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Gram stain and culture of morning and 24 h sputum in the diagnosis of bacterial exacerbation of chronic bronchitis: a dogma disputed

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ABSTRACT: Bacteriological results from microscopic and cultural examinations of morning and 24 h sputum were compared in an attempt to optimize the diagnosis of bacterial exacerbations of chronic bronchitis. Twelve patients collected sputum randomly on two consecutive days, either fresh morning sputum (3 h sputum) first, followed by 24 h sputum or *vice-versa*. Sputa were washed three times in 0.9% NaCl and then examined microscopically before and after homogenization with 2% pancreatin. Cultural examinations were performed on homogenized sputum. *Haemophilus influenzae*, pneumococci and neisseriae were identified more often in Gram stains than in cultures; staphylococci and Gram-negative rods less frequently so. About 50% of organisms microscopically identified as pneumococci failed to grow in cultures. Microscopic examinations revealed a marked reduction of cells and bacteria after homogenization with pancreatin. Gram stains of 24 h sputum show comparable results to those of morning sputum. No differences were found in cultures. Microscopic examination of the Gram stains was found to be superior to cultural examination. The value of microscopic examination was impaired by the addition of pancreatin.

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Gram stain and culture of freshly expectorated morning sputum are standard methods used in the diagnosis of exacerbations of chronic bronchitis. For antibiotic trials, examination of 24 h sputum is preferred by some because its volume is a sensitive criterion of the efficacy of antibiotics [1]. However, microbiologists have maintained that 24 h sputum is unsuitable for cultural examination because of overgrowth during the collection period [2, 3]. Thus, the question of the feasibility of culturing 24 h sputum in bronchitis arises. Furthermore, bacteriological findings of expectorated sputum *per se* have been criticized because of their poor correlation with findings of secretions recovered by transtracheal aspiration or bronchoscopy with protective methods [3].

In order to clarify the issues of collection period and bacteriological methods, we examined fresh morning (3 h) and 24 h sputum aliquots randomly collected from twelve patients with untreated bacterial exacerbation of chronic bronchitis. Findings of Gram stains and cultures following washing, homogenization and quantitative bacterial counts were compared.

Patients and methods

Twelve patients with untreated bacterial exacerbation of chronic bronchitis [4] were included in the study. The diagnosis of exacerbation was based on all of the following criteria established in Laboratory A (Lung Laboratory, Dept of Internal Medicine, University Hospital, Zürich): a) exacerbation of clinical signs and symptoms; b) sputum cytology (crystal violet stain) showing >80% neutrophils and <5% histiocytes [5]; c) total bacterial counts (Gram stain) of >20 microorganisms per oil immersion field (OIF), or haemophilus-like bacilli >12 per OIF, or Gram-positive diplococci >8 per OIF [6]. None of the patients were under antibiotic treatment. Patients collected sputa randomly on two consecutive days: either fresh morning sputum (collected from 7–10 a.m.) followed by 24 h sputum (collected from 7 a.m.–7 a.m. the next day), or *vice versa*. Patients were asked to collect only coughed-up material and to keep it at room temperature during the collection period. The 3 h period was chosen in order to obtain sufficient material. Samples were processed and plated immediately following collection.

Sputum volume was calculated by weight. After inspection, sputa were washed three times in 0.9% saline solution [7]. Crystal violet stains (0.017% aqueous crystal violet solution) were prepared for microscopic examinations of cellular composition [5].

Bacteriological studies included Gram stains and colony counts of specific organisms. Gram stains were made from washed sputa in Laboratory A before and

after homogenization with a 2% pancreatin solution [8]. Homogenization consisted of mixing equal volumes of sputum and 2% pancreatin solution (buffered to pH 7.6) and incubation in a waterbath at 37°C to liquefaction. 0.01 ml loopfuls of homogenized sputum were used for Gram stain and fractional spread on different culture media. Inoculation and incubation at 37°C for 24 h occurred before dispatch to two microbiological laboratories (Laboratory B: Dept of Microbiology, University of Zürich and Laboratory C: Microbiology Laboratory, Pharmaceutical Research Dept, F. Hoffmann-La Roche & Co. Ltd, Basel).

