Postmortem bacteriology of the lung by printculture of frozen tissue

A technique for in situ culture of microorganisms in whole frozen organs

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SUMMARY Lung lobes obtained at necropsy from 100 patients were frozen at -70°C and sectioned as a whole in a sledge microtome as used on complete frozen animals. Prints of freshly cut surfaces were cultured on different solid media in 14 cm Petri dishes. The printcultures showed three patterns of bacterial growth: dense growth in 24%, patchy growth in 43%, and no significant growth in 33%. Forty-two of the 67 positive printcultures showed two or more bacterial species. Besides bacteria known to be pathogens of the lung, streptococci of the viridans group, enterococci, and streptococci of group B were grown, sometimes in pure cultures. The patterns of the printcultures were reproducible in successive sections, and the quantity of the bacterial species could be assessed. Contaminating bacteria on the surface of the lung lobe could be recognised, as these produced colonies restricted to the edges of the print. Because there was no smearing of infecting and contaminating bacteria, printculture offers a method of reference.

The techniques used in postmortem bacteriology consist of taking small samples of tissue by piercing the organ, grinding the suspected tissue, or culturing a frozen section of a small tissue block.¹⁻⁴ We developed a technique for culturing *in situ* microorganisms from whole frozen organs to gain an insight into the quantity, distribution, and number of bacterial species. This technique was applied to frozen lung lobes obtained from 100 necropsies. We chose lungs because pneumonia is one of the major causes of death in patients with serious underlying disease. During life exact data on the bacterial causes of lower respiratory tract infections are difficult to obtain, because it is often impossible to distinguish between pathogens and opportunists in expectorated sputum and the culture results of purulent sputum are not always representative of the bacterial flora in the diseased lung as a whole. Moreover, the processing of sputum and the interpretation of culture results differ.^{5,6}

Material

Portions of lung were taken from 100 necropsies without special precautions to avoid contamination.

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Unless the pathologist decided otherwise, the right lower lobe of the lung was sent for bacteriological investigation. The lung lobes were frozen at -70°C within 2 hours of necropsy and kept at that temperature until used. All specialties were represented except paediatrics. The interval between death and necropsy was less than 24 hours in 67 cases, between 25 and 48 hours in 22 cases, and 49 to 72 hours in six cases. In five cases no data were available.

The data from the necropsy records can be arranged in two groups. Group 1 consists of cases with an infectious process in the lungs: pneumonia, bronchopneumonia, tracheobronchitis, and bronchitis. Group 2 comprises cases without known infection: oedema, congestion, embolus, infarction, atelectases, haemorrhage, tracheostoma, chronic non-specific lung disease (chronic bronchitis, asthma bronchiale, emphysema), and cases where the pathologist did not mention the lungs. The underlying diseases in groups 1 and 2 were: cardiovascular disease, malignancies, cerebrovascular disease, diabetes mellitus, chronic alcoholism, chronic lung disease, and a few multiple traumas.

For every organ a set of five 14 cm Petri dishes were filled with 60 ml of the following media: blood agar containing 43 mg/l paranitrophenylglycerol to prevent swarming of proteus; 7% horse-blood agar

to which 20 mg/l gentamicin was added for culture in a GasPak anaerobic jar; chocolate agar for culture in 5% CO₂ in a candle jar; and CLED and MacConkey agar for aerobic culture. The media were dried and kept at 4°C until used.

A Bright sledge microtome cryostat for sectioning whole frozen laboratory animals was set at -30°C before use.

Methods

PRINTCULTURE

The frozen lung lobe is firmly mounted on the sledge of the microtome using either a mounting medium or wet gauze. It is then sectioned (trimmed) until the cut surface is as large as possible. After carefully cleaning the microtome knife with xylene and removing the frozen debris, a final section is taken before print-culture. During this final section care is taken to clean the knife with a paper tissue before it passes the frozen organ on the return stroke, thus soiling of the cut surface with debris from the microtome knife is avoided. A large Petri dish with a dry solid medium is gently pressed for a moment on the cut surface of the organ. After each printing 50 microns or more is cut off in order to get a fresh surface for the next print. The Petri dishes must be as cold as possible and taken from the refrigerator at the last moment.

