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Oxacillin disk diffusion testing for the prediction of penicillin resistance in *Streptococcus pneumoniae*

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ABSTRACT

Objective. *To 1) describe the correlation between the zones of inhibition in 1-µg oxacillin disk diffusion (ODD) tests and penicillin and ceftriaxone minimum inhibitory concentrations (MICs) of meningeal and non-meningeal strains of* Streptococcus pneumoniae *and 2) evaluate the usefulness of the ODD test as a predictor of susceptibility to penicillin in* S. pneumoniae *and as a quick and cost-effective method easily implemented in a routine clinical laboratory setting.*

Methods. S. pneumoniae *isolates from healthy nasopharyngeal carriers less than 2 years old, obtained in a multicentric cross-sectional study conducted in various Peruvian hospitals and health centers from 2007 to 2009, were analyzed. Using Clinical and Laboratory Standards Institute (CLSI) breakpoints, the correlation between the zones of inhibition of the ODD test and the MICs of penicillin and ceftriaxone was determined.*

Results. *Of the 571* S. pneumoniae *isolates, 314 (55%) showed resistance to penicillin (MIC ≥ 0.12 µg/mL) and 124 (21.7%) showed resistance to ceftriaxone (MIC ≥ 1 µg/mL). Comparison of the ODD test zones of inhibition and the penicillin MICs, using the CLSI meningeal breakpoints, showed good correlation (Cohen's kappa coefficient = 0.8239).*

Conclusions. *There was good correlation between ODD zones of inhibition and penicillin meningeal breakpoints but weak correlation between the ODD results and non-meningeal breakpoints for both penicillin and ceftriaxone. Therefore, the ODD test appears to be a useful tool for predicting penicillin resistance in cases of meningeal strains of* S. pneumoniae, *particularly in low- and middle- income countries, where MIC determination is not routinely available.*

Key words Streptococcus pneumoniae; oxacillin; Peru; Latin America.

Streptococcus pneumoniae is the most common cause of community-acquired bacterial pneumonia, meningitis, and bacteremia and non-invasive diseases such as sinusitis and otitis media (1). Traditionally, the use of penicillin has been recommended for respiratory tract infections, and the administration of third-generation cephalosporins, such as

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ceftriaxone or cefotaxime, has been recommended for meningitis treatments (2). However, this approach has become increasingly restricted by the elevated number of reports of resistance to ß-lactam antibiotics in isolates of *S. pneumoniae* worldwide (2). Given that the identification of a penicillin-resistant isolate of *S. pneumoniae* may modify the empirical antibacterial therapy, early determination of *S. pneumoniae* antimicrobial susceptibility is of great importance in the clinical setting (3).

The Clinical and Laboratory Standards Institute (CLSI) (4) states that the 1-µg oxacillin disk diffusion (ODD) test is an effective screening method commonly used in clinical laboratories for the detection of penicillin-resistant pneumococci. In 2008, the CLSI recommended new breakpoints for the minimum inhibitory concentrations (MICs) of penicillin and ceftriaxone, proposing different interpretive criteria for determining the use of the two antibiotics in treating meningeal and non-meningeal infections. It has been established that isolates with zones of inhibition ≥ 20 mm in the 1-µg ODD test correlate with an MIC of penicillin ≤ 0.06 µg/mL being reported as susceptible. However, in isolates with zones of inhibition ≤ 19 mm, MIC should also be determined because the zones of inhibition may be related to the level of penicillin resistance (high or intermediate) and susceptibility (4). Thus, in cases in which the zone of inhibition is ≤ 19 mm, resistance to penicillin or any other ß-lactam should not be reported before determining the MIC.

This study aimed to 1) describe the correlation between the zones of inhibition in 1-µg ODD tests and penicillin and ceftriaxone MICs of meningeal and non-meningeal strains of S. pneumoniae and 2) evaluate the usefulness of the ODD test as a predictor of susceptibility to penicillin in *S. pneumoniae* and as a quick and cost-effective method easily implemented in a routine clinical laboratory setting.

