohort for immunogenicity).

<table>
<thead>
<tr>
<th></th>
<th>Seroprotection</th>
<th>GMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n</td>
</tr>
<tr>
<td>Primary course</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34–36</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>31–33</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>28–30</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>24–27</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Booster dose</td>
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<td></td>
</tr>
<tr>
<td>34–36</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>31–33</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>28–30</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>24–27</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>
Table 3: Post-primary anti-HBs seroprotection rates and GMCs by birth weight (ATP cohort for immunogenicity).

<table>
<thead>
<tr>
<th>Birth weight (kg)</th>
<th>N</th>
<th>n</th>
<th>% 95% CI</th>
<th>mIU/ml 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥2.0</td>
<td>17</td>
<td>14</td>
<td>82.4</td>
<td>156.6–96.2</td>
</tr>
<tr>
<td>≥1.5&lt;2.0</td>
<td>23</td>
<td>22</td>
<td>95.7</td>
<td>78.1–99.9</td>
</tr>
<tr>
<td>≥1.0&lt;1.5</td>
<td>26</td>
<td>26</td>
<td>100</td>
<td>86.8–100</td>
</tr>
<tr>
<td>&lt;1.0</td>
<td>25</td>
<td>23</td>
<td>92.0</td>
<td>74.0–99.0</td>
</tr>
</tbody>
</table>

Table 4: Post-primary anti-HBs seroprotection rates and GMCs by weight at vaccination and percentile weight at six months (ATP cohort for immunogenicity).

<table>
<thead>
<tr>
<th>Weight at first vaccination (kg)</th>
<th>N</th>
<th>n</th>
<th>% 95% CI</th>
<th>mIU/ml 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥4</td>
<td>19</td>
<td>16</td>
<td>84.2</td>
<td>60.4–96.6</td>
</tr>
<tr>
<td>≥3&lt;4</td>
<td>26</td>
<td>25</td>
<td>96.2</td>
<td>80.4–99.9</td>
</tr>
<tr>
<td>≥2&lt;3</td>
<td>26</td>
<td>26</td>
<td>100</td>
<td>86.8–100</td>
</tr>
<tr>
<td>&lt;2</td>
<td>20</td>
<td>18</td>
<td>90.0</td>
<td>68.3–98.8</td>
</tr>
<tr>
<td>Percentile weight at six months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>29</td>
<td>27</td>
<td>93.1</td>
<td>77.2–99.2</td>
</tr>
<tr>
<td>&gt;25 ≤50</td>
<td>21</td>
<td>20</td>
<td>95.2</td>
<td>76.2–99.9</td>
</tr>
<tr>
<td>&gt;10 ≤25</td>
<td>14</td>
<td>13</td>
<td>92.9</td>
<td>66.1–99.8</td>
</tr>
<tr>
<td>≤10</td>
<td>27</td>
<td>25</td>
<td>92.6</td>
<td>75.7–99.1</td>
</tr>
</tbody>
</table>

Table 5: Anti-HBs concentrations (mIU/ml) after all HBV doses in subjects who were non-responders to hepatitis B at the end of the primary and booster studies.

<table>
<thead>
<tr>
<th>Gestational age (Weeks)</th>
<th>Birth weight (kg)</th>
<th>Percentile at birth</th>
<th>Weight at 1st vaccination (kg)</th>
<th>Percentile at 6 months</th>
<th>Weight at 6 months (kg)</th>
<th>Pre-primary</th>
<th>Post-primary</th>
<th>Pre-booster</th>
<th>Post-booster</th>
<th>Post Extra HBV</th>
<th>Persistence at 4 years (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>2.1</td>
<td>75</td>
<td>4.4</td>
<td>95</td>
<td>8.3</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>690</td>
<td>3485 NG 176</td>
</tr>
<tr>
<td>35</td>
<td>2.0</td>
<td>25</td>
<td>4.0</td>
<td>25</td>
<td>6.7</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>32 128</td>
</tr>
<tr>
<td>32</td>
<td>0.6</td>
<td>&lt;10</td>
<td>1.7</td>
<td>&lt;5</td>
<td>4.4</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>141 NG 64</td>
</tr>
<tr>
<td>31</td>
<td>0.8</td>
<td>&lt;10</td>
<td>1.7</td>
<td>10</td>
<td>5.1</td>
<td>97</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10 NA NG</td>
</tr>
<tr>
<td>33</td>
<td>2.0</td>
<td>50</td>
<td>4.3</td>
<td>&gt;90</td>
<td>8.4</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10 1087 NG NA</td>
</tr>
<tr>
<td>35</td>
<td>1.7</td>
<td>10</td>
<td>3.6</td>
<td>50</td>
<td>6.6</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10 1087 NG NA</td>
</tr>
</tbody>
</table>

NA = Not available; NG = Not given.

One was an infant with IUGR who had anti-HBs antibodies before vaccination and the other although born prematurely at 33 weeks weighed 2000 g and did not have risk factors. These 6 infants did show a good response to the other antigens included in the DTPa-HBV-IPV/Hib vaccine.

4. Discussion

The American Academy of Pediatrics (AAP) recommends that all infants receive hepatitis B vaccine at birth or before discharge from the hospital. In infants weighing less than 2000 g and born to HbsAg negative mothers, the first dose of hepatitis B vaccine is recommended at 30 days [4].