Five prints were obtained from each organ using the five different solid media. The prints on CLED agar, MacConkey agar, and blood agar with para-nitrophenylglycerol added were incubated at 37°C and inspected daily for two days. The print on chocolate agar was incubated at 37°C in a candle jar and also inspected daily for two days. The print on blood agar containing gentamicin was incubated at the same temperature in a GasPak anaerobic jar and inspected after two and seven days. Gram stains were made and pure cultures were obtained. All bacteria were identified according to Cowan and Steel.⁷

TESTING ANTIBACTERIAL ACTIVITY OF FROZEN LUNG TISSUE IN THE ANTIBIOTIC ASSAY

After the last print, a piece of transparent tape (Scotch tape no 3M type 810) is fastened with a roller or a cotton wad on to the cut surface of the organ. The 50 micron section then made is attached to the tape. From the middle of this tape 1 cm² pieces are cut out and layered tissue side down on three antibiotic assay plates containing *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Bacillus subtilis* of known sensitivity. The inhibition zones were read the next day. The pathologist and the microbiologist

examined different parts of the lungs. The interpretation of the data was independent. Where discrepancies were found the frozen parts of the lung were re-examined microscopically.

Results

After printculturing the lung lobes three growth patterns were seen:

(a) no significant growth—growth only along the edges of the print and no growth inside or less than 10 colonies spread evenly (Fig. 1);

(b) patchy growth—clusters of colonies growing within the print surrounded by areas without growth (Fig. 2);

(c) dense growth—the whole print is covered by more or less dense growth. In some cases an area with dense growth is bordered by either confluent growth, patchy growth, or insignificant growth in a neighbouring segment. These were classified as dense growth. In these cases the anatomical borders of the lung lobuli seemed to be retained (Figs 3 and 4).

Consecutive printcultures of one organ showed the same pattern throughout a depth of 300 to 500 microns on the five solid media (Fig. 5 a-e). The same results were obtained after five months' storage at -70°C. Smearing of bacteria from the microtome knife did not occur. The growth of bacteria was seen in the anatomical setting. In a few cases dense growth was restricted to one segment. Differences in species, growth rates, and distribution on the five solid media could be seen. Contaminating bacteria could be recognised as they produced colonies along the edges of the print only (Figs 2 and 5e).

In 100 necropsies (57 men and 43 women), 24 of the lung specimens showed dense growth, 43 showed patchy growth, and 33 showed no significant growth. Of the latter, 25 showed growth only along the edges of the print, and in eight, less than 10 colonies were seen spread evenly on one or two prints. The age distribution in relation to the three patterns of growth is shown in Table 1.

The inhibition of bacterial growth in the antibiotic assay by the lung tissue in the three groups in relation to data from necropsy records is shown in Table 2. In 22 of the 33 lungs showing no significant growth, the pathologist had recorded no signs of inflammation. In two of these cases, inhibition of the antibiotic assay was shown by the lung tissue. Of the 11 cases with recorded inflammation, nine showed inhibition of the antibiotic assay. The remaining two had tracheobronchitis and bronchitis. Of the 43 lungs with patchy growth, the necropsy report mentioned inflammation in 28 cases. In eight of these, the antibiotic assay showed inhibition. Of the 15 cases with-

Fig. 1

Fig. 2

Fig. 3

Fig. 1 No significant growth: contaminating bacteria at the surface of the organ are restricted to the edges of the printculture.

Fig. 3 Dense growth: the whole printculture is covered with more or less dense growth.

Fig. 4

Fig. 2 Patchy growth: bacteria are seen in clusters leaving areas beneath the pleural surface free of growth. Note two colonies of *Proteus* sp. restricted to the edge of the print.

Fig. 4 Dense growth bordered by a segment with insignificant growth.

Fig. 5 Printcultures of the same organ on blood agar (a), MacConkey agar (b), CLED agar (c), blood agar: anaerobic (d) and chocolate agar (e). Note where contaminating bacteria (*Ps. aeruginosa*) can be seen restricted to the edges of the print (e).

Table 1 100 necropsies by age and bacterial growth in lung tissue

Age (yr)	Growth			Total
	None	Patchy	Dense	
20-39	2	3	2	7
40-59	7	11	3	21
60-79	21	26	16	63
80 +	3	3	3	9
Total	33	43	24	100

out inflammation, one showed inhibition. Microscopic examination of the same region showed bacteria and numerous leucocytes in 13 cases, and leucocytes only in two. Of the 24 cases with dense growth, inhibition of the antibiotic assay was shown in five of the 17 cases with inflammation. Of the

remaining seven cases, five were admitted to hospital in coma and two had extensive myocardial infarction. They all died on the first or second day in hospital. Microscopic examination of the same region showed bacteria and leucocytes in all seven cases.