MATERIALS AND METHODS

S. pneumoniae isolates were obtained from a multicentric cross-sectional study conducted in various Peruvian hospitals and health centers from 2007 to 2009 (5). Healthy children 2–24 months old were enrolled in the study during their outpatient well-child visits at their respective Clinics for Growth and Development (*Centros de Crecimiento y Desarrollo*) or Vaccination Centers (*Centros de Vacunación*) (5).

All susceptibility tests were performed according to the methodology proposed by the CLSI (4). Briefly, the oxacillin (Oxoid Ltd., Hampshire, United Kingdom) disk diffusion tests were performed on a Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. The isolates were incubated for 20–24 hours at 35ºC with 5%–7% CO_2. The MIC was determined using broth microdilutions of penicillin G and ceftriaxone (Sigma Aldrich Company, St. Louis, Missouri, United States), spreading 5 x 10^5 CFU/mL onto Mueller-Hinton broth adjusted with divalent cations and supplemented with 2%–5% lysed horse blood containing double serial dilutions of the analyzed antibiotics. The MIC reading was performed after 20–24 hour incubation at 35 ºC.

The *S. pneumoniae* strain ATCC 49619 was used for quality control in both the ODD and MIC assays. In accordance with the CLSI recommendations, the results were interpreted using the breakpoints of penicillin and ceftriaxone for meningeal and non-meningeal strains (4). Isolates with intermediate and high levels of resistance were analyzed together as "non-susceptible" for statistical purposes.

Cohen's kappa coefficient was used to assess the correlation between the ODD and both the penicillin MIC, using CLSI meningeal breakpoints, and the ceftriaxone MIC, using CLSI meningeal and non-meningeal breakpoints. McNemar's chi-squared test was used to assess the correlation between the ODD and the penicillin MIC using CLSI non-meningeal breakpoints.

This study was approved by the ethics committee of the Universidad Peruana Cayetano Heredia (Lima, Peru). Informed consent for the study was obtained from the parents of the participating children. To preserve the confidentiality of patients, all participants were recorded with a study code and without personal identifiers.

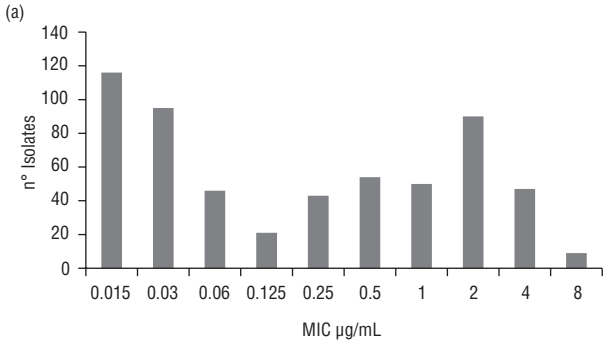
RESULTS

A total of 571 *S. pneumoniae* strains obtained from healthy nasopharyngeal carriers less than 2 years old were analyzed. The most frequent MICs of penicillin were found to be 0.015, 0.03, and 2 µg/mL. The MIC_{50} and MIC_{90} of penicillin were 0.25 and 2 µg/mL respectively (Figure 1A). For ceftriaxone, the most frequent MICs were found to be 0.015, 0.03, and 1 µg/mL; the MIC_{50} and MIC_{90} of ceftriaxone were 0.125 and 1 µg/mL respectively (Figure 1B).

Using the MIC of penicillin and the CLSI breakpoints for meningeal strains, the rate of non-susceptible *S. pneumoniae* isolates was 55.0%. However, when the CLSI breakpoints for non-meningeal strains were used, the rate of non-susceptible *S. pneumoniae* isolates was 9.8%. The rate of non-susceptible *S. pneumoniae* isolates for the MIC of ceftriaxone was 21.7% and 5.8% when the CLSI breakpoints of meningeal and non-meningeal strains, respectively, were used (Figure 2).

