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Controlling an Outbreak of *Pseudomonas aeruginosa* in a Neonatal Intensive Care Unit: Multivariate Analysis of Risk Factors through a Case-Case- Control Study

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Abstract

Background: Neonates are very susceptible to *Pseudomonas aeruginosa*.

Aim: To describe a *P. aeruginosa* outbreak in our Neonatal Intensive Care Unit (NICU), and understand the underlying individual risk factors for colonization or infection, using a case-case-control design.

Methods: Microbiological studies were done routinely in our neonates to identify and instigate contact precautions if *P. aeruginosa* was present. If found, a search was made for possible environmental reservoirs and Random Amplification of Polymorphic DNA (RAPD) was run on all isolated *P. aeruginosa*. We divided our children into two cohorts, (with and without this microorganism). Finally we designed a case-case-control study to evaluate predictive factors in those infants colonized or infected by *P. aeruginosa*.

Results: In 4 months, 14 infants were infected and 16 were only colonized by *P. aeruginosa*. All the *P aeruginosa* isolated from these children were identical on RAPD, but this organism was not found in any environment reservoirs. Separation by status into two cohorts helped to reduce new cases, but global number of cases (sum of the new and existing cases each week) took longer to fall.

In the same time period another 82 NICU-patients had not *P. aeruginosa*, and they were used as controls. Bivariate and multivariate analysis determined the factors associated with colonization or infection by *P. aeruginosa*.

Conclusion: Risk factors of infected neonates: fungal infection, number of cases in previous week and separation into cohorts (protection factor). In colonized, birth malformations were added to above risk or protection factors.

Keywords: *P. aeruginosa* outbreak; NICU; Case-case- control study

Introduction

Pseudomonas aeruginosa is a non fermentative, Gram-negative bacillus, commonly found in soil, water and plants [1]. It seldom causes disease in healthy patients, but is a relatively common pathogen in patients with burns, cancer, immune suppression or newborns [2]. Infection is usually acquired in hospitals [3].

The increase in survival of premature low birth-weight neonates has brought an increase in the rate of hospital acquired *P. aeruginosa* infections [4]. This bacterium is considered responsible for a large number of syndromes in Neonatal Intensive Care Units (NICU), including sepsis, pneumonia, meningitis, diarrhoea, conjunctivitis and skin infections [5]. Traditional external reservoirs of *P. aeruginosa* include sinks, tubs, ventilation devices, incubators, and hand antiseptic solutions. What is more, this pathogen can also be isolated from walls, floors and even phototherapy equipment [6]. There may be different transmission mechanisms, such as binding to catheters, mechanical ventilation, the hands of medical of nurse personnel [7], etc.

Infants are more susceptible to infection for several reasons: they have a less effective skin barrier, an immature immune system (which depends essentially on maternal immunoglobulin) [8], and in many cases, they suffer alterations of intestinal flora [9], due to prolonged antibiotic treatment, and can also have damaged skin or mucous membranes, caused by catheterization or mechanical ventilation. Although *P. aeruginosa* often only colonizes the infants, it sometimes causes infection and, despite improved treatments, *P. aeruginosa* bacteremia is fatal in 20% of cases [10].

In our hospital we maintain a prospective surveillance of colonization and/or infection in neonates with weekly studies of pharyngeal or rectal colonization, etc., as well as analysis of clinical samples, if infection is suspected. Thus, during the period between July and September 2011, we found a larger-than-expected number of infections and colonizations by *P. aeruginosa* in our NICU; the data for the present were drawn from this period in order to assess the factors that influence infection or colonization by this microorganism in the NICU infants, using a different design: case-case-control study (colonized-infected-control cases).

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Materials and Methods

Setting

The Neonatology Department of the Hospital Universitario La Paz, has 80 beds, including the 14 in the NICU. This Level 3 Neonatal Unit attends about 900 patients per year, of which over 90% are born in our hospital´s Maternity ward.

