CAT
Critically Appraised Topic

Title: Gram stain (past, present and future)

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CLINICAL BOTTOM LINE

Gram stain is part of the standard protocol of many clinical specimens. It is used to categorize bacteria as Gram positive or Gram negative and as cocci or bacilli according to the morphology.
In this CAT, Gram stain has been evaluated regarding the analytical aspect, the diagnostic performance, and the impact (clinical/organizational/financial) in primary clinical specimens and in growing culture.
We have sent smears from different types of samples with a questionnaire to a few microbiology laboratories. We have used their answers to evaluate the performance of Gram stain in different laboratories and to compare it with the guidelines.
Based on this work, we conclude the following:

- Gram stain is a poorly controlled test (no external quality control, no frequent internal quality control). The frequency of inter-individual proficiency testing is very different between laboratories.
- Timeline: no Gram stain is performed during weekday night or weekend night in all questioned laboratories, except for emergencies such as meningitis.
- All questioned laboratories correlate direct/indirect Gram stain results with culture results. If they are not concordant, different actions are taken depending on the clinical relevance.
- In all questioned laboratories, Gram stain is always done on positive blood cultures, deep wounds, and body fluids.
- in all questioned laboratories, Gram stain is not performed on catheter tips.
- Different approaches are followed by respiratory tract samples, vaginal swabs for bacterial vaginosis, eye swabs and biopsies.
- The clinical utility of Gram stain for most of microbiological specimens is not worth the time or the effort it requires.
- Gram stain can be considered as a valuable test in the following contexts:
  -Positive blood culture, in order to guide the choice of empirical therapy.
  -Cerebrospinal fluid (CSF) sample in suspected meningitis, if PCR Meningitis/Encephalitis panel is not available or in special context.

-Vaginal samples to detect asymptomatic bacterial vaginosis, which can be important for females who will undergo a gynecological procedure.
-Two other rare indications for Gram stain: suspected *Neisseria* conjunctivitis or *Neisseria* urethritis in the absence of PCR test.

CLINICAL/DIAGNOSTIC SCENARIO

Gram stain is still performed on direct smears of primary clinical specimens or on secondary smears from growth mediums (broths, agar plates, positive blood cultures). After the introduction of matrix-assisted laser desorption-ionization – time of flight mass spectrometry (MALDI-TOF), VITEK 2 identification cards, molecular techniques, and the rapid antigen detection testing, the value of Gram stain as a part of the standard protocol of many clinical specimens is questionable. Omission of Gram stain when not needed could lead to considerable savings in terms of reagent costs and bench work in the laboratory.

QUESTION(S)

1) What is the clinical impact of a direct Gram stain on a clinical specimen?

2) What is the clinical impact of an indirect Gram stain on a subculture of a clinical specimen?

SEARCH TERMS

MeSH Database (PubMed): MeSH term: " diagnosis meningitis", " antibiotic Gram stain fluid", '' Gram stain quality improvement", "sputum Gram stain pneumonia", '' Kopeloff stain'', " Gram sensitivity vaginosis" , " delay Gram stain", "transport Gram stain", "wound epidemiology microbiology", " bacteriology wound", "utility Gram stain wound", "valve Gram stain", " Gram stain biopsy" "Gram stain pleural fluid", "diagnostic pleural fluid", "Gram stain spontaneous bacterial peritonitis", "bacterial conjunctivitis diagnosis Gram", "stool Gram stain", "sonication catheter Gram", catheter tip Gram", "mastoidtis Gram stain", "Vincent angina Gram stain", "epiglottitis Gram stain", "peritonsillar abscess Gram stain", "peritonsillar cellulitis Gram stain", "chronic otitis media Gram stain", "gastric biopsy Gram stain diagnosis", "valve Gram stain", prior antibiotic Gram stain".

1) *PubMed Clinical Queries: gram stain CSF, category: diagnosis, scope: broad. (Diagnosis/Broad [filter]) AND (gram stain blood culture sensitivity).*
2) *PubMed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane,*
3) *National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/)*
4) *Up-to-date*

RELEVANT EVIDENCE/REFERENCES

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APPRAISAL

1. INTRODUCTION

The Gram stain procedure was originally developed in 1884 by the Danish physician Hans Christian Gram to differentiate *pneumococci* from *Klebsiella pneumonia* in a lung tissue from a patient who had died with pneumonia. Gram had discovered that certain stains were taken up and retained by bacterial cells.

Over 100 years later, Gram stain is still in use. The procedure involves the following steps:

The fixed smear is flood with crystal violet for 1 minute, so all cells become purple. Then a solution of iodine (potassium iodide) is applied to these cells. This produces an insoluble crystal violet-iodine complex inside the cell, and this complex is extracted by alcohol (the third step) from Gram-negative but not from Gram-positive bacteria. This is due to the very thick cell wall of the Gram-positive bacteria (several layers of peptidoglycan) which prevent the insoluble crystal violet-iodine complex from escaping. In Gram-negative bacteria, alcohol readily penetrates the lipid-rich outer layer, and the thin peptidoglycan layer. Therefore, crystal violet-iodine complex is easily removed. To visualize the decolorized Gram-negative bacteria, a red counter stain such as safranin is used after decolorization step.

Till now, we still use the historical Gram strain from the year of 1884 with numerous modifications (concentrations of the dyes, length of the staining time and decolorizer decomposition).