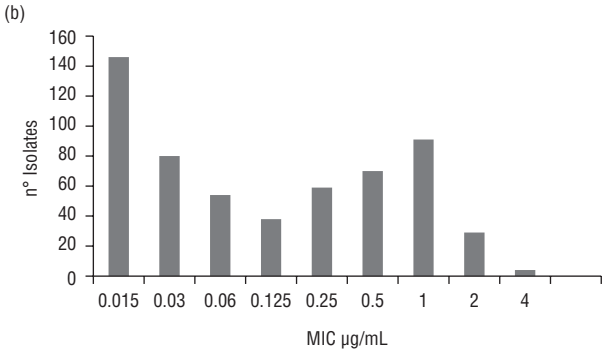
Comparing the zones of inhibition of the ODD test and the MIC of penicillin, the use of meningeal breakpoints showed that of the 257 isolates interpreted as susceptible to penicillin based on the latter criteria (the MIC), 238 (92.6%) also demonstrated zones of inhibition ≥ 20 mm

FIGURE 1A. Distribution of penicillin minimum inhibitory concentrations (MICs) in *Streptococcus pneumoniae* strains isolated from healthy nasopharyngeal carriers in Peruvian children (*n* = 571), Peru, 2007–2009



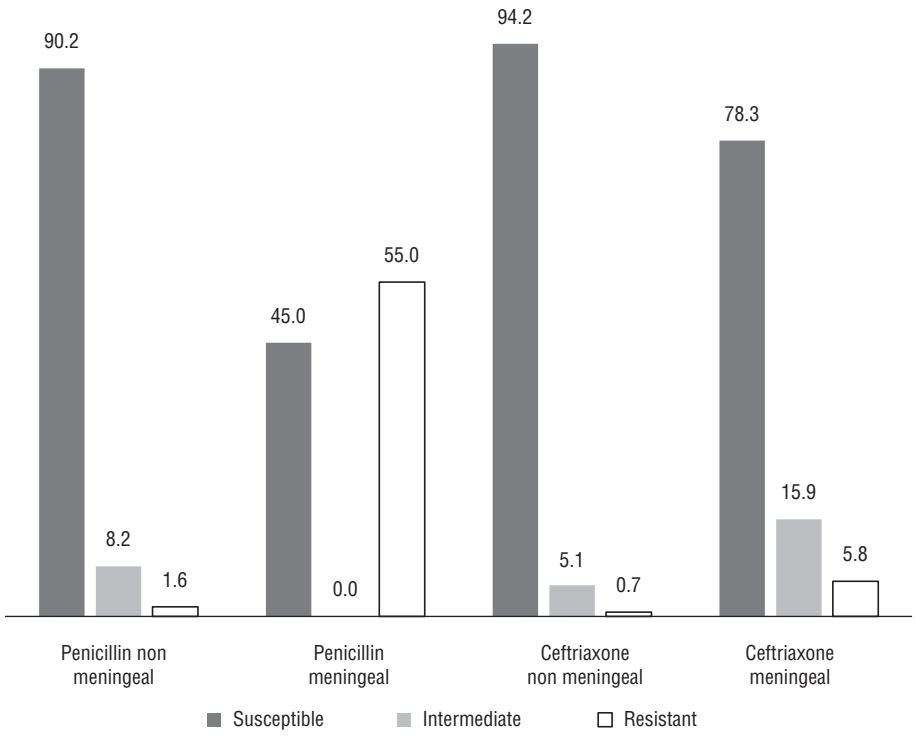
Source: Compiled by the authors based on the study results.

FIGURE 1B. Distribution of ceftriaxone minimum inhibitory concentrations (MICs) in *Streptococcus pneumoniae* strains isolated from healthy nasopharyngeal carriers in Peruvian children (*n* = 571), Peru, 2007–2009



Source: Compiled by the authors based on the study results.

