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Detection of β lactamase in sputum

C Connell, S Aspinall, J Corkill

Abstract

***Aims*—To develop a rapid, sensitive, and reproducible test for the detection of β lactamase in sputum, and to relate these findings to bacteriological culture results.**

***Methods*—One hundred and twenty sputum samples from inpatients were investigated for β lactamase activity using the chromogenic cephalosporin nitrocefin. Sputum samples were sonicated and incubated aerobically at 37°C with nitrocefin for up to two hours. Positive results (production of a red colour) were treated with blue sepharose beads to remove albumin (which can cause non-specific hydrolysis of nitrocefin) and retested. Samples were also cultured for both aerobic and anaerobic bacteria, with all isolates being tested for β lactamase production using nitrocefin. All positive sputum samples and β lactamase producing isolates were further examined by isoelectric focusing (IEF) to determine isoelectric point(s) (pIs).**

***Results*—The process of sonication and albumin removal had no demonstrable effect on β lactamase activity. Forty seven of the 120 sputum samples were positive on initial testing, and of these, 16 remained positive following removal of albumin. These 16 subsequently yielded 19 β lactamase producing bacteria. All sputum samples yielding β lactamase producing bacteria were also positive on direct nitrocefin testing. On no occasion were sputum samples positive in the absence of enzyme producing bacteria—that is, the test was both 100% specific and 100% sensitive. The presence of β lactamase activity in the sputum samples was also confirmed using a microbiological method. In 11 sputum samples the β lactamases detected had similar pI values to the β lactamases obtained from their bacterial isolates.**

***Conclusion*—Detection of β lactamase activity in sputum using nitrocefin, after treatment with blue sepharose beads, is a rapid, reproducible test with high specificity and sensitivity. Analytical isoelectric focusing showed that for 11 of the 16 positive sputa, the source of the β lactamases could be traced to their concurrent bacterial isolates.**

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Department of Medical Microbiology, Royal Liverpool University Hospital, Liverpool
C Connell
S Aspinall
J Corkill

Correspondence to:
Ms Catherine Connell, Microbiology Department, Tameside General Hospital, Fountain Street, Ashton-under-Lyne, OL6 9RW

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Previous studies have concluded that the presence of β lactamases in sputum could inactivate β lactam antibiotics.[1–4] Obtaining a rapid result for the presence of β lactamase could therefore lead to an alternative (and more effective) choice of antibiotic treatment. Various rapid methods have been used for the detection of β lactamase activity in sputum,[1 3 5] including the utilisation of the hydrolysis of the chromogenic cephalosporin nitrocefin.[6] Microbiological tests rely on the inhibition of an indicator organism, thereby delaying the result by up to 24 hours. The objective of this study was to utilise a colourimetric method (based on nitrocefin hydrolysis), previously used for detection of β lactamases in patients with bronchiectasis.[1] A sensitive and specific test for the presence of β lactamases in the sputum of patients with suspected lower respiratory tract infection was developed. It had already been noted that the presence of albumin produced a non-specific colour change with nitrocefin.[1 6] We attempted to resolve this problem by removing albumin from the sample with blue sepharose beads, as used for albumin removal in high pressure liquid chromatography columns.[7] Analytical isoelectric focusing (IEF)[8 9] was used to determine if the β lactamase found in sputum correlated with the enzymes extracted from bacteria isolated from the same sample. From the results obtained the efficacy of such a rapid test could then possibly be assessed with regard to the choice of empiric antimicrobial treatment.

Methods

One hundred and twenty purulent sputum samples (1 ml) from inpatients were sonicated (in a Class 1 safety cabinet) using a Kontes cell disrupter for up to one minute until they were homogenised. Fifty microlitres of the sonicated sample were then pipetted into a 96-well flat-bottomed microtitre tray (Sterilin, Hounslow, Middx) to which an equal volume of nitrocefin (Unipath Ltd, Basingstoke, Hants) was added. Nitrocefin was reconstituted according to the manufacturer's instructions and diluted 1 in 5 with saline to obtain a 200 μM solution. The tray was covered with a transparent lid and incubated aerobically at 37°C for up to two hours. Wells were examined every 15 minutes for a colour change from yellow to red. Control wells were included in each test; these consisted of a β lactamase producing *Staphylococcus aureus* (NCTC 10724) (positive control) and *S aureus* (NCTC 6571) (negative control). Control organisms were emulsified in 3 ml of

distilled water (to McFarland's No 5 Standard) and sonicated by the same method as the sputum samples.