Stain and use	Hucker's		Carbol fuchsin		Kopeloff's	
	Reagent	Time	Reagent	Time	Reagent	Time
Initial stain	Crystal violet	30 s	Crystal violet	30 s	Alkaline crystal violet: flood with solution A; add 5 drops of solution B	2–3 min
Iodine	Gram's iodine	30 s	Gram's iodine	30 s	Kopeloff's iodine	≥2 min
Decolorizer	Acetone-alcohol	~1–5 s	95% ethanol	~30 s	3:7 acetone-alcohol: rinse immediately after applying	
Counterstain	Safranin[a]	30 s	Carbol fuchsin or 0.8% basic fuchsin	≥1 min	Kopeloff's safranin	10–30 s
Recommended use	General bacteriology		*Bacteroides* spp. *Fusobacterium* spp. *Legionella* spp. *Campylobacter* spp. *Brucella* spp. and other faintly staining gram-negative organisms		Anaerobes Diagnosis of bacterial vaginosis (Appendix 3.2.1–3)	

[a] Or, preferably, use 0.1 to 0.2% basic fuchsin as a counterstain (5).

Table 1: Gram stain modification, recommended reagents, timing and uses (Clinical Microbiology Procedures Handbook, Leber 2016).

To answer the two main questions of the CAT, we have discussed direct smears and indirect smears apart. We started with the analytical performance, diagnostic performance, then the clinical, organizational and financial impact.

2. Analytical performance:

2.1 Pre-analytical factors:

Patient- related:

- *Prior use of antibiotic*: Prior use of antibiotic may have an adverse effect on Gram stain result. This has been shown in a few studies about sputum, CSF and pleural fluid. According to Greene JL et al. the sensitivity of CSF Gram stain in patients with meningitis is 60%–80% without antibiotic treatment and 40%–60% in patients who have received antibiotic treatment **(1).** Prior use of antibiotics may hinder but not prevent the bacteriological diagnosis of meningitis. This was the conclusion of Bohr V. from his 3 part series study from Denmark 36 years ago. In this study, the bacterial diagnosis of meningococcal meningitis was affected by treatment **(2).**

  Significant decrease in the proportion of positive CSF Gram stain after antibiotic therapy in patients with *Haemophilus influenzae* meningitis has also been documented in a prospective study (120 pediatric patients)**(3).** Gram staining of the skin lesion in patients with meningococcal meningitis can also be helpful to establish the diagnosis because skin lesions are less affected by pre admission antibiotic therapy **(4-5).**

  According to Lise E. Nigrovic et al. the rate of positive CSF Gram stain results did not differ according to pretreatment status (see table 2). In this study, the causative pathogens and the antibiotic groups were not mentioned **(6).**

  In the study of Mucher DM et al., Gram stain was positive in one of seven samples and culture was positive in two of seven sputum samples among patients with bacteremic pneumococcal pneumonia who had been treated with antibiotics for over 24 hours before submitting a sputum sample **(7).** The morphology of bacteria may also be affected if the patient has received antibiotics prior to specimen collection. For example, some Gram-negative bacteria may become longer with appearance of ultra structures of *Staphylococcus aureus* in sputum after treatment with beta-lactam antibiotics **(8-9).** The changes appeared microscopically rather than in the subculture.

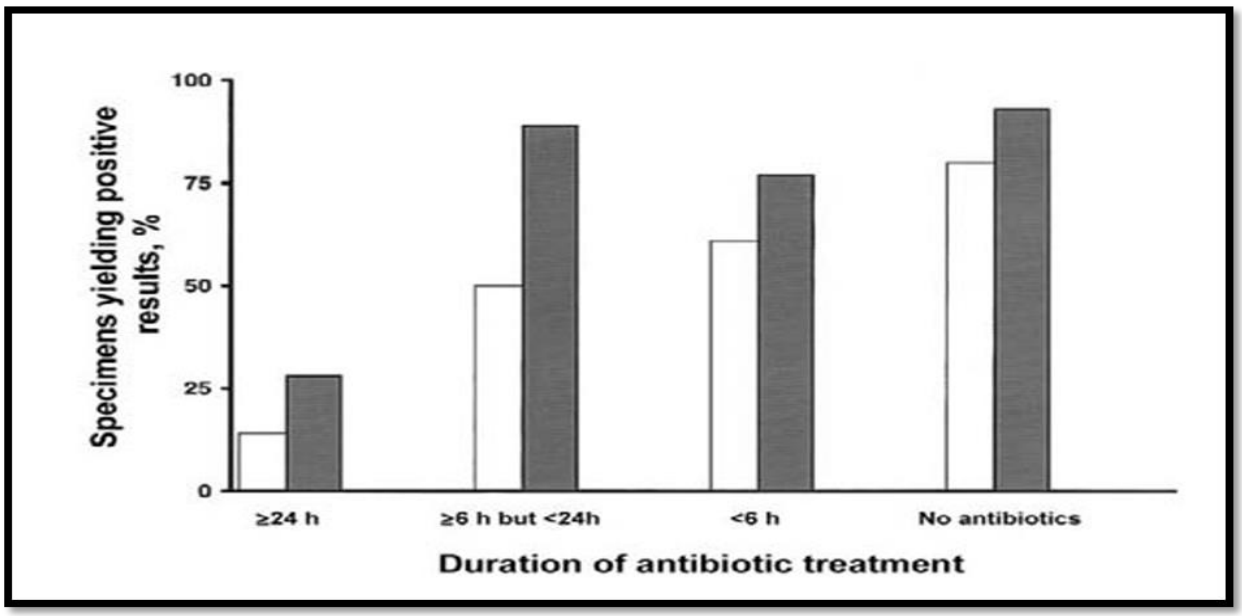


Figure 1: Relationship between the duration of antibiotic treatment and results of Gram staining And culture. The yield of sputum Gram staining (open bars) and culture (shaded bars) for detection of *Streptococcus pneumoniae* in patients with proven pneumococcal pneumonia, shown on the vertical axis, is inversely proportional to the duration of antibiotic treatment, shown on the horizontal axis (Musher DM et al.)[7].