We have a system of Early Outbreak Detection: Every Wednesday, samples are taken from all children admitted to the NICU. These are taken from: pharynx, rectum, water and respirator tubing, and sent for microbiological study. In addition, on any day of the week, other samples are sent to Microbiology, if infection is clinically suspected according to have temperature instability (>38°C or <36.2°C), alteration of skin or mucouses, increasing apnea, feeding intolerance, abdominal distension, increased respiratory support, lethargy and hypotonia, immature/total neutrophil (I:T) ratio >0.2 etc (these samples include catheter tips, bronchoalveolar exudates, blood, conjunctiva secretions, etc). For all children with a clinical suspicion or a diagnosis of infection, empirical antibiotic treatment begins, and therapy is then adapted according to the antibiogram susceptibility of the isolated microorganism.

As universal precautions in the NICU we encourage the use of the alcoholic solutions available in the hospital (Alco-Aloe ®: 70º isopropyl-alcohol and chlorhexidine 0.5%) after contact with patients, rather than traditional hand washing (unless hand are visibly soiled, in which case they should be washed with soap and water).

Outbreak

A "case" is determined by the isolation of *P. aeruginosa* in any biological sample from patients (catheter tip, bronchoalveolar exudates, blood, conjunctiva, throat, rectal, etc.), regardless of the presence of symptoms. The frequency of infection and/or colonization was measured each week, expressed as the number and percentage of new cases and total cases (new plus existing cases). The "percentage of new cases" in a week was the number of new neonates diagnosed with *P. aeruginosa*, divided by the total number of children in our NICU that week multiplied by 100, and "percentage of total cases", was the total number of cases present that week (new cases together with the cases from previous weeks), divided by the total number of neonates admitted to the NICU (this week) and after, multiplied by 100.

During this outbreak, various complementary interventions were carried out:

1) All children with *P. aeruginosa* in any positive sample: "contact precautions" were established and we insisted on thorough disinfection of surfaces with a disinfectant that was active against *P. aeruginosa*. The method to study this activity was designed in a previous paper [11]. If these measures were not sufficient to prevent the continuation of the outbreak, it became necessary to separate into two "patient cohorts" (with and without *P. aeruginosa,* dividing our NICU in two physical areas) and, when possible, the patients with this microorganism were attended by different nurses of the rest of neonates (cohort specific nurses).

2) In addition, weekly cultures were performed on samples from the water and tubing of the children on respirators, and at the beginning of the outbreak, we looked for other environmental reservoirs of the microorganism, culturing samples from all water taps in the unit, as well as endoscopes, antiseptics and milk from the Paediatric Nutrition Service. These samples were seeded in McConkey and Blood-agar plates (0.1 ml of each of these liquids were extended over the entire surface of these plates). After that, they were cultured at 37ºC, for two days and any microorganisms were identified.

3) All isolated *P. aeruginosa* were further analyzed by Random Amplification of Polymorphic DNA (RAPD) in the Microbiology Laboratory

Study of risk factors of colonization and infection by *P. aeruginosa*

After the outbreak was resolved, we designed a case-case-control study.

Inclusion criteria: All children admitted more than 3 days in our NICU from July to October of 2011 and having had, at least, one study of their microbiota. All children with *P. aeruginosa* in a biological sample, (regardless of the presence of symptoms), were classified as "cases". During outbreak analysis they were divided into "colonized-cases" (without infection) or "infected-cases" (colonization plus infection, or only infection, diagnosed from clinical samples taken due to suspicion of infection). The control group were all the children fulfilling these inclusion criteria, except for the presence of *P. aeruginosa*.

Statistical method

We studied the data on the weekly frequency of children infected/colonized, (new and total cases), plotting two epidemic curves. In addition, we collected information from the clinical histories of each patient (infected, colonized or controls) with respect to different variables present at admission (Apgar, weight, sex, cardiovascular problems [12], malformations, etc.), as well as others that arose during their NICU stay (arterial or venous catheter, surgery, etc.).