FIGURE 2. Comparison of penicillin and ceftriaxone susceptibility using meningeal and non-meningeal breakpoints for minimum inhibitory concentration (MIC) interpretation in *Streptococcus pneumoniae* strains isolated from healthy nasopharyngeal carriers in Peruvian children (*n* = 571), Peru, 2007–2009



Source: Compiled by the authors based on the study results.

and were thus reported as susceptible to penicillin (MIC ≤ 0.06 µg/mL). A total of 19 strains (7.4%) had zones of inhibition ≤ 19 mm and were thus reported as resistant to penicillin based on the ODD test but were subsequently reported as susceptible based on the MIC of penicillin (Figure 3A). The use of meningeal breakpoints also showed that of the 314 isolates interpreted as resistant to penicillin based on the penicillin MIC criteria, 283 (90.1%) demonstrated zones of inhibition ≤ 19 mm and were thus reported as resistant to penicillin (MIC ≥ 0.12 µg/mL). A total of 31 isolates (9.9%) demonstrated zones of inhibition ≥ 20 mm and were thus reported as susceptible to penicillin based on the ODD test but resistant to penicillin based on the penicillin MIC.

Using the meningeal breakpoints, the comparison between the zones of inhibition of the ODD test and the MIC of penicillin had a good correlation value (Cohen's kappa coefficient (*k*) = 0.8239). However, when the non-meningeal breakpoints were used, the correlation between the two methodologies was weak. Furthermore, ODD test zones of inhibition ≤ 19 mm with a respective resistance to penicillin (MIC ≥ 0.12 µg/mL) were found to a have a positive predictive value (PPV) of 0.937 when the meningeal breakpoints were used. In contrast, the use of non-meningeal breakpoints had a low PPV of 0.162 (Table 1).

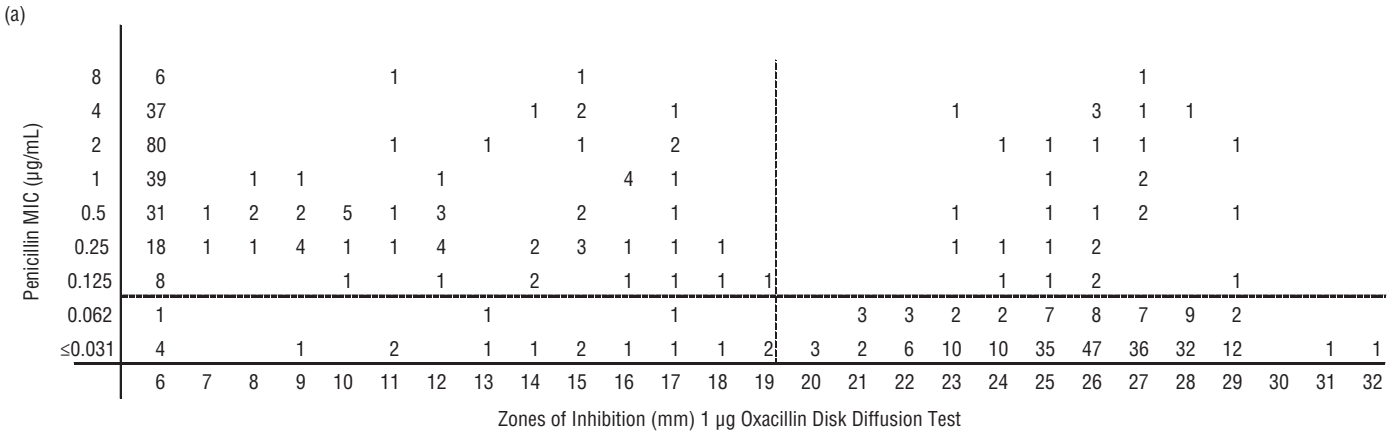
The MIC of ceftriaxone was also compared with the zones of inhibition of the ODD test using the meningeal breakpoints (Figure 3B). Of the 447 isolates interpreted as susceptible to ceftriaxone based on its MIC, 260 (58.2%) demonstrated zones of inhibition ≥ 20 mm and were thus reported to be susceptible to ceftriaxone (MIC ≤ 0.5 µg/mL). However, 187 isolates (41.8%) demonstrated zones of inhibition ≤ 19 mm and were thus reported as resistant to ceftriaxone based on the ODD test. Using the meningeal breakpoints, 115 of the 124 isolates (92.7%) interpreted as resistant to ceftriaxone based on its MIC showed zones of inhibition ≤ 19 mm and were thus reported as resistant to ceftriaxone (MIC ≥ 2 µg/mL), whereas the remaining 9 (7.3%) showed zones of inhibition ≥ 20 mm and were thus reported as susceptible to ceftriaxone based on the ODD test.