	n/N (%)		*P*
	No Antibiotic	Any Antibiotic	
Positive CSF Gram-stain results[a]	95/150 (63)	46/74 (62)	.86
Positive blood culture results[b]	123/187 (66)	16/33 (48)	.05
Positive CSF culture results[c]	136/154 (88)	53/76 (70)	.001

Table 2: Frequency of Positive Gram-Stain and Blood + CSF bacterial culture results for patients with befinite Bacterial meningitis, according to antibiotic pretreatment status (Lise E. Nigrovic et al.)[6]

In a prospective study including 110 children with parapneumonic effusion, 50% had received antibiotics at least 48 hours before pleural fluid analysis. This has a negative impact on the results of Gram, culture, and blood culture tests (see table 3). Nevertheless, it did not interfere significantly with biochemical parameters of pleural fluid (pH, glucose, and LDH) **(10).**

The biochemical CSF parameters (glucose and protein) are also not significantly altered by previous use of antibiotics in patients with bacterial meningitis **(11).** Similar results were shown by Blazer and colleagues in the assessment of cellularity, glucose, proteins, and the percentage of polymorphonuclear cells in CSF **(12).**

Characteristic	Previous use of antibiotics (n = 55)	Without previous antibiotics (n = 55)	*P*
Biochemical analysis			
pH ≤7.1[c,d]	11 (23.4)	20 (41.7)	.093
Glucose ≤40 mg/dL[c,d]	19 (36.5)	30 (56.6)	.062
LDH ≥1.000 UI/L[c,d]	37 (78.7)	29 (74.4)	.825
Microbiological analysis			
Gram-positive[c,d]	8 (14.5)	19 (34.5)	.027
Positive PE culture[c,d]	7 (12.7)	18 (32.7)	.023
Positive hemoculture[c,d]	5 (9.4)	15 (27.3)	.033
WBC count (cells/mm³)[a,b]			
Blood	15.190 (11.530-20.367)	16.460 (9.400-22.200)	.964
PE	4.500 (1.082-9.370)	5.400 (563-20.590)	.568

Table 3: comparison of children with or without previous use of antibiotic therapy (Becker A et al.)[10]

No data are available about the effect of pre antibiotic treatment on Gram stain results of other microbiological samples.

- *Time of specimens collection*:
  No data are available about the effect of sampling time on Gram stain result for different microbiological samples.

- *Inappropriate specimen sampling*:
  Poor quality sputum samples are more likely to yield positive Gram stain and negative culture than good to fair samples. Good quality sputum samples are more likely to yield Gram stain results that agreed with culture result (ie, both culture and Gram stain are positive or both are negative) **(13).** It is not simple to obtain good quality respiratory samples. In a cohort of 1669 patients with community acquired pneumonia CAP, only 14% of all sputum samples of these patients were of good quality and with predominant bacteria **(14).**
  No evidence are available about the effect of this pre-analytical factor on the Gram stain results of samples other than sputum samples.

- *Effect of transport medium*:
  Microscopic examination of genital and wound swabs (using ESwab) showed superior results to those obtained in the Amies gel Transystem **(15).**

Specimen types	Results are expressed as: no. of slides presenting differences in microscopic observation of human cells and/or of microbial elements/no. of samples tested		
(no.)	ESwab Volumes for slides preparation		
	100 µl§	50 µl§	Amies Gel slides
Vaginal Swab (32)	32/32	26/32	16/32
Cervical Swab (27)	27/27	25/27	15/27
Urethral Swab (11)	11/11	11/11	8/11
Wound Swab (10)	10/10	10/10	7/10
Total (80)	80/80 (100%)	72/80(90%)	46/80 (57.5%)
P value		P = 0.16	P = 0.04

§ = the results were the same even after 24 and 72 h storage

Table 4: Microscopic examination of ESwab gram-slides vs Amies gel slides (Fontana C et al) [15]

No evidence are available about the effect of transport medium on direct Gram stain result of other microbiological samples.

- *Delayed transport*: It has been mentioned that the interpretation of urine analysis is dependent on the quality of the urine samples and the conditions of transport to the laboratory **(16).** No evidence are available about the effect of this factor on the Gram stain result of urine samples or other samples.

- *Sample contamination due to bad collection technique and handling:* Specimens should not be processed if received in inappropriate containers or improper transport medium, or if received after a prolonged delay. No data are available about the effect of contamination on Gram stain result.

- *Specimen processing*: centrifugation of body fluids improves the sensitivity of Gram stained smear about 2 log and improve the WBCs morfology **(17).**

- *Smear preparation*: very thick smears may lead to under-decolorization and misinterpretation. No data are available about the effect of this pre-analytical factor on Gram stain result.