In addition to the above variables obtained from the infants´ clinical histories, three "ecological variables" were included:

-"Number" of patients colonized or infected in the week preceding the one in which the patient was colonized or infected. In each control, we assumed that the number of cases in the "previous week" was the median of the number of cases colonized or infected during the total period of admission to the NICU for that control.

- "Percentage" of cases in previous week was calculated by dividing the above absolute number by the total number of children admitted to our NICU in this same week, multiplied by 100. In a control, the above personnel median was divided by the median of all admitted children during it NICU stay.

-Existence of "separation into cohorts". Neonates are included in the "no" group, if they were admitted to the unit before August 1st, and the "yes" group, if admitted after this date, on which the cohorts were formed.

All of them were analyzed using the Statistical Package for the Social Sciences (SPSS) program (19 version). Given the case-case-control design we were able to perform a bivariate and multivariate analysis in 3 groups:

1. Infected vs. controls,

2. Colonized vs. controls,

3. Infected vs. colonized (this analysis was restricted to the cases).

Quantitative variables were analyzed with Analysis of Variance (ANOVA) and Bonferroni post-hoc tests. For variables that did not follow a normal distribution, nonparametric tests (Kruskal-Wallis) were used.

Qualitative variables were studied with Pearson's chi-squared test. To determine risk factors for colonization or infection, multiple logistic regression was performed, controlling for different confounders and using the Hosmer-Leme show test for goodness of fit. The cut-off point for the variables entering into the equation determination was $p<0.2$ in the bivariate analysis. Variables related to administration of antibiotics were excluded (except ampicillin and gentamicin, given as prophylaxis), since most of the antimicrobials were administered in each case after identification of *P. aeruginosa*.

Results

A total of 30 infants had some isolation of an identical strain (RAPD) of *P. aeruginosa* during the outbreak. Of these, 16 were only colonized and 14 were considered infected, as they were symptomatic (5 conjunctivitis, 1 conjunctivitis + dermatitis, 1 sepsis + dermatitis, 3 bronchopneumonia, 1 bronchopneumonia + sepsis, 1 meningitis, 1 surgical site infection, 1 urinary tract infection).

One of them died from bronchopneumonia due to the bacterium. In 9 of the 14 infected cases, the infection started one or more weeks after colonization was detected, while in the rest, infection and colonization were detected in the same week, or even, infection was diagnosed some days before the colonization was detected.

Outbreak description

The outbreak began in the first week of July 2011, (Table 1) with one case of colonization with *P. aeruginosa*. However, the outbreak was not established until seven new cases (all colonized) were identified during the next two weeks. We arranged a meeting with the Neonatology Service to study the situation, proposing they intensify contact precautions. During the entire outbreak, Biosanit®, (a mixture of three quaternary ammonium compounds), was used because it was demonstrated to be effective on the *P. aeruginosa* isolated from neonates, according to our evaluation of disinfectants against bacteria on germ-carriers.

Despite this, the number of cases increased over the next week, so we decided to separate patients into two cohorts, depending on whether they had or did not have *P. aeruginosa* (infecting or colonizing). The number of new cases began to decline until October (one or two cases per week) except for the middle of August, when they rose to three new cases in one week. We saw that some children remained colonized for several weeks, as happened with a case, detected the week of July 12th, that remained colonized whom *P. aeruginosa* until October 4, when he was discharged. Like this child, there were three others (10% of those colonized/infected) in which *P. aeruginosa* was found in throat or rectum after a period of three weeks of negative controls. However, there was no problem of dissemination of this bacterium to other children, as they were still considered or treated as "colonized", even though they had had two negative controls. Therefore, they remained in the cohort of children with *P. aeruginosa*. The outbreak was considered over on October 11th, as this strain of *P. aeruginosa* was not found again. There were two cases of *P. aeruginosa*, as well as those found in subsequent weeks, that were different by RAPD from the *P. aeruginosa* found during the outbreak and they are not included in this description.