The *k* values for the correlation between the ODD test and the MIC of ceftriaxone were lower than that for the MIC of penicillin when using either the meningeal or non-meningeal breakpoints (0.3352 and 0.0770 respectively). The PPV of the ODD test for resistance to ceftriaxone was found to be 0.381 when the meningeal breakpoints were used, whereas using the non-meningeal breakpoints resulted in a PPV of 0.096 (Table 2).

DISCUSSION

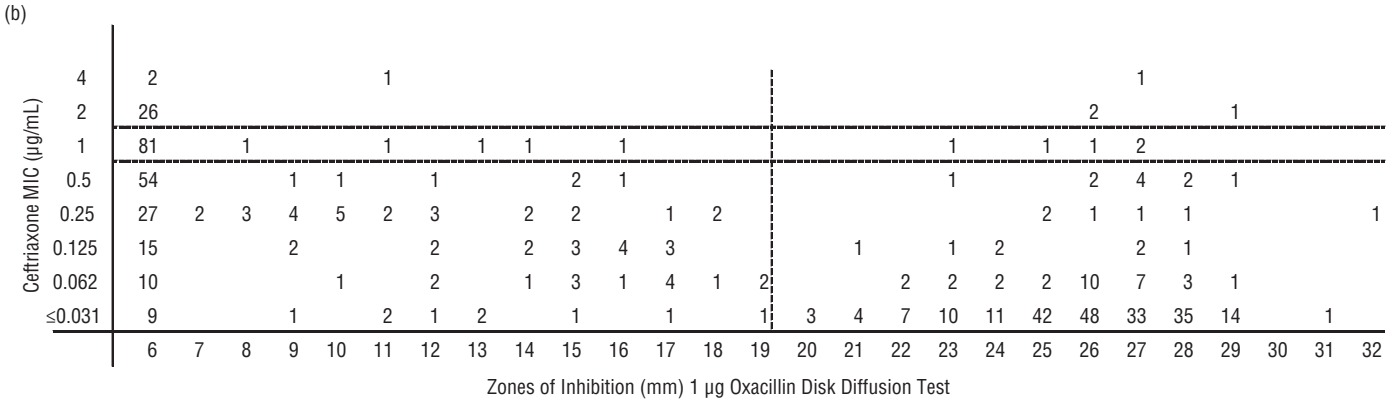
One of the most important findings of this study was the elevated correlation value (*k* = 0.8239) obtained by comparing ODD zones of inhibition ≤ 19 mm and the MIC of penicillin when the meningeal breakpoints were applied. Furthermore, comparison of these two tests showed a high PPV, providing more evidence that

FIGURE 3A. Comparison of minimum inhibitory concentrations (MICs) of penicillin to zones of inhibition of the 1-µg ODD test, using meningeal breakpoints (susceptible MIC ≤ 0.06 µg/mL, resistant MIC ≥ 0.125 µg/mL), for *Streptococcus pneumoniae* strains isolated from healthy nasopharyngeal carriers in Peruvian children (*n* = 571), Peru, 2007–2009



Source: Compiled by the authors based on the study results.

FIGURE 3B. Comparison of minimum inhibitory concentrations (MICs) of ceftriaxone to zones of inhibition of the 1-µg ODD test, using meningeal breakpoints (susceptible MIC ≤ 0.5 µg/mL, intermediate MIC = 1-µg/mL, resistant MIC ≥ 2 µg/mL), for *Streptococcus pneumoniae* strains isolated from healthy nasopharyngeal carriers in Peruvian children (*n* = 571), Peru, 2007–2009



Source: Compiled by the authors based on the study results.

with proper application of meningeal breakpoints ODD tests predict *S. pneumoniae* resistance to penicillin almost as accurately as an MIC assessment.