For Gram stains, 0.01 ml of washed sputum was spread over a glass-slide using a calibrated platinum loop. Average numbers of each bacterial morphotype per oil immersion field were determined for 20 adequate, randomized fields and expressed in micro-organisms per ml. An adequate field was defined as one with >five cells originating from the bronchopulmonary system but no more than would present as a single layer. Squamous epithelial cells had to be absent both from the field under evaluation and for a distance of three oil immersion fields in all directions. Bacterial types were classified presumptively by their morphological characteristics and listed as haemophilus-like, pneumococcus-like, neisseria-like, staphylococcus-like, and Gram-negative rods [6]. The diagnosis of pneumococcus-like organisms was confirmed by a quelling reaction with Omniserum®.

The number of non-bacterial sputum cells per ml was determined from selected pancreatin-homogenized and non-homogenized sputum aliquots. Liquefaction of the latter was carried out using the technique of OPPENHEIMER *et al.* [9], with counts performed in a haemocytometer. In the cultures we focused on *Streptococcus pneumoniae*, *Haemophilus spp.*, *Branhamella catarrhalis*, *Staphylococcus aureus*, Enterobacteriaceae, and nonfermentative Gram-negative rods. Laboratory B used: blood agar (GC agar base (BBL*)+7% human blood), CNA (Columbia Colistin-Nalidixic acid agar (BBL*)+5% sheep blood), Crowe agar (GC agar base (BBL*)+1% IsovitaleX (BBL*)+8% boiled sheep blood + 10 $U \cdot ml^{-1}$ Bacitracin**), and MacConkey agar (BBL*). Laboratory C used blood agar (Blood Agar Base (Oxoid***) + 7.5% human blood) and chocolate agar (Blood Agar Base (Oxoid***) + 7.5% human blood kept at 80°C for 1 h before pouring). All media were incubated for a total of 48 h at 37°C in 10% CO_2. Standard microscopical and biochemical methods were used for identification [10].

Data were statistically analysed using a nonparametic two-sided Wilcoxon matched-pairs test [11]. Because of the extremely asymmetric distribution of the observations, median non-zero values were calculated instead of arithmetic means. Non-zero values were used throughout since the mean incidence of bacteria had to be determined.

* BBL: Baltimore Biological Laboratories, Cockeysville, Md, USA.
** Bacitracin: Dr. A.G. Grossenbacher, Allschwil, Switzerland.
*** Oxoid: Oxoid Ltd, Basingstoke, UK.

Results

Results are seen in tables 1–3 and in figure 1. On Gram-stained specimens of 3 h and 24 h sputa (table 1), counts of haemophilus-like and pneumococcus-like micro-organisms were similar whether fresh morning sputa or 24 h sputa were examined, *i.e.* the medians of the non-zero observations did not differ significantly. High numbers of bacteria were evenly distributed, indicating no overgrowth in the 24 h specimens. This was also true for "other" Gram-negative rods and neisseria-like organisms. Staphylococcus-like micro-organisms were not found.

Counts of *Haemophilus* on Crowe, blood and chocolate agar (table 2) did not differ significantly between sputa of the two collection periods, medians being identical. There were also no differences with regard to culture media. On the other hand, culture counts of pneumococci differed, but only in regard to the culture media, chocolate agar being superior to CNA agar. The small number of positive findings precluded any statistical evaluation.

