Prematurity and protracted mechanical ventilation as risk factors for *Pneumocystis jiroveci* infection in HIV-negative neonates in an intensive care unit

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This work was undertaken to elucidate some aspects of the epidemiology of Pneumocystis pneumonia (PP). We studied 42 mechanically ventilated, human immunodeficiency virus (HIV)-negative, severely ill neonates treated at an intensive care unit. The study group included 40 premature neonates and two mature neonates with lethal congenital defects. Progressive respiratory dysfunction in PP necessitated mechanical ventilation. Infection was usually noticeable on the 22nd day of life or after 12 days of ventilation. The usual manifestations included apnea, pallor, copious frothy sputum, seizures, and feeding difficulties. The diagnosis was established by detecting *Pneumocystis jiroveci* cysts in bronchial lavage fluid specimens (88.1% sensitivity). PP was managed with cotrimoxazole and pentamidine combination therapy administered over 14 days. No clinical improvement was noted in four neonates and three of them died during therapy. Prematurity and protracted mechanical ventilation are two risk factors for *P. jiroveci* infection in severely ill neonates in an intensive care unit.

Key words: Pneumocystis jiroveci, newborn infant, pneumonia, mechanical ventilation, prematurity.

Pneumocystis jiroveci, previously known as *Pneumocystis carinii* forma specialis hominis, is an atypical fungus responsible for Pneumocystis pneumonia (PP) in patients with immune deficits that accompany acquired immunodeficiency syndrome (AIDS), immunosuppression, transplantation, or steroid therapy[1-5]. Also at increased risk of *P. jiroveci* infection are severely ill and immature neonates whose cellular ($CD4^+$ cells) and humoral (interferon) responses are compromised[6-7].

Molecular studies have revealed that the genus Pneumocystis is a heterogeneous group of opportunistic fungi with exceptional host specificity[8]. This feature of Pneumocystis is linked with differences as to phenotype and genotype[9,10]. Infections in humans are caused by *P. jiroveci*[1,2]. Healthy humans can serve as a reservoir for the pathogen[11,12]. Vargas et al.[13] identified *P. carinii* DNA in nasopharyngeal secretions of healthy infants and Chabé et al.[14] demonstrated the ability of Pneumocystis to replicate in the lungs of immunocompetent hosts. Thus, transmission of the pathogen among medical personnel and patients is likely and transmission from the mother to the neonate remains possible[11,12,15]. Extrapulmonary sites of pneumocystosis in the liver, lymph nodes, muscles, and rarely in kidneys, thymus and pancreas have been reported in terminally ill AIDS patients[16-18].

The literature on PP in children without coexisting human immunodeficiency virus (HIV) infection is scarce[19-21]. Therefore, we

decided to undertake a clinical observational study of *P. jiroveci* infections in HIV-negative, severely ill, mechanically ventilated neonates.

Material and Methods

We analyzed 234 severely ill, mechanically ventilated neonates at the Clinic of Obstetrics and Perinatology, Pomeranian Medical University, between 1999 and 2003. Neonates were assigned to two groups: A (n=42) with PP, and B (n=192) without PP. Assignment was based on microscopic examination of bronchial lavage fluid and additionally on clinical symptoms, chest X-ray, and laboratory findings.

Intrauterine bacterial infection was recognized during the first 72 hours of life based on maternal history [chorioamnionitis defined as intrapartum fever >38°C, tachycardia (maternal ≥100 beats/min; fetal ≥160 beats/min), uterine tenderness, purulent or foul smell of amniotic fluid or increased total leukocyte count (>15x10^9/L)], clinical symptoms in the neonate (skin color: pallor, jaundice, cyanosis; respiratory dysfunction: apnea, tachypnea >60/min, grunting, nasal flaring, intercostal or sternal retractions, need for high ventilator settings or oxygen; cardiovascular symptoms: brady/tachycardia, poor peripheral perfusion, hypotension; neurologic symptoms: hypotonia, irritability, lethargy, seizures; gastrointestinal dysfunction: abdominal distension, green or bloody residuals, vomiting; temperature instability), and/or positive blood culture. Nosocomial infections were diagnosed when appearing after three days of life. Pneumonia was diagnosed in neonates presenting with characteristic findings in chest X-ray (diffuse, asymmetric or localized, alveolar or interstitial disease) and with respiratory distress[22].