Most *P. aeruginosa* we found showed no acquired resistance, except for five cases. Three of these were sensitive and became resistant to third generation cephalosporins and piperaziline-tazobactam four or five weeks after their first isolation. The other two cases had similar resistance to the three above, only that in these, the resistance was detected on the first week of their admission, indicating transmission of the *P. aeruginosa* strain from one of the three previous cases. However, despite this acquisition of antibiotic resistance, the strain in these five cases was the same as in the outbreak, as determined by RAPD.

After determining there was a possible outbreak, in July we investigated, twenty-five possible environmental reservoirs: all NICU water faucets, antiseptics, soaps, endoscopes used on children and milk from the Lactodiet Unit. *P. aeruginosa* was isolated on one of the taps, but it was a different strain (by RAPD), from the one causing the outbreak.

Figure 1 shows the curve of percentage of cases, each week, expressed as "new" and "total" cases (new plus existing cases).These data are from Table 1 (3st and 5th columns). When assessing numbers relative to children admitted each week, between August 9 and 23, the percentage of total cases increased, not only because of the permanence of cases, but also because of the reduction in incoming patients, so if we were to only observe this percentage of prevalence, we would not immediately appreciate the effect of the division into two cohorts; the opposite to what is seen when the percentage of new cases is considered.

Week No.	Date of the week	number of children new cases (and % vs total children)	number of existing cases	total cases (and % vs total children)	total (each in our NICU (each week)
1	5-11-Jul	1 (6.6%)	0	1 (6.6%)	15
2	12-18-Jul	3 (18.7%)	1	4 (25%)	16
3	19-25-Jul	3 (17.6%)	3	6 (35.3%)	17
4	26-Jul to 1 Aug	7 (43.7%)	3	10 (62.5%)	16
5	2-8 Aug	3 (11.5%)	8	11 (42.3%)	26
6	9-14-Aug	1 (7.1%)	8	9 (64.3%)	14
7	15-22-Aug	3 (21.4%)	8	11 (78.6%)	14
8	23-29-Aug	1 (8.3%)	5	6 (50%)	12
9	30-Aug to 5-Sept	1 (6,2%)	6	7 (43.7%)	16
10	6-12-Sept	2 (13.3%)	5	7 (46.7%)	15
11	13-19-Sept	2 (16.7%)	4	6 (50%)	12
12	20-26-Sept	2 (13.3%)	4	6 (26.7%)	15
13	27-Sept to 3 Oct	1 (6.2%)	3	4 (25%)	16
14	4-10-Oct	0 (0%)	3	3 (18.7%)	16
15	11-17-Oct	0 (0%)	2	2 (11.8%)	17
16	18-24-Oct	0 (0%)	0	0 (0%)	18
17	25-31-Oct	0 (0%)	0	0 (0%)	16

Table 1: Temporally (weekly) description of an outbreak of *P. aeruginosa* in a NICU.

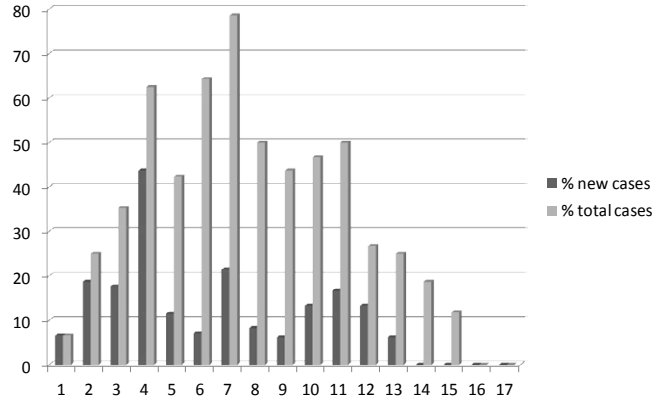

Figure 1: Epidemic curve of an out break of *P. aeruginosa* in a NICU.

Case-case-control study

Our study was conducted with 14 infants infected with *P. aeruginosa*, 16 colonized with this organism and 82 controls. Tables 2 and 3 show these variables and their level of significance, when comparing infected or colonized cases versus controls.