Data on *B. catarrhalis*, "other" Gram-negative bacteria and *S. aureus* are given in table 3. *B. catarrhalis* was cultivated in the 3 h and 24 h sputa of two patients by Laboratory C which used chocolate and blood agar only. "Other" Gram-negative bacteria were demonstrated in the 3 h and 24 h sputa of five identical patients in about the same numbers by Laboratory B, indicating no overgrowth. Laboratory C, however, showed higher counts culture in the 24 h sputa of two patients. *S. aureus* was isolated on CNA and blood agar in higher numbers in the 24 h sputa of two patients than in the 3 h samples. Most patients showing bronchopathogenic bacteria in one collection period were accurately identified even when sputum of the other collection period was examined. This was true for microscopy as well as for culture. Although microscopy of fresh morning and 24 h sputa detected an equal number of patients with haemophilus-like organisms (table 1), these bacteria were not present in corresponding sputum specimens of two patients (Nos 1 and 8). Cultural examination of 3 h sputa detected only one less patient than did microscopical examination. For 24-h sputa the yield of *Haemophilus* was the same (Laboratory B) or even better (Laboratory C) than in the 3 h samples. No patient positive in the 3 h sample was negative in the 24 h sample. Gram stains of fresh morning sputa revealed one additional patient with pneumococcus-like organisms and three additional patients with neisseria-like organisms compared to microscopy of 24 h sputa. These bacteria were not identified in the corresponding sputa of three patients (nos 1, 3, 5 and 4, 5, 12, respectively). On the contrary, cultural examination gave a much lower yield, especially in the case of pneumococci. Using CNA agar pneumococci were cultivated in the 3 h and 24 h sputa of only two patients (Laboratory B), whereas cultivation on chocolate agar detected pneumococci in the sputa of four and five patients, respectively (Laboratory C, table 2). Likewise,

Table 1. – Quantitated Gram stains of bronchopathogenic bacteria in nonhomogenized 3 h and 24 h sputa of twelve chronic bronchitics with bacterial exacerbations

	Haemophilus-like		Pneumococcus-like		Neisseria-like		other Gram Negative like		Staphylococcus-like	
Patients no.	3 h sputum	24 h sputum	3 h sputum	24 h sputum	3 h sputum	24 h sputum	3 h sputum	24 h sputum	3 h sputum	24 h sputum
1	18†	0	6	0	341	1118	0	0	0	0
2	24	290	84	11	0	0	335	11	0	0
3	13,301	6901	0	645	0	0	0	0	0	0
4	5835	2166	624	15	129	0	0	0	0	0
5	1703	2344	1165	0	18	0	0	0	0	0
6	24,444	7601	1649	1163	0	0	0	0	0	0
7	0	0	1323	1425	0	0	0	0	0	0
8	0	609	2882	2742	0	0	0	30	0	0
9	0	0	1548	1016	0	0	0	0	0	0
10	323	12,449	74	526	0	0	0	0	0	0
11	19,892	25,044	1269	2268	0	0	0	0	0	0
12	2715	3182	20	670	13	0	114	0	0	0
Median*	2715	3182	1165	843	73,5	1118	22.5	20.5	0	0
p**	NS		NS		ND		ND		ND	

†: x10³ per ml sputum; *: median of non-zero observations; **: two sided Wilcoxon matched-pairs test; NS: not significant (p>0.05); ND: not determined.

Table 2. – Semiquantitative counts of cultures of *H. influenzae* and pneumococci in 3 h and 24 h sputa of twelve chronic bronchitics with bacterial exacerbations

	H. influenzae				Pneumococci			
	3 h sputum		24 h sputum		3 h sputum		24 h sputum	
	Lab B	Lab C	Lab B	Lab C	Lab B	Lab C	Lab B	Lab C
Culture median†	CR	CA	CR	CA	CNA	CA	CNA	NA
Patients								
1	0	0	2.4††	0	0	0	0	0
2	45.3	63.9	90.0	90.0	0	0	0	0
3	90.0	90.0	90.0	90.0	0	0	0	0
4	90.0	90.0	90.0	90.0	0	0	0	2.1
5	90.0	90.0	90.0	90.0	0	0	0	0
6	90.0	90.0	90.0	90.0	0	0	0	0
7	36.6	0	43.2	0	0	90.0	0	90.0
8	0	0	0	0	77.4	90.0	90.0	90.0
9	0	0	0	0	90.0	90.0	39.6	90.0
10	0	90.0	90.0	90.0	0	0	0	0
11	90.0	90.0	90.0	90.0	0	0	0	0
12	90.0	90.0	90.0	90.0	0	21.9	0	57.9
median*	90.0	90.0	90.0	90.0	83.7	90.0	64.8	90.0
P**	NS		NS		ND			
	NS							
	NS							

Lab B: Dept of Microbiology, University of Zurich; Lab C: Microbiology Laboratory, Pharmaceutical Research Dept, F. Hoffmann-La Roche & Co, Ltd Basel; †: reported is the culture medium with optimal growth. In case of zero growth no culture is mentioned; ††: x10³ per ml sputum; CR: Crowe agar; CA: chocolate agar; CNA: Colistin-nalidixic acid-agar; *: median of non-zero observations; **: two-sided Wilcoxon matched-pairs test; NS: not significant (p>0.05); ND: not determined.