- *Smear staining*: number of studies have shown that methanol fixation gives more reliable Gram staining results and more cells per field than heat fixation. Methanol-fixed Gram-positive bacterial cells were less

sensitive to decolorization during the Gram staining procedure than heat-fixed cells **(18-19-20).**

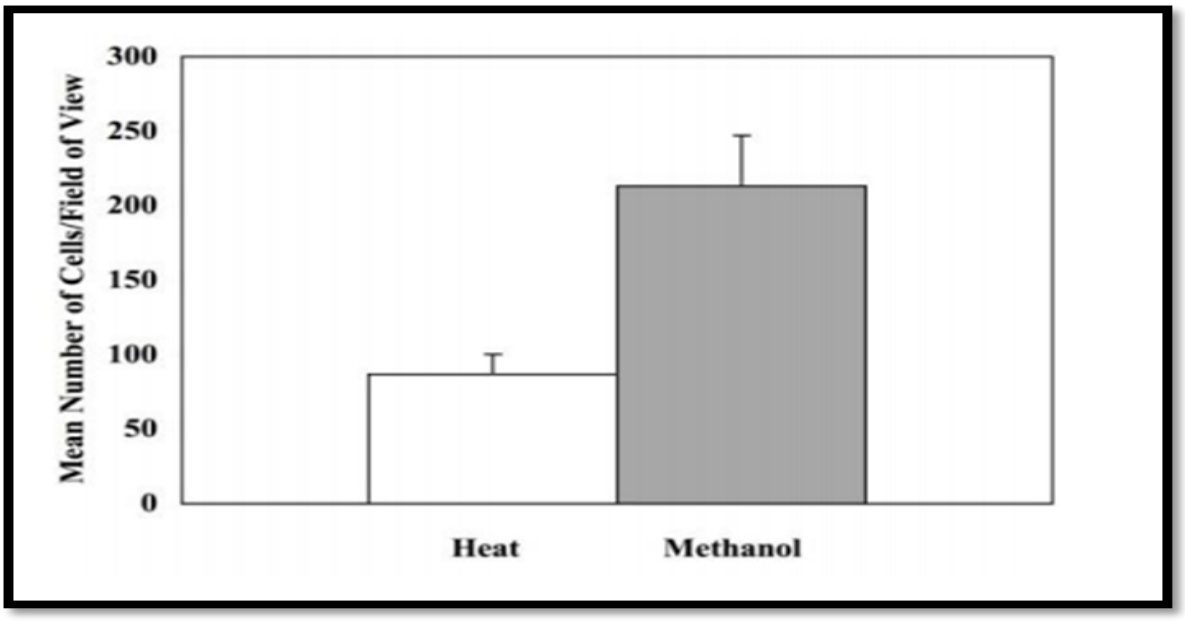


Figure 3: (Jeanne M. Minnerath et al.)[20]

2.2 Analytical factors:

- *Detection limit*: to be visible on a slide, organisms that stain by the Gram method must be present in a minimum concentrations of 10^4 to 10^5 organisms/ml fluid. At lower concentrations, the Gram stain will rarely reveal organisms even if the culture is positive.

- *Accuracy*: in a multicenter assessment of Gram stain errors, 24% of discrepant results were due to reader error. This varied significantly between the sites A, B, C, and D (9% to 45%). The samples were respiratory fluid, biopsy tissue, and wound samples (positive blood culture smears were excluded) **(21).** Gram stain misinterpretation was mostly with mixed infection or Gram positive cocci. There were also misinterpretations in the positive blood cultures but in low percentages **(21-22).**
  In Q-probe study in 2015, the median discrepancy rate in Gram stain interpretation of blood cultures was 1%. The highest discrepancy rate was 20.8% for mixed culture **(23).**

Site	No. (%) of reader errors	Projected no. (%) of total errors	Projected errors/ 1,000 smears	Z-score
A	6/67 (9)	7 (0.4)	3.8	−3.1042
B	24/78 (31)	25 (1.5)	15.3	1.0884
C	12/74 (16)	15 (0.9)	9.1	−0.9565
D	20/44 (45)	26 (2.7)	26.6	3.6351
Total	62/263 (24)	73 (1.2)	6.9	

Table 5: Analysis of discrepant results and Gram stain error rate (Samuel LP. et al.)[21]

- *Correlation with culture:*
  High correlation rate 94% was observed between Gram stain and specimens cultures that are rich with colonies (3+, 4+). Less correlation (76%) was observed with specimens' cultures with few (2+) colonies. The lowest correlation (29%) was found for specimen cultures with rare (1+) colonies **(21).**

- *Precision*: To monitor reproducibility, Bartlet RC and his group have made preparation of suspensions of cells and bacteria that yielded identical smears for subsequent examination (examiners as unknowns) **(24).** This method has shown the following results:
  - Neutrophils conformance: 72-78%
  - Squamous cells conformance: 68-78%
  - Bacterial identification category 100%
  - Bacterial enumeration 45-96%

Table 6: Results of Gram stain from 6 different microbiology laboratories to which 11 Gram stain slides from 11 samples with relatively high counts of bacteria were sent to each of them for analysis.