Immunogenicity studies indicate that premature children generally respond less well to hepatitis B vaccination than full-term infants, both in terms of seroprotection rates [10–15] and GMCs [14, 16–21]. Our results are consistent with these findings, although the seroprotection rate in preterm subjects was not significantly different to that in full-term subjects.

Analysis according to birth weight in premature infants has suggested a relationship between reduced immunological response and low birth weight [16, 20, 22, 23]. Interestingly, in our study, babies under 1000 g achieved higher seroprotection rates than those children with a birth weight in excess of 2000 g, but their GMC response was lower.
Gestational age seems to be a much more objective parameter than weight for assessing immune response in premature babies. After birth dosing, both seroprotection rates and GMCs are generally lower in babies with a reduced gestational age [16, 20, 21, 23] but this trend reverses as the chronological age at the time of starting vaccination increases [24]. In our study, all children with a gestational age below 31 weeks responded to immunisation in the two groups with a greater gestational age. The response in terms of GMCs was however irregular.

Our findings strengthen the notion that the immunological response in premature babies depends on postnatal age of vaccination and not on birth weight or gestational age. Indeed, studies in term infants have demonstrated that very young infants (up to 2 months) achieve lower GMCs after three doses of hepatitis B vaccine compared to infants who are at least 3 months of age at the time of first vaccination [25].

Low-birth weight has been reported to be associated with an inadequate immune response to early hepatitis B vaccination in premature infants by some workers [16], but others have either shown no effect [19] or the opposite [26]. Similar anomalies have been seen when considering gestational age [19, 26] and the postnatal use of corticosteroids [16, 26, 27]. In our study all of the nonresponders had a gestational age of at least 31 weeks and, except for two babies with severe IUGR, were of appropriate weight. Nine infants received postnatal corticosteroids and although they responded to a lesser extent to hepatitis B vaccination, the differences were not significant. Only one of these babies was a non-responder. Two children in our cohort received transfusions (1 or 6) in the neonatal period, coinciding with severe intrauterine restriction, but no differences in response were identified between the transfused and nontransfused infants.

Other factors linked to nonresponse to hepatitis B vaccination in premature babies include hyperbilirubinaemia [28], sepsis [16, 19, 26], and the presence of specific antibodies transferred by the mother through the placenta. All of the children in our study who did not respond to HBsAg developed jaundice, but all were within physiological limits for bilirubin. Sepsis was recorded in only one of the infants classified as a non-responder. Only one of our non-responding babies had prior antibodies, but in conjunction with severe intrauterine restriction and the receipt of multiple transfusions.

We found no differences in immune response in the infants with either IUGR, or in the different groups stratified according to percentile weight at 6 months, consistent with previous findings [15, 17]. However, at the time of vaccine initiation, GMCs were lower in those children who weighed less than 2 kg but this is logical given that they were smaller at birth.

Three doses of HB vaccines are generally recommended in preterm babies, although 4 doses have been suggested [10, 15, 17, 22, 29, 30], as well as an antibody test after the third to assess the suitability of a booster dose [19, 20, 22]. In a study of 29 preterm infants who were non-responders, 14 received additional doses of vaccine; 7 seroconverted after the first additional dose, 3 required a second additional dose and 1 required a third [16]. In another study although only three of the nine non-responding pre-term infants returned for a fourth vaccine dose, all subsequently seroconverted [26]. We decided to administer 4 doses, but according to the schedule recommended for full-term babies, and include a booster dose at 18 months with the hexavalent vaccine used for primary vaccination. All six infants that did not respond to the primary course also did not respond to a fourth dose; 1 infant responded to the fifth, 2 to the sixth, and 1 after seven doses. Only two infants remained non-responders.

Few authors have studied the persistence of anti-HBs in premature infants; however, Kesler et al. [27] reported that similar declines in antibody titres are seen in pre-term and full-term babies after primary vaccination, although preterm infants generally have a lower titre. We found similar results prior to the booster dose and observed a good immune response with similar GMCs among preterm and full-term infants after the booster.

Many long-term studies of infants indicate that immunisation against hepatitis B induces both antibody-producing cells and memory cells, and the rapid development of anamnestic responses [31]. Booster doses of vaccine do not seem necessary to ensure long-term protection [32–34], although some factors might be important. Delaying vaccination until infants achieve a weight of 2000 g results in a significantly higher GMC at 3–3.5 year of age as compared to early vaccination in both pre- and full-term infants [35]. Most children vaccinated at birth retain immunological memory to hepatitis B vaccine for 15 years, but those who did not were more likely to have received HB Ig immunoglobulin at birth, suggesting that passive antibody may interfere with the induction of immunological memory [36].

In summary, preterm babies born to HBsAg-negative mothers and vaccinated with a hexavalent DTPa-HBV-IPV/Hib vaccine at 2, 4, and 6 months responded well immunologically and similarly to children born at full term. The response in terms of seroprotection rates was not influenced either by gestational age or birth weight, and these findings strengthen the notion that the immunological response in premature babies depends on postnatal age and not on birth weight or gestational age. Six infants, all with a gestation age above 31 weeks, did not respond, but we did not find any associated risk factors, other than severe intrauterine malnutrition in two babies. After a further vaccination cycle, only 2 remained “true” non-responders.

The vaccination schedule used here for preterm babies born to HBsAg-negative mothers offers a single schedule for all infants, with fewer injections and better acceptance.

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