In cases, only variables existing before *P aeruginosa* diagnostic, were included in the logistic regression. In controls, their global NICU-stay were considered "before *P aeruginosa*", and all variables were included without restriction.

Multivariate analysis (Table 4) is divided according to two type of cases:

1) "Infected versus Control": This equation have a good fit with the Hosmer- Lemeshow test, and we found three significant associations with infected cases (two variables that behaved as risk factors and

VARIABLE (yes/no)	Infected N=14 Yes : n (%)	Controls N=82 Yes : n (%)	Colonized N=16 Yes : n (%)
Premature	11(78.6%)	49(59.8%)	12(75%)
Sex female	5(35.7%)	25(34.1%)	9(56.3%)
Respiratory Distress	12(85.7%)	64(78%)	11(68.8%)
Apnea	5(35.7%)	19(23.5%)	4(26.7%)
Caesarean section	8(57.1%)	52(64.2%)	9(60%)
Total Malformations	11(78.6%)	54(65.9%)	14(87.5%)
Malf. without Persistant Ductus Arteriousus	8(57.1%)	33(40.7%)	12(75%) *
Other malf. (non cardiac or Persistent Ductus Arteriosus)	5(35.7%)	15(18.3%)	8(50%) *
Cardiopathy	5(35.7%)	25(30.9%)	6(37.5%)
Persist. Ductus Art	4(28.6%)	26(31.7%)	5(31.3%)
Hyper-Bilirrub	10(71.4%)	49(59.8%)	10(62.5%)
Hyperglycemia	2(14.3%)	9(11.1%)	4(25%)
Hypoglycemia	3(21.4%)	13(16%)	4(25%)
NasoGastric Tube	6(42.9%)	25(30.9%)	3(18.8%)
Mechanical Ventilat	10(71.4%)	64(78%)	14(87.5%)
Central Venous Cath.	13(81.3%)	60(73.2%)	8(57.1%)
Umbilical Artery Cath.	4(25%)	40(48.8%)	5(35.7%)
Venous Umbil. Cath	4(28.6%)	34(41.5%)	6(37.5%)
Parenteral Nutrition	13(81.3%)	69(84.1%)	10(71.4%)
Surgical chemo-prophylaxis	7(50%)	37(45.1%)	10(62.5%)
Chorioamnionitis	3(21.4%)	18(22%)	2(12.5%)
Anemia	8(57.1%)	49(59.8%)	11(68.8%)
Leucocyte Alteration	10(71.4%)	51(62.2%)	10(62.5%)
Cefalosp. 1 Gen	1(7.1%)	7(8.5%)	1(6.3%)
Cefalosp. 3 Gen	8(57.1%)	53(64.6%)	12(75%)
Carbapenem	6(42.9%)	17(20.7%)	5(31.3%)
Aminoglycosides	10(71.4%)	57(69.5%)	7(43.8%) *
Glycopeptides	8(57.1%)	46(56.1%)	10(62.5%)
Ampicillin	6(42.9%)	54(65.9%)	7(43.8%)
Other antibiotics	11(78.6%)	21(25.6%)	10(62.5%) *
Infection virus or bacteria ≠ *P aeruginosa*	9(64.3%)	36(43.9%)	8(50%)
Fungal Infection	6(42.9%) **	5(6.1%)	4(25%) *
Exitus	1(7.1%)	10(12.2%)	0(0%)
NICU stay after separation into two cohorts	8(57.1%) **	67(81.7%)	8(50%) *

Table 2: Bivariate analysis. Qualitative variables (chi-squared). Case-case-control of *P. aeruginosa* in a NICU. (*= p<0.05 colonized vs controls , ** = p<0.05 infected vs controls. There were not significant differences between infected and colonized neonates by *P. aeruginosa*).