Slide	Sample type	Gram stain (Lab 1)	Gram stain (Lab 2)	Gram stain (Lab 3)	Gram stain (Lab4)	Gram stain (Lab 5)	Gram stain (Lab 6)	Culture
1	Perianal abscess	WBC+++ GNR+++ GPC rare	WBC ++ RBC ++ mixed flora +++	WBC+++ RBC++ GNR+++ GPC+++	WBC ++ RBC + GNR++ GPC +	WBC +++ RBC ++ GNR+++ GPC++	WBC+++ RBC+++ GNR+++ GPC +	S.anginosus+++ S. agalactiae++ C. freundii (enrichment) H.parainfluenzae afew B. fragilis+++
2	Sputum	PLEC ++ WBC++ Mixed flora +++ GNR rare GPC ++ Pneumococci?	PLEC ++ WBC ++ Mixed flora ++	PLEC+ WBC++ GNR++ GPC+++	PLEC + WBC+ Yeast + GNR++	PLEC >10 WBC >100/field (Sample is not representative for deep airways)	PLEC ++ WBC+++ Mixed flora +++	Mixed flora ++ P.aerogenosa++ Yeast a few
3	Sputum	No PLEC WBC +++ GPC +++ Pneumococci?	PLEC rare WBC +++ GPC +++	PLEC+ WBC++ GPC+++ GNR++	PLEC rare WBC+ GPC+	WBC +++ GPC +++ GNR ++	PLEC + WBC+++ GPC +++	S. pneumoniae +++ Mixed flora ++ H.parainfluenzae

		Mixed flora +++			Mixed flora+ **Mononuclear cells+?**			**+**
4	**Blood culture**	GPR	GPR or GNR (repeat it again)	GPR and GNR or Gram variable?	GPR and GNR? (repeat it again)	GPR	GNR	**Clostridium ramosum**
5	**Blood culture**	Streptococcus	Streptococcus	Streptococcus	Streptococcus	Streptococcus	Streptococcus	**E. faecalis**
6	**Deep wound (swab) : stoma**	GNR+ GPR+	WBC ++ Mixed flora ++	WBC+++ Mixed flora+++ **(anaerob.?)**	WBC + RBC+ GNR + GPR + GPC +	WBC ++ RBC ++ GNR+++ GPR ++ GPC++	WBC +++ RBC +++ GNR + GPR rare GPC +	**P. mirabilis (enrichment)** **E. coli+++** **S.vestibularis +++** **S.lutetiensis +++** **E. faecium +** **S.anginosus +** **P.pentosaceus +** **C.perfringens ++**
7	**Biopsy (bilioma)**	GNR GPR **GPC (Staph.)**	WBC ? Mixed flora +++	WBC++ GNR+++ GPC+ GPR++	WBC+- GNR +++, GPC+ GPR+	No WBCs GNR +++ GPC ++ GPR ++	GNR +++ GPC + GPR +	**C.freundii** **L.johnsonii** **E. faecium** **E. faecalis** **B.fragilis** **P.denticola** **C. tropicalis (enrichment)**
8	**Vaginal swab**	PLEC+++ Clue cells +++ Gram var. rods +++ GPC rare WBC rare	PLEC +++ Clue cells ++ Gramvariale rods +++ Mixed flora+ WBC rare	Microscopic: BV Bacterial vaginosis	PLEC+ clue cells + Gram variable rods +++ GPC+ lactobacillus+	PLEC++ Clue cells + **Nugent score: 8** Suggestive for bacterial vaginosis.	Microscopic : BV Clue cells 1+	**Gardnerella vaginalis +++** **Normal vaginal flora+** **K. pneumoniae ++**
9	**Ear discharge (swab)**	WBC ++ GNR +++	PLEC + WBC ++ Mixed flora rare **yeast +**	WBC+ GNR+++	WBC rare GNR ++	WBC + GNR +++	WBC + GNR +++	**P.aeruginosa +++** **S.epidermidis (enrichment)**
10	**Sputum**	PLEC +++ WBC+ Mixed flora +++ **Yeast, pseudomycelim +++**	PLEC ++ WBC ++ Mixed flora +++ Yeast rare	PLEC + No WBC GND++ GPR+ GPC+	broken	**PLEC** >10/field (sample is not representative for deep airways)	PLEC +++ WBC +++ Mixed flora +++	**Mixed flora +++** **Yeast ++**
11	**Jackson - Pratt drain**	WBC+++ No bacteria	WBC rare No bacteria RBC +++	PMN+++ No bacteria	broken	WBC + No bacteria RBC +++	WBC + No bacteria RBC +++	**Negative**

There are some observations on the Gram stain results in the table above:

- **Sample 1**: Irregular staining, round ended or pleomorphism of *Bacteriodes fragilis* (as mentioned in microbiology books and literature) were not noticed by all of the six laboratories.

- **Sample 2** and the same for **sample 10**: These were sputum sample with a lot of plate like epithelial cells. Such samples are worked out in five of the six laboratories and is rejected by one laboratory. According to IDSA 2018, poor-quality sputum specimens give misleading results and should be rejected because the interpretation would be compromised. The predominance of Gram negative rods (*Pseudomonas aeruginosa*) in sample 2 was not observed on Gram stain by most of the six laboratories. Two of the six laboratories have observed predominance of Gram positive cocci (possibly *Pneumococci*) on the Gram stain (which did not grow on culture).

- **Sample 3**: Sputum with a few or rare plate-like epithelial cells and rich with Gram positive cocci *(Streptococcus pneumoniae).* Five of the six laboratories have not seen or mentioned that there are Gram positive diplococci (or possibly *pneumococci*) In the Gram stained smear.

- **Sample 4**: This was a positive blood culture with Gram positive or Gram variable rods (*Clostridium ramosum*). In some answers, it was thought that there are 2 types of bacteria Gram positive and Gram negative rods.

- **Sample 5, 6, and 7**: were concordant or almost concordant.

- **Sample 8**: microscopic diagnosis of bacterial vaginosis was reproducible. The lack of standardization in reporting here is remarkable.

- **Sample 9**: one of the six laboratories had missed the predominance of Gram negative rods (*Pseudomonas aeruginosa*).

- **Sample 11**: was concordant. No bacteria were seen in Gram stained smear.

- Discrepancies of Gram results with final culture results are mostly noticed in cultures from non sterile sites.