Fifteen saliva samples from healthy volunteers were also collected (by sluicing with water) and sonicated for 30 seconds, and then tested by the above protocol. All sputum samples were mixed with an equal volume of Sputasol (Unipath Ltd) and vigorously shaken and centrifuged for 20 minutes at room temperature, then cultured on Columbia blood agar plates (Columbia agar base (Unipath Ltd) containing 7% (v/v) whole horse blood) and chocolate agar plates (using Columbia agar base) in an atmosphere of 5% carbon dioxide in air for 24 hours at 37°C. They were also cultured on Columbia blood agar plates and incubated in an anaerobic cabinet (Don Whitley, Shipley, Yorks) at 37°C for 48 hours. All isolates were screened for β lactamase production using nitrocefin,[10] and all positive isolates fully identified using commercial kits and standard tests.[11]

EFFECT OF SONICATION ON β LACTAMASE ACTIVITY

The effect of sonication was determined on two previously characterised β lactamases, one plasmid mediated (*Escherichia coli*, isoelectric point (pI) 5·4) and one chromosomal (*Pseudomonas paucimobilis* pI 4·6). Beta lactamase preparations were obtained by culturing the isolates in brain-heart infusion broth (Unipath Ltd) for 24 hours at 37°C. Broths were sonicated four times for 15 seconds and centrifuged to clarify at 13 000 rpm for 10 minutes. Using the Kontes microcell disrupter, the enzymes were sonicated for 10, 20, 30, 60 and 90 seconds immersed in ice. Nitrocefin (100 μM) was added to 25 μl of both sonicated and unsonicated β lactamase, and the rate of hydrolysis measured in a Pye Unicam 1800 ultraviolet spectrophotometer (Pye Unicam, Cambridge, England) at an absorbance wavelength of 482 nm.[6]

The temperatures achieved in 1 + 1 ml of purulent and mucopurulent sputum were measured using a thermocouple after 10, 30, 60 and 90 seconds of sonication.

TOTAL PROTEIN AND ALBUMIN ESTIMATION IN SPUTUM

Ten sputum samples ranging from mucoid to purulent were diluted 1 in 10 in distilled water, vortexed, and centrifuged at 13 000 rpm. Total protein estimation was then determined on the supernatant fluids according to the method of Lowry *et al.*[12] Albumin estimations were determined on the sputum supernatant fluids using a microassay performed on a Microstat 3 (Instrumentation Laboratories INC, Lexington, USA).

REMOVAL OF ALBUMIN FROM SPUTUM

To those sputum samples which produced a colour change with nitrocefin, an equal volume (1 ml) of blue sepharose beads (Pharmacia Ltd, Milton Keynes, UK) was added, mixed, and centrifuged for five minutes at 13 000 rpm to remove any albumin present. Supernatant fluids were then retested for the presence of β lactamase using nitrocefin. Various albumin concentrations of 4, 3, 2 and 0·1 g/l were also mixed with the beads and the test repeated to assess the effectiveness of albumin removal. To determine that the blue sepharose beads did not remove the β lactamase, a broad spectrum β lactamase solution (Unipath Ltd) was diluted to give a range of concentrations from 1 to 500 IU/ml. An equal volume of beads (500 μl) was added to 500 μl of each dilution, mixed, centrifuged and the supernatant fluid retested with nitrocefin.

ANALYTICAL ISOELECTRIC FOCUSING OF SPUTUM SONICATES AND THEIR ISOLATES

All *S aureus* isolates were grown overnight in brain-heart infusion broth (Unipath Ltd) in the presence of 0·5 mg/l of methicillin as an inducer to increase the yield of enzyme.[13] The broths were then centrifuged at 13 000 rpm for 10 minutes and supernatant fluids concentrated 10-fold in a Minicon B15 concentrator (Grace & Co, Dawers) for 18 hours at 4°C. Heavy suspensions of Gram negative isolates (*Haemophilus influenzae*, *Moraxella catarrhalis*, *Escherichia coli* and *Ps aeruginosa*) were made in 3 ml of distilled water and sonicated for one minute in 15 second bursts, followed by centrifugation at 13 000 rpm for five minutes. Sputum samples were also concentrated 10-fold overnight in a minicon B15 concentrator (Amicon, Stonehouse) at 4°C before focusing. Isoelectric focusing was performed on a series of molecular weight markers with known pI values (LKB, Bromma, Sweden), sputum samples, and extracted β lactamases from enzyme positive bacteria, using an Ampholine Pagplate (1804–103) pH 3·5–9·5 (LKB). Bands of β lactamase activity were visualised by soaking the gel with 100 μM solution of nitrocefin for up to 15 minutes at 37°C. Actual pIs were calculated from a standard curve obtained with the LKB control proteins of known pI (LKB).