Diagnostic criteria for respiratory distress syndrome (RDS) included oxygen supplementation exceeding 40%, demand for alveolar surfactant, typical chest radiograph (uniform reticulogranular pattern and air bronchograms), and no evidence of systemic infection in laboratory tests[23].

Diagnosis of bronchopulmonary dysplasia (BPD) was made when the infant still required oxygen after 28 days of age or at 36 weeks of postconceptional age, and continued to show signs of respiratory problems at that time. Chest X-ray film may facilitate the diagnosis by demonstrating lungs that resemble ground glass (RDS) or appear spongy as in BPD[23].

Infection (bacterial pneumonia and sepsis) was further confirmed with cultures and biochemical tests of the blood. We determined the concentration of C-reactive protein (CRP values <10 mg/L were considered normal), leukocyte (>25 or <5x10^9/L), and platelet counts (<100x10^{12}/L), and the immature to total neutrophil ratio (I:T <0.2)[24].

In all cases, bronchial lavage fluid specimens were stained with Giemsa reagent and searched for *P. jiroveci* cysts at the start and after 7-10 days of treatment. Microscopy was done by the same, highly-experienced person. Specimens were collected during routine nursing care of the ventilated airways.

We also searched for *P. carinii* DNA in the lavage fluid but the result had no impact on the diagnosis. Polymerase chain reaction (PCR) was done with 130 randomly selected samples which were stored at -24°C prior to the diagnosis or microscopic examination.

DNA was isolated with the Masterpure DNA purification kit – Epicentre. Single PCR was performed with the PC41/PC22 primer pair. Product size was 420 bp consistent with the targeting by these primers of the small ribosome subunit 18S rRNA gene sequence. The following conditions were applied: initial denaturation at 94°C for 5 minutes; 40 cycles consisting of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. We also performed nested PCR with pAZ102H/pAZ102E and pAZ102X/pAZ102Y primers targeting the mitochondrial large subunit (mtLSU) rRNA gene sequence. Product size with these primer pairs was 346 bp and 263 bp, respectively. We used 40 cycles of denaturation at 94°C for 1.5 minutes, annealing at 50°C for 1.5 minutes and extension at 72°C for 2 minutes; other PCR conditions were the same as for PC41/PC22 primers. Positive control was done with DNA isolated from the lung of inoculated rat and supplied by the Polish Institute of Hygiene in Warsaw. Amplification products were electrophoresed in 2% agarose gel and stained with ethidium bromide.

Statistics were done with χ^2-test and Student's t-test and the level of significance was taken as p=0.05.

Results

Pneumocystis pneumonia was diagnosed in 42 (17.9%) neonates (group A). The remaining neonates served as controls (group B). The mothers were Caucasian whites, with an average or good socio-economic status, normal body weight, no immune impairment or systemic disease, HIV-negative, and not on immunosuppressive therapy.

Two children with PP were born at term (40 and 41 weeks of gestation, 2250 and 3000 g), but both had lethal congenital defects (trisomy 18; complex defect of the central nervous system with tracheo-esophageal fistula) and required mechanical ventilation from birth till death (68 and 30 days, respectively). The remaining children in this group were born prematurely.

We found that both groups of neonates (Table I) differed as to maturity and weight at birth. For these reasons, the study and control groups differed significantly as to duration of mechanical ventilation and hospital stay. Immaturity and low birth weight are factors predisposing to serious complications postnatally. Neonates with PP were less mature, had a lower birth weight, and more often demanded steroids to accelerate lung maturation. In this group, there were more cases of RDS necessitating treatment with exogenous surfactant, and likewise, criteria for BPD were more often fulfilled. Bacterial and fungal infections in the form of sepsis or pneumonia were more often diagnosed in PP neonates.