Previous studies comparing ODD test zones of inhibition with MICs of penicillin and cefixime found that 98% and 99% of pneumococcal strains with no zones of inhibition were not susceptible to penicillin and cefixime respectively. Based on those results, the ODD test was used as a predictor of non-susceptibility to penicillin and cephalosporins (6, 7). It should be noted that these studies did not take into consideration whether the isolates were meningeal or non-meningeal strains when establishing the penicillin MIC breakpoints, whereas the study reported here showed that the ODD test is only a good predictor of *S. pneumoniae* resistance to penicillin when meningeal breakpoints are applied.

In this study, applying either the meningeal or the non-meningeal breakpoints reduced both the level of correlation between the ceftriaxone MIC and the ODD indicator of susceptibility (zones of inhibition ≤ 19 mm) and the PPV of the ODD zones of inhibition for cases of *S. pneumoniae* strains non-susceptible to ceftriaxone. These results contradict those from a study in Colombia that found that when the zone of inhibition of the ODD test was correlated to the pneumococcal resistance to ceftriaxone, all isolates showing some degree of resistance to ceftriaxone had a zone of inhibition of 6 mm (3). In light of the current results, ODD should not be used to predict ceftriaxone resistance. There is still no accepted disk diffusion method for detection of resistance to extended spectrum cephalosporins, mainly because of the reported severity of error (minor) of more than 15% for cefotaxime and ceftriaxone (8).

In the current results, which were based on isolates from Peruvian children under 2 years old, the rates of resistance to penicillin and ceftriaxone were high (55.0% and 21.7% respectively) with the use of meningeal breakpoints. In contrast, using the non-meningeal breakpoints for both antibiotics, the percentage of isolates resistant to penicillin and ceftriaxone was 9.8% and 5.8% respectively. Therefore, the strains used in

TABLE 1. Correlation between 1-µg oxacillin disk diffusion (ODD) test zones of inhibition and minimum inhibitory concentrations (MICs) of penicillin, using meningeal and non-meningeal breakpoints, for *Streptococcus pneumoniae* strains isolated from healthy nasopharyngeal carriers in Peruvian children (*n* = 571), Peru, 2007–2009

ODD test zones of inhibition	MIC				
	Meningeal breakpoints for penicillin[a]		Non-meningeal breakpoints for penicillin[b]		
	Susceptible (*n* = 257) No. (%)	Resistant (*n* = 314) No. (%)	Susceptible (*n* = 515) No. (%)	Intermediate (*n* = 47)[c] No. (%)	Resistant (*n* = 9)[c] No. (%)
Resistant	19 (7.4)	283 (90.1)	253 (49.1)	41 (87.2)	8 (88.9)
Susceptible	238 (92.6)	31 (9.9)	262 (50.9)	6 (12.8)	1 (11.1)

Source: Compiled by the authors based on the study results.

[a] Cohen's kappa coefficient (*k*) = 0.8239.

[b] McNemar's chi-squared coefficient (X^2_{Mc}) = 230.86.

[c] Intermediate and resistant isolates were analyzed together as "non-susceptible" for statistical purposes.

TABLE 2. Correlation between 1-µg oxacillin disk diffusion (ODD) test zones of inhibition and minimum inhibitory concentrations (MICs) of ceftriaxone, using meningeal and non-meningeal breakpoints, for *Streptococcus pneumoniae* strains isolated from healthy nasopharyngeal carriers in Peruvian children (*n* = 571), Peru, 2007–2009

ODD test zones of inhibition	MIC					
	Meningeal breakpoints for ceftriaxone[a]			Non-meningeal breakpoints for ceftriaxone[b]		
	Susceptible (*n* = 447) No. (%)	Intermediate (*n* = 91) No. (%)	Resistant (*n* = 33) No. (%)	Susceptible (*n* = 538) No. (%)	Intermediate (*n* = 29)[c] No. (%)	Resistant (*n* = 4)[c] No. (%)
Resistant	187 (41.8)	86 (94.5)	29 (87.9)	273 (50.7)	26 (89.7)	3 (75.0)
Susceptible	260 (58.2)	5 (5.5)	4 (12.1)	265 (49.3)	3 (10.3)	1 (25.0)

Source: Compiled by the authors based on the study results.