Variable (days)	Infected N=14 X SD	Control N=82 X SD	Colonized N=16 X SD
Stay in NICU	49.57 ± 28.452	42.59 ± 35.367	57.19 ± 51.502
Gestational age (days)	217.29 ± 34.217	233.93 ± 41.116	229.13 ± 37.47

Table 3(a): Quantitative variables. Mean (X) and Standard deviation of mean (SD). Bivariate analysis. Case-case-control of *P. aeruginosa* in a NICU (ANOVA –Bonferroni) (There were no significant difference between infected, colonized or controls regarding gestational age or length of stay**).**

Variable	Infected (N=14) M (p25 - p75)	Control (N=82) M (p25 -p75)	Colonized (N=16) M (p25 - p75)
Number of cases previous week	3 (1.75 - 3) **	1 (0 - 2)	1 (1 - 3) *
Percentage of cases previous week	35.3 (32.725 -58.375) **	11 (0 - 37.05)	35.3 (6.15 - 49) *
Apgar: 1min	7.50 (5.25-9)	6 (5-8)	9 (4.25-9)
Apgar : 5min	8 (7.25-9)	9 (7-9)	9 (8-9)
Birth Weight	1.275 (0.866-2.552)	1.687 (0.9875-3.096)	2.01 (0.793-2.895)
Days Mec. Ventilation	2 (0-9)	4 (1-8.75)	2 (1-13)
Days Centr.Venous Catheter	12 (0-15.50)	8 (0-15.50)	11 (0.75-20.50)
Days Umbilical Artery Catheter	0 (0-5.25)	0 (0-5)	0 (0-5.25)
Days Umbilical Vein Catheter	0 (0-1)	0 (0-4.75)	0 (0-6.25)
Days with Parenteral Nutrition	14 (0-20)	10 (4-21)	8.50 (1.25-21.75)
Number of surgeries	0 (0-1)	0 (0-1)	1 (0-1)

Table 3(b): Quantitative variables: Median (M), percentiles 25 and 75 (p25-p75). Bivariate analysis: case-case-control of *P. aeruginosa* in a NICU. Non-parametric test (Kruskal-Wallis) (=p<0.05 colonized vs controls, ** = p<0.05 infected vs controls. There were no significant difference between infected and colonized neonates by *P. aeruginosa*).**

Variable	B coefficient	Standard Deviation of B coef	OR	95% OR Confidence Intervals	p
Fungus Infecion	3.170	0.929	23.801	3.857-147.01	0.001
Separation of cohorts	-1.893	0.840	0.151	0.03-0.781	0.024
Num. cases in the previous week	0.655	0.187	1.925	1.334-2.776	<0.001

Table 4(a): Multivariate analysis: case-case-control of *P. aeruginosa* in a NICU. (4-a: **Infection vs control** (Hosmer-Lemeshow: 0.561)).

Variable	B coefficient	Standard Deviation of B coef	OR	95% OR Confidence Interval	P
Total Malformations	2.182	0.928	8.866	1.437-54.694	0.019
Separation of cohorts	-3.528	0.979	0.029	0.004-0.200	<0.001
% cases in the previous week	0.065	0.022	1.067	1.022-1.113	0.003
Fungus Infection	1.494	0.899	4.453	0.764-25.934	0.097

Table 4(b): Colonization vs control (Hosmer-Lemeshow: 0.526) (4 (c): **Colonization vs infection**: no equation with a significant goodness of fit was obtained).

one protective factor) : Odds-Ratio (OR) and 95% confidence limits (between parenthesis after OR) are calculated.

Risk factors:

"fungal infection" Odds-Ratio (OR) was 23 (3.8-147)

"number of cases in the previous week" (OR = 1.9 x number of cases, eg. if there were two cases, OR=1.9 x 2 = 3.8).

-The protective factor was the "separation into cohorts" with an OR of 0.15 (0.002-0.78), indicating a large decrease in risk that resulted from applying this measure (more than six times less risk: 1/OR = 1/0.15=6.6).