Table 3. – Semiquantitative counts of cultures of *Branhamella catarrhalis*, other Gram-negative bacteria and *Staphylococcus aureus* in 3 h and 24 h sputa of twelve chronic bronchitics with bacterial exacerbations

	Branhamella catarrhalis				Other Gram-negative bacteria				*Staphylococcus aureus*			
	3 h sputum		24 h sputum		3 h sputum		24 h sputum		3 h sputum		24 h sputum	
	Lab B	Lab C	Lab B	Lab C	Lab B	Lab C	Lab B	Lab C	Lab B	Lab C	Lab B	Lab C
Culture median†		CA		BA	BA	BA	MC	BA	CNA	BA	BA	BA
Patients												
1	0	87.3††	0	90.0	0.6	0	0.3	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	6.9	0	13.5
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	9.0	0.9	5.7	26.4	0	1.8	0	0
6	0	0	0	0	40.5	55.2	30.0	32.7	0	0	0	0
7	0	0	0	0	1.5	0.9	1.2	3.3	0	0	0	0
8	0	0	0	0	1.8	3.9	18.0	30.0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	10.2	6.3	34.2	38.1
12	0	33.6	0	33.0	0	0	0	0	0	0	0	0
Median*	0	60.5	0	61.5	1.8	2.4	5.7	28.2	10.2	6.3	34.2	25.8
P**		ND				ND				ND		

Lab B: Dept of Microbiology, University of Zurich; Lab C: Microbiology Laboratory, Pharmaceutical Research Dept, F. Hoffmann-La Roche & Co, Ltd Basel; †: reported is the culture medium with optimal growth. In case of zero growth no culture is mentioned; ††: $x10^3$ per ml sputum; *: median of non-zero observations; **: two-sided Wilcoxon matched-pairs test; CA: chocolate agar; BA: blood agar; MC: MacConkey agar; CNA: CNA-agar; ND: not determined.