The initial suspicion of PP was based on clinical findings (dyspnea, pallor, copious frothy or mucous sputum, episodes of apnea, feeding difficulties, absence of weight gain, seizures, pulmonary bleeding) and radiologic evidence of interstitial pneumonia. PP was confirmed in 37 out of 42 neonates (sensitivity = 88.1%, specificity = 100%) by the presence of cysts in bronchial lavage fluid. In the remaining five cases with negative staining, the clinical course and radiologic evidence were clearly in favor of the diagnosis. Worth noting is the fact that bronchial fluid was obtained after all five neonates were started on cotrimoxazole. Thus, the final diagnosis of PP was confirmed by positive microscopic examination of bronchial lavage fluid and positive response to specific pneumocystosis therapy.

None of the 130 samples of bronchial fluid from mechanically ventilated neonates tested positive for *P. jiroveci* DNA.

In group A, mechanical ventilatory support was started in the first day of life and continued over three days in 33 neonates (78.6%). Five premature neonates (11.9%) with RDS I/II°

Table I. Demographic and Clinical Data of Mechanically Ventilated Neonates

	Group A (PP Group) n=42	Group B (Control Group) n=192	p
Gestational week; median (range)	27 (23-41)	32 (30-39)	<0.05
Birth weight (g); median (range)	1080 (520-3000)	1570 (830-3860)	<0.05
Males; n (%)	20 (47.6)	78 (40.6)	NS
Emergency cesarean section; n (%)	36 (85.7)	119 (62)	NS
Apgar score at 5 min.; mean±SD	3±2	4±2	NS
Hospitalization in days; mean±SD	64±23	33±19	<0.05
Mechanical ventilation in days; mean±SD	37±18	19±12	<0.05
Prenatal steroid therapy; n (%)	33 (78.6)	90 (46.9)	0.05
NRDS managed with surfactant; n (%)	38 (90.5)	83 (43.2)	<0.01
BPD; n (%)	35 (83.3)	23 (12)	<0.001
Congenital infection; n (%)	28 (66.7)	106 (55.2)	NS
Nosocomial pneumonia; n (%)	26 (61.9)	142 (74)	NS
Bacterial sepsis; n (%)	23 (54.8)	54 (28.1)	<0.05
Mycotic sepsis; n (%)	5 (11.9)	5 (2.6)	<0.05
Antibiotics; n (%)	38 (90.5)	122 (63.5)	<0.01
Days on oxygen; mean±SD	58±14	25±8	<0.05

BPD: Bronchopulmonary dysplasia. NRDS: Neonatal respiratory distress syndrome.

received a single dose of surfactant postnatally and were ventilated during less than 24 h. In the remaining four cases, symptoms of dyspnea appeared during the first 10 days of life. In group B, 99 neonates (52%) required ventilation during less than three days. PP led to further deterioration of respiratory function in all neonates. All neonates with PP required continuous positive airway pressure (CPAP) or intermittent mandatory ventilation (IMV).

Infection with *P. jiroveci* was diagnosed between the 6th and 58th day of life (mean 22±12) or between day 6 and day 27 of ventilation (mean 12±8). PP was the first pulmonary infection in five neonates. In three cases, PP coincided with bacterial infection [Klebsiella oxytoca (n:1), Serratia marcescens (n:2)]. Initial symptoms of *P. jiroveci* infection included apnea and reduced saturation recorded with the oximeter. Symptoms appeared either spontaneously or during nursing procedures, were unaccompanied by slowing of heart action, and were absent in four neonates only. Cyanosis or pallor was usually seen in spite of normal hematology, saturation, or oxygen partial pressure (paO_2) in arterial blood. Frothy or mucous exudate appeared in the airways in large or very large quantities. Pulmonary bleeding of varying intensity was noted in eight neonates. Due to aggravating symptoms of dyspnea, 12 out of 13 previously extubated neonates required re-intubation and assisted ventilation and one required nasal CPAP. The remaining neonates developed PP during mechanical ventilation for RDS, BPD or infection (bacterial or fungal). When infection coincided with mechanical ventilation, an upregulation of the respirator was necessary. Seizures appeared or intensified in 16 neonates. Feeding difficulties, regurgitation, slow gastric emptying, and flatulence were noted in 29 cases (Table II).