MICROBIOLOGICAL METHOD

Amoxycillin Adatabs (Mast Laboratories, Liverpool, England) were added to Columbia base agar plates to give a final amoxycillin concentration of 60 μg/l. Dried plates were then seeded before use with the fully sensitive *S aureus* (NCTC 6571). Sputum or a suspension of the sputum isolate (100 μl) was inoculated on to the surface and the plate was incubated aerobically at 37°C for 24 hours. A β lactamase producing *S aureus* and a non-β lactamase producing *S aureus* were used as positive and negative controls. Growth of the *S aureus* at the site of sample inoculation indicated the presence of β lactamase(s).

Results

The rate of hydrolysis of nitrocefin by the two control β lactamases before sonication was taken as 100% and used for comparison of hydrolysis rates after sonication. As the sonication times increased, hydrolysis rates

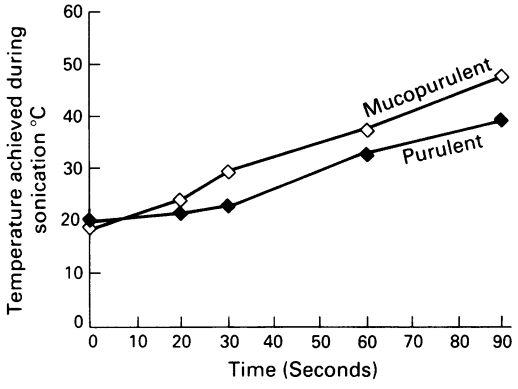

Figure Temperature achieved during sonication of sputum samples.

for both enzymes remained constant at 100%.

Sonication of two sputum samples for 10, 30, 60 and 90 seconds led to an increase in temperature in both samples (figure). The highest temperature achieved in the sputum sample after 90 seconds of sonication was 48°C and therefore did not seem to be sufficient to cause excess denaturation of any β lactamase(s) present.

In 10 varied sputum samples the albumin concentrations ranged from 0·1 to 1·38 g/l and protein from 0·66 to 4·2 g/l (table 1). The more purulent samples had the highest concentrations of protein and albumin.

The sepharose beads removed albumin concentrations up to 2 g/l (indicated by a lack of colour change from yellow to red with nitrocefin). This value exceeded the highest concentration of albumin measured in the sputum samples (table 1).

Table 1 Total protein and albumin content in 10 sputum samples

Sputum No	*Total protein (g/l)*	*Albumin (g/l)*
1	1·54	0·55
2	1·41	0·28
3	1·63	0·10
4	0·84	0·14
5	2·20	0·16
6	2·56	0·19
7	4·20	1·38
8	0·66	0·17
9	1·04	0·1
10	1·41	0·12

Table 2 Comparison of pIs from 16 sputum sonicates and 19 bacterial isolates obtained from sputum samples

Sputum sonicate	*pI of sputum sonicate*	*pI of isolates*	*Sputum isolates*
1	5·9	6·0	*M catarrhalis*
2	5·9, 5·7	ND	*E coli*
		ND	*M catarrhalis*
3	5·6	6·0	*M catarrhalis*
4	5·8	ND	*E coli*
5	5·9	5·6	*M catarrhalis*
6	ND	ND	*S aureus*
7	ND	4·6	*S aureus*
		4·7	*S aureus*
8	5·7, 5·5	5·5, 5·4	*H influenzae*
		6·0	*M catarrhalis*
9	ND	6·3, 5·9, 5·2	*M catarrhalis*
10	5·6	7·5	*S aureus*
11	5·5	5·6, 5·1	*M catarrhalis*
12	7·8, 7·4, 6·95	7·7, 7·3, 6·85	*Ps aeruginosa*
13	5·4, 5·2	5·4, 5·5	*M catarrhalis*
14	5·75, 5·7	5·75	*M catarrhalis*
15	7·95, 7·4, 6·8	8·2, 7·4, 6·85	*Ps aeruginosa*
16	5·6	5·7, 5·8	*H influenzae*

ND = not detected

All concentrations of the β lactamase (from 1 to 500 IU/ml) retained their hydrolytic activity towards nitrocefin after treatment with blue sepharose beads.