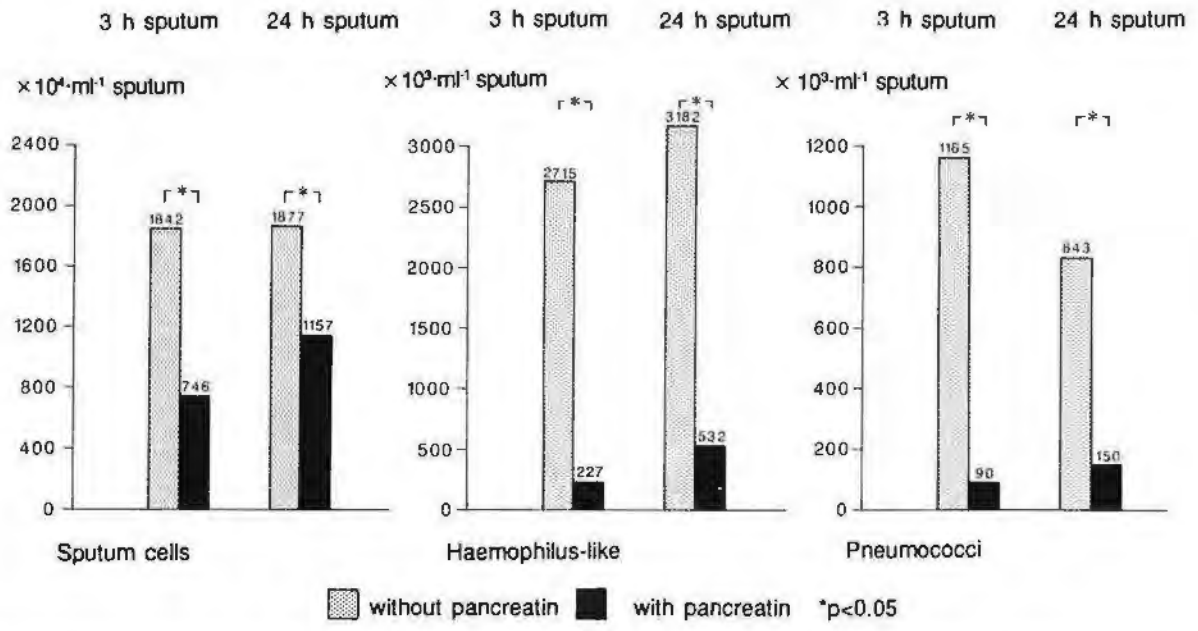

Fig. 1. – Effect of homogenization with 2% pancreatin on sputum cells and bacteria. Reported are the medians of non-zero observation. The differences were tested using two-sided Wilcoxon matched-pairs test.

B. catarrhalis was cultivated in the sputa of both collection periods in only two patients (Laboratory C, table 3).

Microscopic examinations of Gram-stained sputa revealed "other" Gram-negative rods in only two patients, table 1;staphylococcus-like organisms were never found. However, "other"Gram-negative bacteria were cultivated in the sputa of both collection periods in the same four and five patients. (Laboratory B and C respectively) *S. aureus* was detected in the 3 h and 24 h sputa of only one patient by Laboratory B, whereas Laboratory C cultivated this organism on blood agar in the 3 h and 24 h sputa of three and two patients, respectively.

The effects of homogenization with pancreatin are shown in figure 1. Cell and bacterial counts of haemophilus-like and pneumococcus-like micro-organisms were reduced by 90% or more.

Discussion

Bacteriological methods used in the clinical laboratory should be simple, reliable and as rapid as possible. Many clinicians and bacteriologists argue that sputum collected during 24 h is unsuitable for bacteriological examination in patients with chronic bronchopulmonary diseases. The reasons usually given are death of bronchopathogenic bacteria due to autolysis, antibody activity, and non-specific anti-bacterial substances on the one hand, and overgrowth of contaminating micro-organisms on the other [2, 3, 12]. Under our trial conditions no difference was found between bacteriological findings in fresh morning sputum and 24 h sputum with regard to Haemophilus and pneumococci, the two important pathogens in chronic bronchitics [7, 13]. Our findings contradict those of MAY and DELVES [12], who reported loss of viability of both micro-organisms when cultivation was delayed.

There was a statistically unprovable overgrowth of Gram-negative rods and *S. aureus* in the 24 h sputa which, however, did not substantially interfere with growth of *Haemophilus* or pneumococci. Our findings suggest that 24 h sputum of chronic bronchitics can be used for bacterial examination, either for Gram stains or for cultures, provided that optimal cultivation methods are employed for *Haemophilus* and pneumococci. Blood agar gave the same results as Crowe or chocolate agar for the cultivation of *Haemophilus*. Pneumococci were cultivated more often on chocolate agar than on CNA agar.

Gram stains of sputa of both collection periods revealed more patients with bronchopathogenic bacteria and higher bacterial counts than did cultures. The reduction of bacteria observed may be an effect of the pancreatin used in homogenization of sputum before culture which, however, would have worked on all specimens alike. Another reason for the difference may have been that bacterial counts over 90,000 CFU·ml sputum could not be recognized in culture because of confluent growth.

The successful cultivation of *Haemophilus* was impressive in view of the report of LEVINE et al. [14] in which more than 50% of patients with *Haemophilus* pneumonia, diagnosed in Gram stains and substantiated by positive blood cultures, had negative sputum cultures. Recently, CHODOSH [15] reported slightly better bacteriological results, the Gram stain of sputum of infected chronic bronchitis being positive in 62.8% of the specimens, and cultures in 88.5%. Our good recovery of *Haemophilus* was probably due, in part, to the washing procedures as reported by LAPINSKI *et al.* [16] On the other hand, difficulties in the cultivation of pneumococci have frequently been documented [13, 15–19]. We failed to cultivate pneumococci in over 50% of patients who had microscopically diagnosed pneumococci in their sputa. This corresponds to the findings of LEPOW *et al.* [17] and Chodosh [15], who showed that pneumococci could not be identified on culture from nearly 50% and 44%, respectively, of sputum specimens showing many pneumococci on Gram stained smears. Pancreatin may have contributed to this phenomenon.