Table II. Clinical Findings in the Course of Pneumocystis Pneumonia

Clinical findings	n=42
Dyspnea	42/42
Apnea, reduced Hb saturation	38/42
Pallor	35/42
Copious frothy sputum	35/42
Feeding difficulties	29/42
Seizures	16/42
Pulmonary hemorrhage	8/42

Biochemical determinants of infection (leukocyte, neutrophil, and platelet counts, I:T ratio) were normal. CRP was modestly elevated (between 18.2 and 29.8 mg/L) and thrombocytopenia (85 and $53x10^9$/L) was determined in two children. In both cases, however, pneumocystosis coincided with bacterial infection. Radiologic evidence of interstitial pneumonia was obtained in 37 neonates. Diffuse infiltrates were seen in five children.

Bacteriologically confirmed intrauterine infection was diagnosed in 27 neonates of both groups [positive blood cultures: *Streptococcus agalactiae* (n=7), *Enterococcus faecalis* (n=5), *Escherichia coli* (n=9), *Staphylococcus aureus* (n=2), *Staphylococcus epidermidis* (n=4)]. Nosocomial infections were caused by *Serratia marcescens* (n=5), *Klebsiella pneumoniae* (n=7), *Klebsiella oxytoca* (n=8), and *Staphylococcus haemolyticus*, methicillin-resistant *Staphylococcus aureus* (MRSA) (n=12). Cultures were negative in the remaining cases and the diagnosis of bacterial infection was based on clinical symptoms, characteristic laboratory findings, and response to antibiotics (cephalosporins, penicillin, aminoglycosides, imipenem, vancomycin). Fungal sepsis caused by Candida albicans (n=6) or Candida glabrata (n=6) was diagnosed only in neonates with extremely low birth weight chronically on antibiotics and was managed with fluconazole.

Combined treatment with cotrimoxazole and pentamidine was continued for 14 days in most cases. Cotrimoxazole 40 mg/kg/24h was administered in two doses. A single 4 mg/kg dose of pentamidine was infused during 30 minutes. Microscopic examination was repeated after 7-10 days in five neonates, disclosing the presence of *P. jiroveci*. Treatment was continued and a negative result of microscopy was demonstrated after 28 days. Clinical improvement was noted in 38 neonates after 8.7±2.2 days of therapy (range: 5 to 12 days). In spite of treatment and negative results of microscopy, four neonates failed to improve clinically, showing symptoms of chronic lung disease or other pathology. Three of these neonates died - one (2.4%) due to massive pulmonary hemorrhage evidently associated with pneumocystosis, and the other two (mature) due to congenital defects.

Discussion

The risk of *P. jiroveci* infection is of particular concern in immature or sick neonates. Infection in a Neonatal Intensive Care Unit may be sporadic among children with primary or secondary immune deficiency but may also take the form of an epidemic[25,26]. Sources of infection remain unclear. Stringer[27] reported on long-term colonization by Pneumocystis of immunocompetent hosts. Although Pneumocystis DNA has been detected in air samples, person-to-person transmission remains the most probable route of infection. According to Stringer and Dei-Cas[27,7], symptomatic infection in adults may be the outcome of colonization during infancy or early childhood by genetically variant Pneumocystis strains. Vargas et al.[28] suggested that Pneumocystis lacks the ability to colonize the airways. Thus, PP would be the result of a recent infection. Infection is airborne but the need for isolation of infected patients remains disputable.