[a] Cohen's kappa coefficient (*k*) = 0.3352.

[b] *k* = 0.0770.

[c] Intermediate and resistant isolates were analyzed together as "non-susceptible" for statistical purposes.

this study, which were from healthy nasopharyngeal carriers, might have been causative agents of meningitis, for which high rates of resistance to penicillin have been observed. In a previous Peruvian study that tested 101 *S. pneumoniae* isolates causing invasive pneumococcal disease obtained from the same age group, the rates of resistance to penicillin and ceftriaxone in meningeal strains were considerably lower—34.4% and 17.4% respectively (9)—but this could be attributable to the fact that nasopharyngeal strains are usually more resistant to penicillin than invasive strains. Based on these results, penicillin would not be optimal for meningeal infections but might still be the drug of choice in non-meningeal infections such as pneumonia or other non-invasive infections.

With the use of the meningeal breakpoints in isolates exhibiting ODD zones of inhibition ≤ 19 mm, this study showed that 7.4% of the isolates were susceptible to penicillin. These results concur with previous data (6) that reported that 8.2% of *S. pneumoniae* strains with ODD zones of inhibition ≤ 19 mm were susceptible to penicillin (MIC ≤ 0.06 µg/mL). For isolates showing ODD zones of inhibition ≤ 19 mm, the MIC should also be determined. The reason for confirmatory testing is twofold: 1) to determine the level of penicillin resistance (intermediate or high, a distinction that is not made in the ODD test criteria) and 2) to identify penicillin-susceptible strains that showed false positives for resistance based on the ODD test (i.e., major errors) (7). Even though there is good correlation between ODD test results and penicillin MICs for penicillin resistance, using meningeal breakpoints, the use of penicillin has dropped and ceftriaxone has become the drug of choice for treatment of meningitis in many parts of the world. However, both penicillin and chloramphenicol are still considered empiric treatments for meningitis in several low-income countries (10). Therefore, an important conclusion that can be drawn from this study, based on the high rates of penicillin resistance in meningitis that were found, is that ceftriaxone should be considered first-line empiric therapy in treatment of meningitis.

Other methodologies that can be used to complement the ODD test include the E-test, which is more affordable than MIC panels for testing penicillin and ceftriaxone. Microdilution is another reliable and relatively inexpensive method for determining MICs, but it is also cumbersome and time-consuming, and thus might not be a viable technique for analysis of outbreaks or large amounts of clinical samples. In addition, microdilution might be difficult to implement in rural areas, especially in low-income countries. Given those limitations, use of the E-test is preferable as an alternative assay to complement the ODD test (11). E-test methodology can be implemented easily and has shown good correlation with the agar dilution method. When penicillin resistance rates were determined using both agar dilution and the E-test, the results had a rate of agreement of 88.6% to 92%, with no major errors (11, 12). Despite the good correlation, analysis of penicillin MICs using the E-test technique tends to result in 1 dilution lower than broth microdilution, increasing the need for extra caution when the results are within the breakpoint boundaries (13).

It has been proposed that susceptibility to penicillin (MIC ≤ 0.06 µg/mL) with reduced ODD zones of inhibition is related to alterations in the penicillin binding protein 2X (PBP2X) that can produce a visible effect on ODD results. The alterations would only cause a slight effect in the penicillin MICs, which would be expected to remain below the breakpoints (14). The exact mechanism of resistance was described in a study by Dowson et al. (14) in which six isolates that were susceptible to penicillin (MIC ≤ 0.06 µg/mL) had reduced ODD zones of inhibition.