Although our investigation suggests that Gram stains are superior to cultures for detecting bronchopathogenic bacteria in sputum, culture examinations retain their value for antibiotic testing of resistant pathogens.

The introduction by RAWLINS [8] of a method for homogenization of sputum before culturing was a considerable technical advance as it overcame the variations inherent in any method while examining small portions of heterogenous material. There remained, however, the problem of destruction of micro-organisms and cells by the homogenizing agent. As our results show, this was in fact occurring: 2% pancreatin had a highly detrimental effect on bronchopathogenic bacteria and sputum cells reducing the number of both microscopically determined bacteria and cells 5–100 times (fig. 1). Since sputa were diluted before as well as after liquefaction (dilution factor 2–3), the low number of cells and micro-organisms after liquefaction was probably caused by destruction and not by dilution. These results were unexpected and differed from the findings of RAWLINS [8, 20]. He showed that pancreatin, in 1% solution with pH 7.6 at 37°C, liquefied sputum without destroying sputum cells and interfering with bacterial viability; colony counts being the same before and after liquefaction. In contrast to Rawlins, we used a 2% pancreatin solution, which completely liquefied most sputa in 20 min. When we used a 1% solution, certain sputa did not liquefy within hours or at all. In fact, it took RAWLINS one to one and a half hours to liquefy sputum with 1% pancreatin [8]. Interestingly, he did not mention any difference in the rate of liquefaction by 1% and 2% pancreatin solutions. These contradictory results need clarification since Rawlins' method is a "standard procedure" for liquefying sputum prior to cultural examination.

For our bacteriological investigation all sputa were washed according to the method of MULDER [7] for two reasons: 1) washing of sputa eliminates the oral and pharyngeal flora, which contaminates tracheobronchial secretions [7, 21]; 2) washing of sputa facilitates the isolation of *Haemophilus* and pneumococci as reported by LAPINSKI *et al.* [16].

Although the number of patients in our study was small, the quantitative approach provided sufficient data to conclude that:

1) bacteriological examination of 24 h sputum yielded comparable results to those of fresh morning sputum as far as bronchopathogenic bacteria are concerned;
2) microscopic examination of 24 h sputum was comparable to fresh morning sputum;
3) Gram stains detected more patients with bronchopathogenic bacteria than cultures;

4) pancreatin was an inappropriate homogenizer as it destroyed bacteria and cells.

These conclusions are, however, restricted to sputa of chronic bronchitics examined with the methods described and should not be referred to other sputa.

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Coloration de Gram et culture de l'expectoration matinale et de 24 h pour le diagnostic des exacerbations bactériennes de la bronchite chronique: un dogme remis en question. T. Medici, A. Von Graevenitz, H. Shang, E. Böhni, M. Wall.

RÉSUMÉ: Les résultats bactériologiques provenant d'examens directs et de cultures de l'expectoration matinale et de l'expectoration de 24 h. ont été comparés pour tenter d'améliorer le diagnostic des exacerbations bactériennes de la bronchite chronique. Douze patients ont produit des échantillons de crachats pendant deux jours consécutifs, selon une répartition au hasard, soit d'abord l'expectoration matinale fraîche (expectoration de 3 h), suivie de l'expectoration de 24 h, soit la séquence inverse. Les crachats ont été lavés trois fois dans une solution de NaCl à 0.9%, et examinés au microscope avant et après homogénéisation au moyen de pancréatine à 2%. Les cultures ont été ensemencées avec l'expectoration homogénéisée. *Haeophilus influenzae*, Pneumocoques et Neisseria, ont été identifiés plus souvent dans les examens au Gram que dans les cultures. Les Staphylocoques et les Gram (-) le furent moins fréquemment. Environ 50% des organismes identifiés à l'examen microscopique comme Pneumocoques n'ont pas poussé à la culture. Les examens microscopiques ont révélé une réduction marquée des cellules et des bactéries après homogénéisation à la pancréatine. Les colorations de Gram de l'expectoration de 24 h montrent des résultats comparables à ceux de l'expectoration matinale. On n'a pas trouvé de différence non plus dans les cultures. L'examen microscopique des colorations de Gram s'avère supérieur au processus de culture. La valeur de l'examen microscopique est réduite par l'addition de pancréatine.
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