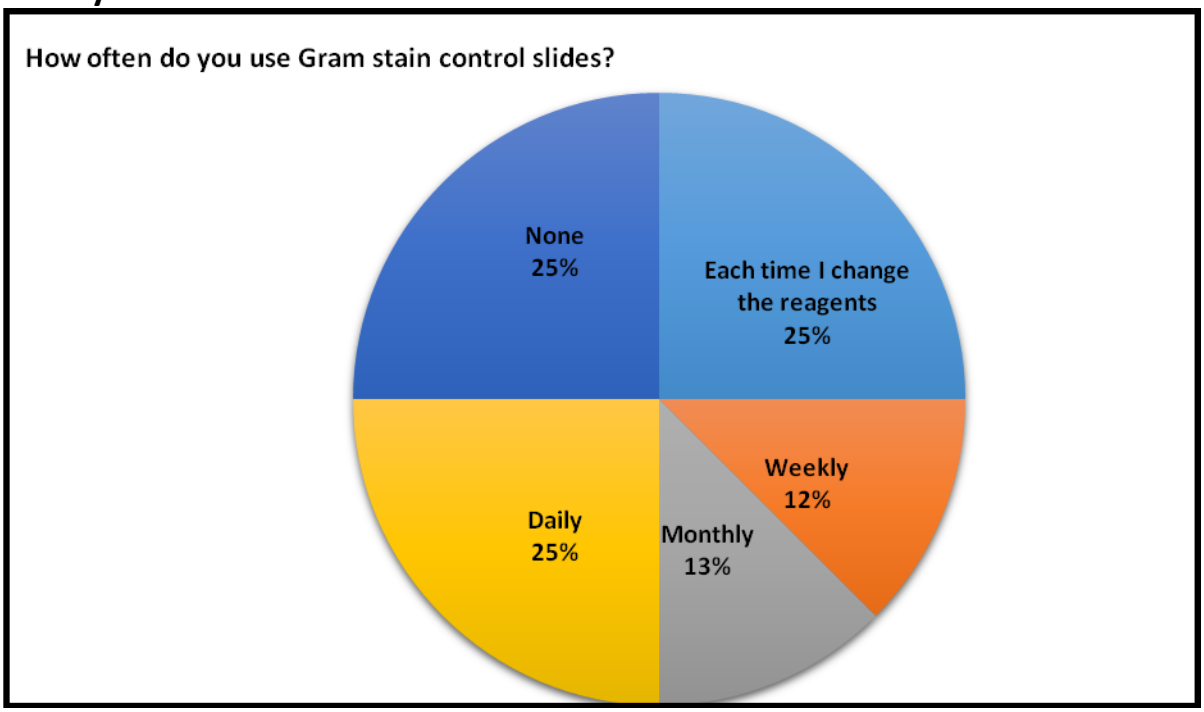
- The Gram variable nature of some organisms can be misleading (example: *Bacillus spp*. may appear Gram negative while *Acinetobacter spp.* may stain Gram positive).