2) "Colonized versus Controls": This equation also shows a good fit by Hosmer-Lemeshow and identified four factors associated with colonization: three risk factors and one protective factor:

-Risk factors:

"birth malformations" , OR= 8.8 (1.4-54.7),

"percentage of cases in the previous week "(OR= 1.07 x percentage of cases, eg if the previous week we had 40%, OR= 1.07 x 40 = 42.8)

"fungal infection", OR= 4.4 (0.76-25.9).

-The protective factor is the same as in the above equation, "separation into cohorts", with an OR of 0.029 (0.004-0.2), implying a reduction in risk for being colonized by *P. aeruginosa* of 34 times (1/OR; 1/0.029=34), with respect to when there was no separation.

3) Finally, we did not get any equation with a good fit to compare colonized with infected cases, and this means there were no relevant variables that would indicate a risk for evolving from colonization to infection in our 30 cases.

Discussion

Technological advances in hospital neonatal units have made a significant improvement in survival and the quality of life of premature infants with birth defects. However, they also produce a higher risk for nosocomial infection in infants, due to the immaturity of their immune system and to the increased need for invasive techniques [13,14]. In this respect we have seen a significant relationship between colonization and the presence of congenital malformations in our study, although we found no significant association between malformations and infection.

In the bivariate analysis the association of *P. aeruginosa* with carbapenem antibiotics might be the results of their typically being used for the treatment of infections caused by Pseudomonas, so their use would be more a consequence than a risk factor. This is why this variable was not introduced into the multivariate analysis. However, in our NICU ampicillin and gentamicin are often used empirically, (as in other NICUs, [15]) and this could be a risk factor, so it was included in the multivariate analysis, although it did not remain significant and was removed from the final equation.

The longer hospital stays of infected children can be both a cause of infection, as children are exposed more time, or a result of infection, and, also, those with more severe disease are more easily infected or colonized [14].

This study found no significant relationship between the use of central venous catheters, mechanical ventilation, umbilical catheters, low birth weight or prematurity and infection or colonization by *P. aeruginosa*, although there are numerous studies linking infections with these factors [16-18].

The control measures taken during the epidemic are consistent with the available literature regarding the management of Pseudomonas outbreaks in NICUs. So far, the measures that have proven most useful in controlling these situations are: early detection of cases, as well as instigating additional contact precautions for all cases (strict hand hygiene [19-21], correct use of gloves and gown) and cohort cases and staff (the last, if possible). It was important that these measures were taken early (even though, at the beginning, colonization was by a microorganism without acquired resistance).

Sometimes it is difficult to demonstrate the efficacy of making these cohorts of patients, but this case-case-control design allowed us to evaluate the reduction in the risk of colonization (34 times) or infection (6.6 times) by multivariate analysis, after separating patients into two cohorts (with and without *P. aeruginosa*).

This design is suitable for the analysis of epidemics in which many of the subjects involved are a reservoir for the organism but do not suffer the infection and it is possible that being colonized or becoming infected may result from different risk factors. In our study the risk and protective factors were very similar for both groups, but fungal colonization was a stronger risk factor in infected versus colonized patients (OR 23 vs 4), which probably implies that intestinal microbiota alteration by antibiotics, which facilitates colonization and subsequent infection by *P. aeruginosa*, was greater in infected neonates. This is logical because infected children normally receive more antibiotic treatment. Finally, using the case-case-control design we were able to demonstrate another risk factor that was specific to colonized cases: the presence of malformations. This could be explained by these children usually needing to longer stays and more invasive instrumentation and, probably, having a less mature immune system than controls without these risk factors.

Conclusions

1. Multivariant analysis was carried out and identified fungus infection, separation of colonized or infected cases (cohort of cases), number or percentage of cases in previous week as risk or protection factors for both infected and colonized neonates, while birth malformations were a risk factor only for colonized neonates.
2. The case-case-control design seems very appropriate in circumstances where there are two situations of different severity of cases, since it can assess risk or protection factors that, in the classic case-control analysis, could appear to be different or non-relevant. Example: neonate malformations were relevant only in colonized cases, or fungus infection was greater risk in infected than colonized cases, or separation into cohorts was more interesting protection factor in colonized cases, etc.

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