All 15 samples of saliva from healthy control volunteers did not possess β lactamase using both nitrocefin and microbiological testing, and no β lactamase producing organisms were isolated.

From the 120 samples of sputum obtained from patients with suspected chest infections, nitrocefin hydrolysis was detected in 47, of which 16 had β lactamase after removal of albumin by the blue sepharose beads. All 16 yielded β lactamase producing bacteria, while no β lactamase producing organisms (either commensals or pathogens) were isolated from any of the other sputa tested.

Sixteen sputum sonicates and 19 β lactamase preparations from bacterial isolates of the sputa were subjected to isoelectric focusing (table 2). For 11 of the sputum samples, similar pIs were obtained for the sputum and the sputum isolate. No similarities were obtained for the remaining five. All of the *H influenzae* and *Ps aeruginosa* isolates had similar pIs to those of their corresponding sputum samples, as did seven of the nine *M catarrhalis* isolates (table 2).

All sputum samples shown to possess β lactamase activity after blue sepharose treatment by nitrocefin hydrolysis were also confirmed positive by the microbiological method. All other sputum samples showed no enzyme activity by either method.

Discussion

The aim of this study was to develop a rapid test for the detection of β lactamase in sputum based on nitrocefin hydrolysis, which would not result in non-specific colour changes due to interference from albumin in the sample. With a microtitre based assay, the test was used to detect β lactamase in sputum samples obtained from inpatients. False colour changes caused by the presence of albumin[16] led to the inclusion of a step to remove albumin. This was achieved by using blue sepharose beads, which removed up to 2 g/l of albumin from solution, a value which exceeded the highest concentration of albumin detected in 10 varied samples of sputum (1·38 g/l). The beads did not effect the β lactamase. Before testing the sputum samples with nitrocefin, they were sonicated to release any β lactamase that may have been contained in the mucus. Sonication can lead to localised heating, resulting in β lactamase inactivation.[14] Therefore, to ensure that the temperature achieved by this would not affect β lactamase activity, temperature changes were monitored using a thermocouple. The maximum temperature achieved was 48°C which was insufficient to cause degradation in β lactamase activity. Reports of inactivation of some β lactamases as a result of sonication have been described.[15,16] Therefore, its effect was investigated by determining the rates of nitrocefin hydrolysis of both a plasmid and

chromosomally encoded β lactamase. No change in rates of hydrolysis of either β lactamase following sonication were found.

Due to the possibility of β lactamase producing organisms being present in saliva, 15 saliva samples from healthy adults were examined using the nitrocefin hydrolysis test. All were negative, and on culture no β lactamase producing bacteria were isolated.

From 120 sputum samples, obtained from patients with suspected chest infections, 47 specimens produced a colour change with nitrocefin. After treatment with blue sepharose beads to remove albumin, 16 of the 47 sputum samples still retained the ability to hydrolyse nitrocefin (indicating that 31 initial positive assays were due to non-specific colour changes caused by albumin). The 31 samples producing non-specific colour changes were confirmed to be negative by the microbiological method while the 16 positive specimens were confirmed to possess β lactamase activity using the same method. All 16 positive specimens harboured potentially pathogenic β lactamase producing organisms, while none of the remaining 104 sputum samples had such isolates. Analytical isoelectric focusing was used to determine the possible bacterial origin of sputum β lactamase. Sputum and enzyme preparations from their β lactamase producing isolates were concentrated before focusing and then compared visually on the same gel. The 16 positive sputum samples together with their bacteria derived β lactamase extracts were tested, and of these, 11 similar pIs were obtained, thereby indicating the source of the β lactamase. For the remaining five samples, no pIs were obtained. This could be due to several factors—for example, the concentration of β lactamase in the samples may have been insufficient to be detected by focusing or the pI of the β lactamase may have been out of the range covered by the gel (pH 3·5–9·0).

This rapid test was suitable for the detection of β lactamases produced by *Haemophilus influenzae*, *Mycobacterium catarrhalis* and *Pseudomonas aeruginosa* and by incorporation of a novel method for removal of albumin, resulted in 100% sensitivity and specificity. These observations, therefore, could be of benefit clinically when deciding on appropriate antimicrobial chemotherapy.

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