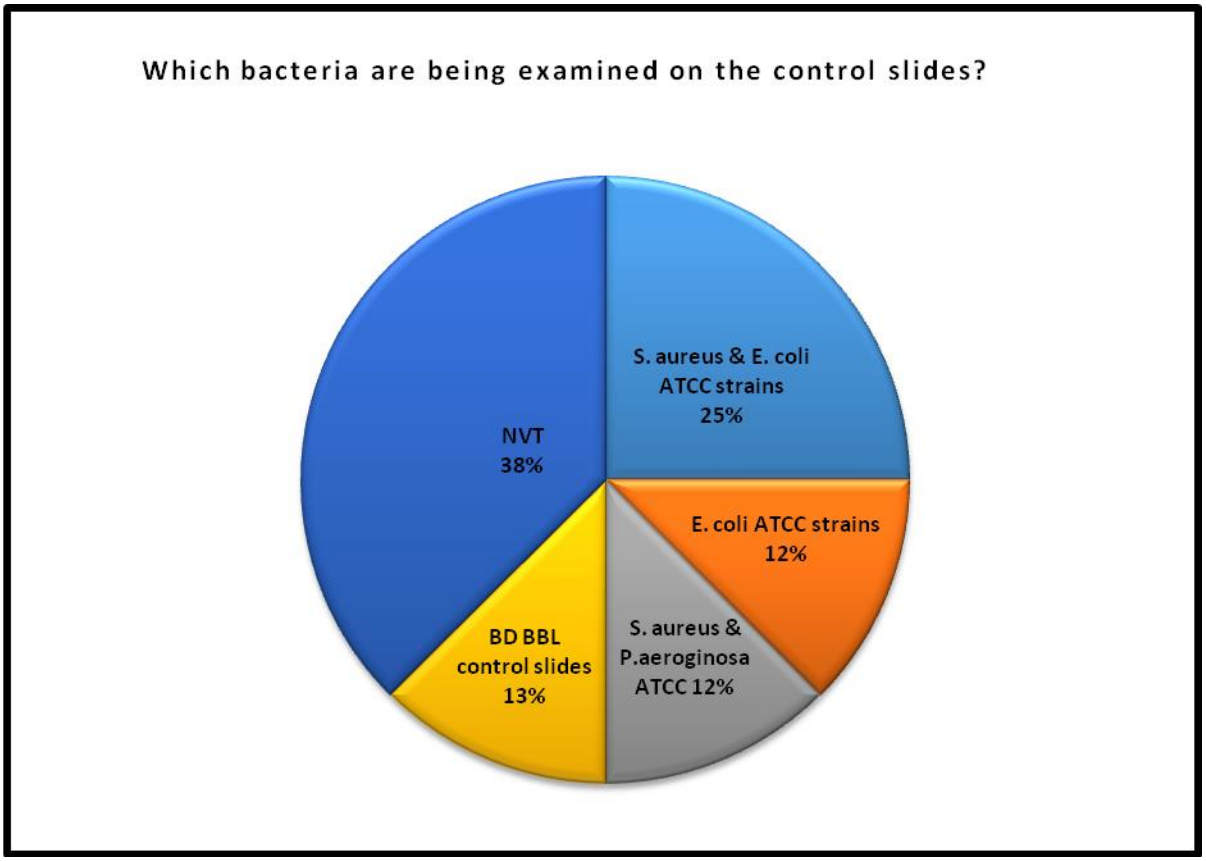
3. Quality factors

3.1. *Internal quality control*:

According to the Clinical Microbiology Procedures Handbook (Leber et al.,2016), The College of American Pathology (CAP), and ISO 15189: Gram stain reagents should be tested with control organisms (known Gram-positive and Gram negative), with each batch of reagents, lot number and shipment and weakly thereafter.

Survey results:

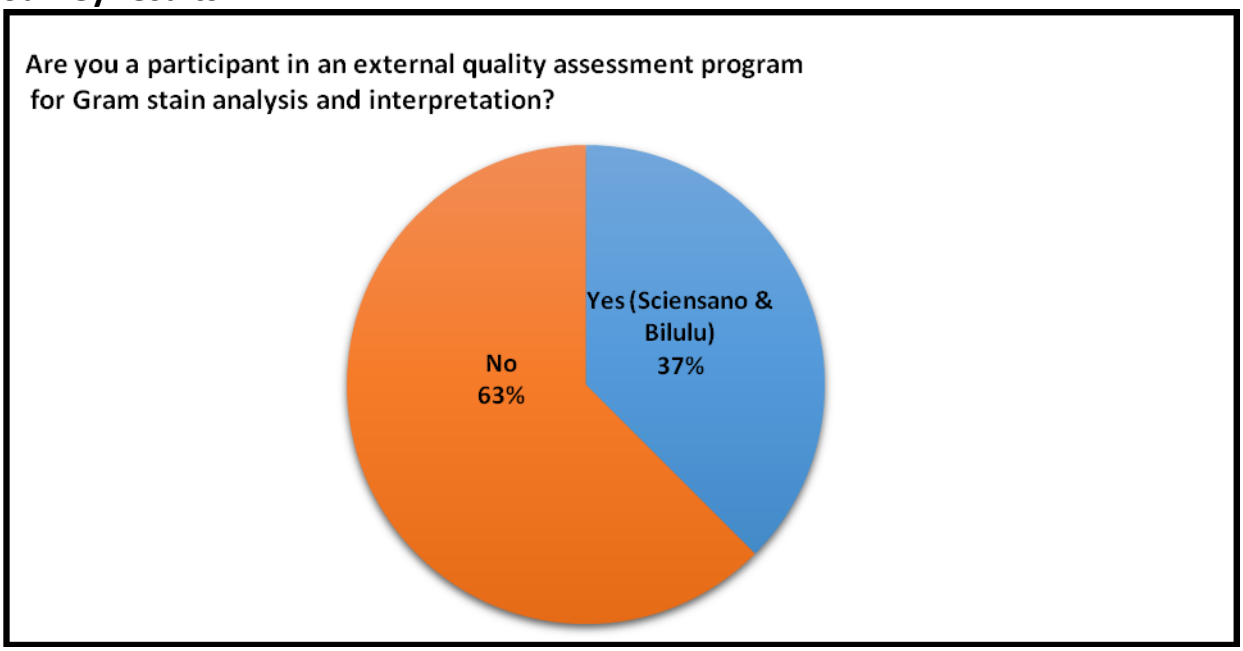


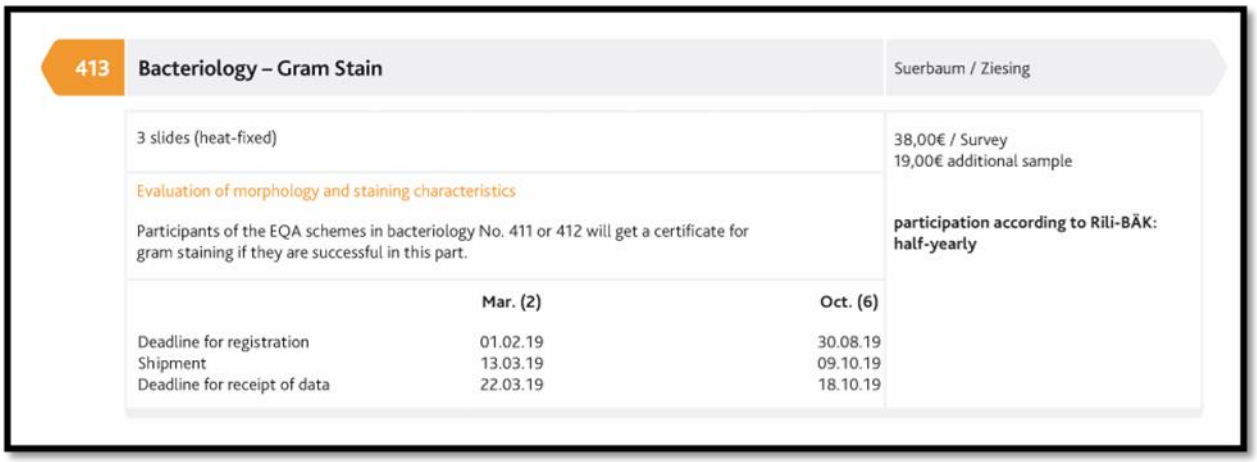


After analysis of the survey, it appears that Gram is a poorly controlled test. All of the 7 clinical laboratories use an automatic stainer (Aerospray, RAL-stainer, Mira stainer, Polystainer). The frequency of usage Gram stain control slide was extremely variable.