The relation between the pathological findings and the number of bacterial species isolated from the printcultures is summarised in Table 3. A pure culture was seen, of either dense or patchy growth, in 15 cases with recorded inflammation and in 10 cases without known inflammation. Two or more species were seen in 30 cases with recorded inflammation and in 12 without known inflammation.

The bacterial species isolated in pure culture, or in combination with one or more species, in the groups with dense and patchy growth are shown in Table 4. Either dense or patchy growth was observed in 67 cases. In 25 of these printcultures, bacteria grew

Table 2 Pattern of growth by pathological findings and inhibition of antibiotic assay (IAA) in 100 lungs

Pathological findings	Growth						Total	IAA
	None	IAA	Patchy	IAA	Dense	IAA		
Infectious process present	11	9	28	8	17	5	56	22
No infectious process present	22	2	15	1	7	0	44	3
Total	33	11	43	9	24	5	100	25

Table 3 Percentage of cases with a pure culture (1), a combination of two species (2), and more than two species (3) in the groups with dense and patchy growth in relation to pathological findings in 100 lungs

Pathological findings	100 lungs from 100 necropsies								
	No growth (n = 33)			Patchy growth (n = 43)			Dense growth (n = 24)		
				1	2	3	1	2	3
Infectious process present	11			9	7	12	6	5	6
No infectious process present	22			6	4	5	4	2	1
Total	33			15	11	17	10	7	7

Table 4 Number of cases with species growing in pure culture (1), or in combination with one other species (+1), or with more than one other species (+ > 1)

Isolated species	Dense growth			Patchy growth			Total
	1	+1	+ > 1	1	+1	+ > 1	
<i>Staph. aureus</i>	1	5	4	1	4	10	25
<i>Klebsiella</i> sp.	3	1	4	3	3	6	20
<i>E. coli</i>	2	2	2	3	3	5	17
<i>Strep. pneumoniae</i>	1	1	2	2	2	8	16
<i>Strep. agalactiae</i> group B	0	1	3	0	0	9	13
<i>Strep. viridans</i> group	1	0	2	1	4	5	13
<i>Proteus</i> sp.	1	1	3	1	2	3	11
<i>Enterococcus</i>	0	2	2	2	1	3	10
<i>Haemophilus</i> sp.	0	0	0	0	1	3	4
<i>Pseudomonas</i> sp.	0	0	1	1	0	1	3
<i>C. perfringens</i>	1	0	0	1	0	1	3
<i>Corynebacterium</i> sp.	0	0	0	0	1	1	2
<i>Branhamella catarrhalis</i>	0	0	0	0	1	1	2
<i>Strep. pyogenes</i> group A	0	1	0	0	0	0	1
Other species	0	0	0	0	0	6	6

in pure culture. In these cases *Klebsiella* species were seen most frequently, followed by *Escherichia coli* and *Streptococcus pneumoniae*. In the remaining 42 cases, more than one bacterial species were seen in the printcultures. In these cases, *Staphylococcus aureus* was seen most frequently, followed by *klebsiella*, *Strep. pneumoniae*, and *Strep. agalactiae*. Considering the isolated bacterial species as a whole in the 67 cases, the order of frequency is: *Staph. aureus*, *klebsiella*, *E. coli*, *Strep. pneumoniae*, *Strep. agalactiae*, *Strep. viridans* group, *proteus* and *enterococcus*. Of the 13 cases with tracheostoma, nine were reported to have an infectious process in the lungs.

Discussion

Different methods of collecting specimens post mortem for bacterial culture have been used by previous workers.¹⁻⁴ As stated by Koneman *et al.*,⁸ the technique of De Jongh *et al.*¹ is the most practical. Briefly, this consists of searing a small surface area of an organ to dryness with a red hot metal instrument, and either piercing this area with a sterile swab or removing 1 cm³ tissue blocks with separate sets of sterile instruments. These tissue blocks are then ground in a tissue grinder, and a loopful of the supernatant is cultured. The results of the latter are comparable to those of the most elaborate of these methods,^{2,3} that closely parallels a careful operative procedure, during which pieces of approximately 1 g are removed from the organs with separate sets and treated in the same way. In some laboratories tissue blocks, taken without aseptic precautions during necropsy, are dipped for a few moments in boiling water before being homogenised for culture. Others have taken small tissue blocks of 2.5 cm² and 0.5 cm thick, of which frozen sections of 25-30 microns were laid with sterile cold forceps on solid media.⁴

The disadvantages of the aforementioned methods are:

- (1) sampling errors when only a small tissue sample is examined, and
- (2) the possibility of contamination during the several steps of the procedure.