Dutz et al.[29] found pneumocystosis in infants aged 10 to 24 weeks. In the present study, symptoms of *P. jiroveci* infection appeared on the average after 20 days from birth. This rapid course of pneumocystosis may be attributed to immaturity of our neonates and of their immune system, surfactant deficiency, or coexisting infection. Iatrogenic causes, like protracted mechanical ventilation, antibiotics, and steroids, may also be considered. The well-known predilection of *P. jiroveci* for cells of the alveoli explains why respiratory symptoms dominate. Growth of Pneumocystis in the infected host is inhibited when the lung contains normal amounts of surfactant[7] and conversely, alterations in the composition and quantity of surfactant contribute to PP. Congenital deficiency of surfactant was noted in 90.5% of neonates in our PP group and in only 43.2% of the control group. Apparently, immaturity-related surfactant deficiency implicated in RDS, as well as protracted mechanical ventilation and oxygen supplementation (leading to BPD), are important risk factors for PP.

Intermittent apnea was the first symptom of PP[19]. Copious amounts of frothy sputum are the result of hypertrophy and proliferation of type II alveolar cells and accumulation of macrophages and eosinophils in the alveolar lumen[7]. Vargas et al.[30] observed sudden infant death associated with *P. carinii* infection. Extrapulmonary symptoms include diarrhea and weight loss[31]. We observed feeding difficulties and gastrointestinal disorders.

Pneumocystis pneumonia is usually managed with cotrimoxazole, pentamidine, or both. Dautzenberg et al.[32] and Jules-Elysee et al.[33] reported on the effectiveness of pentamidine inhalation. Berrington et al.[5] found cotrimoxazole to be effective in all 50 infants with primary immune deficiency. PP is a serious complication in severely ill patients and is marked by a high mortality rate. Identification of high-risk groups and effective prevention remain part of the clinical approach to PP[34]. Cotrimoxazole was found ineffective in preventing PP among HIV-positive patients and children of HIV-positive mothers[4,35]. In our experience, administration of cotrimoxazole to manage an unknown infection will produce false-negative results in bronchial fluid histopathology done to confirm pneumocystosis. However, we always used combination therapy to manage PP.

Our single and nested PCR proved of little value for the diagnosis of *P. carinii* infection. Sing et al.[3] reviewed 27 papers on the diagnosis of PP with traditional microscopy and PCR applied to broncho-alveolar lavage fluid, but failed to find any distinct advantage of PCR. They demonstrated that single or nested PCR has a marginal advantage over microscopy, even in HIV-positive patients. Of greater importance for the diagnosis of PP are the quantity, quality, and moment of specimen collection. Perhaps our disappointing results with PCR were due to the small volume of fluid that can be obtained from an immature neonate. Nevez et al.[36] suggested that the lungs are colonized by too few *P. jiroveci* organisms for a reliable application of PCR. It should also be emphasized that Pneumocystis strains demonstrate marked host specificity. Until recently, the human pathogen *P. jiroveci* was believed to be a strain of *P. carinii*[2,8]. Durand-Joly et al.[10] convincingly demonstrated that transmission of pneumocystosis from animal to man is impossible. Nevertheless, a review of the literature revealed that the same PCR protocol is used to detect *P. carinii* and P. jiroveci[37-40]. One may presuppose that there is no acceptable PCR protocol for the detection of *P. jiroveci* and that the investigator is left with sequencing as the only way to confirm or

exclude amplicon specificity. Further advances in this field await the development of a highly sensitive and specific PCR method that will provide for a rapid and reliable detection of *P. jiroveci* DNA in bronchial lavage fluid from premature neonates. When routinely available, this test shall certainly facilitate the diagnosis of *P. jiroveci* infection.

In conclusion, we believe that prematurity and protracted mechanical ventilation are risk factors for *P. jiroveci* infection in severely ill neonates in an intensive care unit.

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