Cephalosporins are known to mainly interact with the PBP2X. Consequently, the sequential acquisition of alterations in the PBPs and other genetic determinants are likely the cause of high levels of resistance to the aforementioned antimicrobial agents (15, 16). These initial alterations in PBP2X are the first step to high levels of resistance to antimicrobials. Therefore, the use of cephalosporin therapy might have played a role in the creation of a selection pressure that allowed for the development of pneumococci with reduced susceptibility to oxacillin (17).

Limitations

One limitation of this study was the use of nasopharyngeal strains of *S. pneumoniae* from healthy carriers. Nasopharyngeal strains are usually more resistant to penicillin than invasive strains, so the resistance percentages for the strains used in this study are most likely slightly higher than resistance percentages for invasive strains in the same population. Nevertheless, the information gathered from this study is useful for proper empiric therapy for *S. pneumoniae* in Latin America. Other study limitations were 1) the use of convenience sampling for selection of the health care centers and 2) the consecutive enrollment of the healthy carriers of *S. pneumoniae*, resulting in a sample that might not be representative of all children in Peru. However, these limitations would not have affected the correlation between the ODD zones of inhibition and the MICs.

Conclusions

Use of meningeal breakpoints resulted in good correlation between the ODD zones of inhibition and the penicillin MIC for penicillin resistance, whereas use of meningeal breakpoints for ceftriaxone, and non-meningeal breakpoints for both penicillin and ceftriaxone, did not. Therefore, the authors consider the ODD test a useful tool in predicting penicillin resistance in cases of meningeal strains of *S. pneumoniae*, particularly in low- and middle-income countries, where MIC determination is not routinely available.

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Conflicts of interest. None.

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RESUMEN

Prueba de difusión con discos de oxacilina para predecir la resistencia a la penicilina de *Streptococcus pneumoniae*

Objetivo. 1) Describir la correlación entre las zonas de inhibición observadas en la prueba de difusión con discos de oxacilina de 1 µg y la concentración inhibitoria mínima (CIM) de penicilina y ceftriaxona frente a cepas meníngeas y no meníngeas de *Streptococcus pneumoniae* y 2) evaluar si la prueba de difusión con discos de oxacilina permite predecir la sensibilidad de *S. pneumoniae* a la penicilina y sirve como método rápido y eficaz en función de los costos, y resulta fácil de aplicar en los laboratorios clínicos ordinarios.

Métodos. Se analizaron colonias de *S. pneumoniae* aisladas de la nasofaringe de portadores sanos menores de 2 años obtenidas en un estudio transversal multicéntrico realizado en diversos hospitales y centros de salud del Perú entre los años 2007 y 2009. Se determinó la correlación entre las zonas de inhibición observadas en la prueba de difusión con discos y la CIM de la penicilina y la ceftriaxona utilizando los valores críticos definidos por el Instituto de Estándares Clínicos y de Laboratorio..

Resultados. De las 571 colonias aisladas de *S. pneumoniae*, 314 (55 %) presentaron resistencia a la penicilina (CIM ≥ 0,12 µg/ml) y 124 (21,7%), resistencia a la ceftriaxona (CIM ≥ 1 µg/ml). Se observó una buena correlación (coeficiente κ de Cohen = 0,8239) entre las zonas de inhibición de la prueba de difusión con discos y la CIM de la penicilina utilizando los valores críticos del Instituto respecto de las cepas meníngeas.

Conclusiones. Se encontró una buena correlación entre las zonas de inhibición de la prueba de difusión con discos y los valores críticos de CIM de la penicilina respecto de las cepas meníngeas, pero una correlación débil entre los resultados de la prueba de difusión y los valores críticos tanto de la penicilina como de la ceftriaxona respecto de las cepas no meníngeas. Por consiguiente, la prueba de difusión con discos es un método de utilidad para predecir la resistencia a la penicilina de las cepas meníngeas de S. pneumoniae, en particular en los países de ingresos bajos y medianos, donde no suele ser posible determinar la CIM.

Palabras clave Streptococcus pneumoniae; oxacilina; Perú; América Latina.