(1) With printculturing it has been shown that, in some cases, areas of dense growth border areas without growth (Fig. 4) and that clusters of patchy growth are often centered around bronchioles leaving areas beneath the pleural surface without growth (Fig. 2). With the techniques currently in use, sampling errors might be possible in these cases. Culturing of tissue sections larger than 2 cm² is practically not feasible. With printculturing a cross-section of a lung lobe surrounded by pleura is

explored (50-150 cm²), and this diminishes sampling errors.

(2) With most of the current methods, pieces of tissue are ground in separate sterile tissue grinders. If during the many steps of the procedure contamination takes place, these contaminating bacteria are mixed with infecting bacteria in the tissue grinder. After grinding, only a loopful of the supernatant is cultured. Differences in the growth rates of contaminating and infecting bacteria or their interaction might further confuse the culture results. Lung lobes, free of bacteria but with a surface contaminated during necropsy or in the laboratory, showed growth only along the edges of the print (Fig. 1). With printculturing no smearing of bacteria occurred from the microtome knife either from the surface of the lung or from the inside. This phenomenon was reproducible. In contrast to the grinding techniques, contamination can be recognised in printculture.

Several workers have found no correlation between ante mortem infection and post mortem culture results.⁹ Others have postulated the existence of an indigenous microflora to explain the presence of bacteria that were not expected to be there.³ They noted also that in one patient different species of bacteria were cultured from different organs and theorised that every organ must have its own indigenous microflora, as organs could differ in their ability to cope with microorganisms.¹⁰ However, with the current techniques, contamination cannot be ruled out with certainty, and this is necessary to decide on the existence of an indigenous microflora. According to Newhouse *et al.*,^{11,12} the lung is normally sterile from the first bronchial division to the terminal lung units.

Postmortem bacterial transmigration is no longer believed to be of great importance because the isolation rate does not increase with lengthening of the interval between death and necropsy, provided that the bodies are cooled after death.^{2,3,13}

The growth pattern seen in the printculture shows the bacteria in relation to their anatomical settings at the time of death. This is supported by the observation that in some cases it was possible to correlate the pattern of the printculture with typical clinical and pathological data, for example, an infected infarction (Fig. 5) after multiple emboli, or dense growth of a pure culture of *E. coli*, after catheterisation of the bladder just before death (Fig. 3). Sometimes we saw a mixture of bacterial species in one segment and almost a pure culture of one of them in a neighbouring segment. By trimming the organ with the microtome, printcultures of cross-sections at different levels can be compared. In our hands, printcultures were reproducible through a depth of at least 500 microns (Fig. 5 a-e).

Oropharyngeal commensals behaving as opportunists are trapped *in situ* along with bacteria known to be pathogens. When printcultures were repeated after five months' storage at -70°C we got the same results.

Another advantage of printculturing in post-mortem bacteriology is that separate sets of sterile instruments for taking samples of organs are not needed.

In 61% (42 of the 67 cases with dense and patchy growth) two to six bacterial species were isolated. In the remaining 39% (25 cases), a pure culture was obtained. However, due to technical shortcomings anaerobic culture was not optimal and so the number of the isolated species might be higher.

Bacteria may enter the lower respiratory tract by aerosol inhalation, haematogenous spread, direct extension from a neighbouring site of infection, or by aspiration. The last possibility is thought to be the most important.¹⁴ Aspiration of pharyngeal secretions occurs in normal adults during deep sleep and in 70% of patients with depressed sensorium.¹⁵ In healthy individuals, aspirated bacteria are effectively cleared. In patients with serious underlying diseases, however, depressed sensorium and impaired clearance mechanisms are often combined. Moreover, colonisation of the pharynx with Gram-negative bacteria increases with the level of care¹⁶ and severity of illness.¹⁷ Lowering gastric acidity seems to have the same results,¹⁸ with a reported high mortality¹⁹ when this is followed by pneumonia. We isolated Gram-negative bacteria in 48% of cases. As our series consisted of inpatients with serious underlying disease, the high percentage (67%) of positive printcultures agrees with the findings of others.

The common practice of looking for the predominance of one or more anticipated pathogens in sputum cultures needs reconsideration. The isolation of pure cultures of bacterial species from lungs, considered to be commensals of the pharyngeal cavity, for instance, streptococci of the viridans group, merits further investigation.

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