3.2. *External quality control*:

Survey results:





413	Bacteriology – Gram Stain	Suerbaum / Ziesing
3 slides (heat-fixed)		38,00€ / Survey 19,00€ additional sample
Evaluation of morphology and staining characteristics Participants of the EQA schemes in bacteriology No. 411 or 412 will get a certificate for gram staining if they are successful in this part.		**participation according to Rili-BÄK: half-yearly**

	Mar. (2)	Oct. (6)
Deadline for registration	01.02.19	30.08.19
Shipment	13.03.19	09.10.19
Deadline for receipt of data	22.03.19	18.10.19

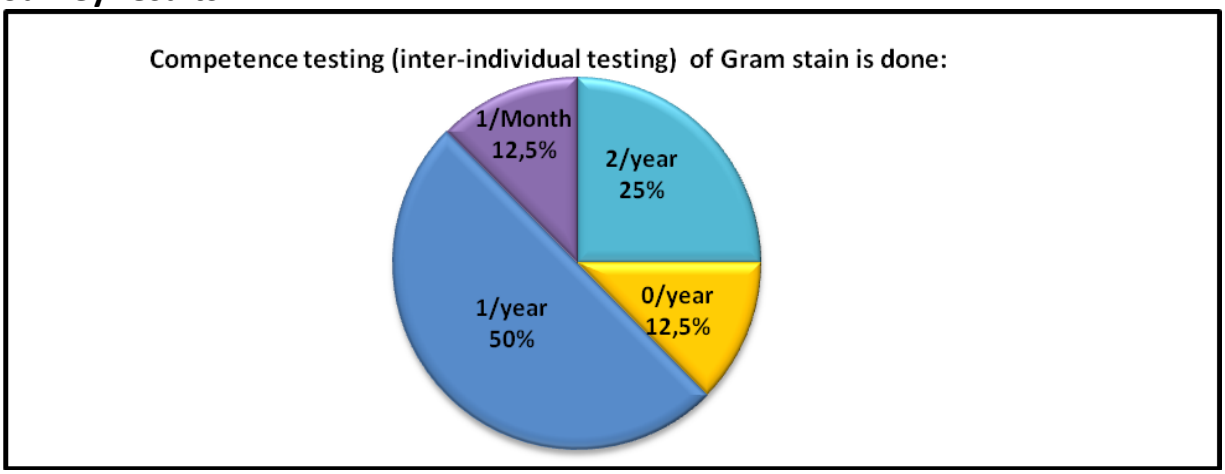
The only available program which is specific for Gram stain quality control is INSTAND society 2 times a year. Sciensano (WIV) external quality control is actually for identification and susceptibility testing of bacteria, and not for Gram stain.

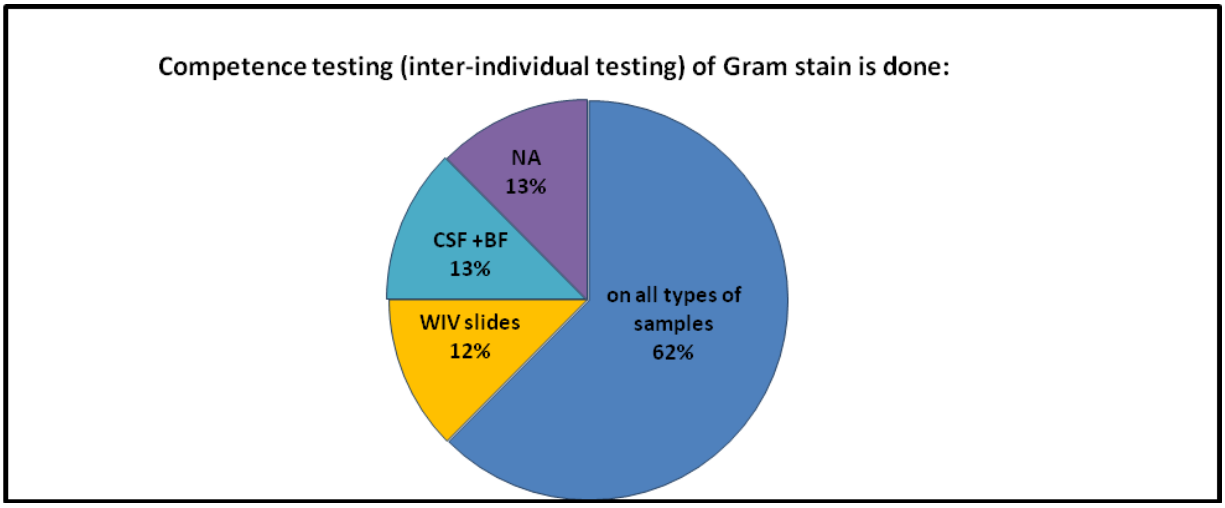
Also the circulation of special samples between the laboratories of the BILULU study group, can be considered as a kind of quality control. But this is not specific for Gram stain either.

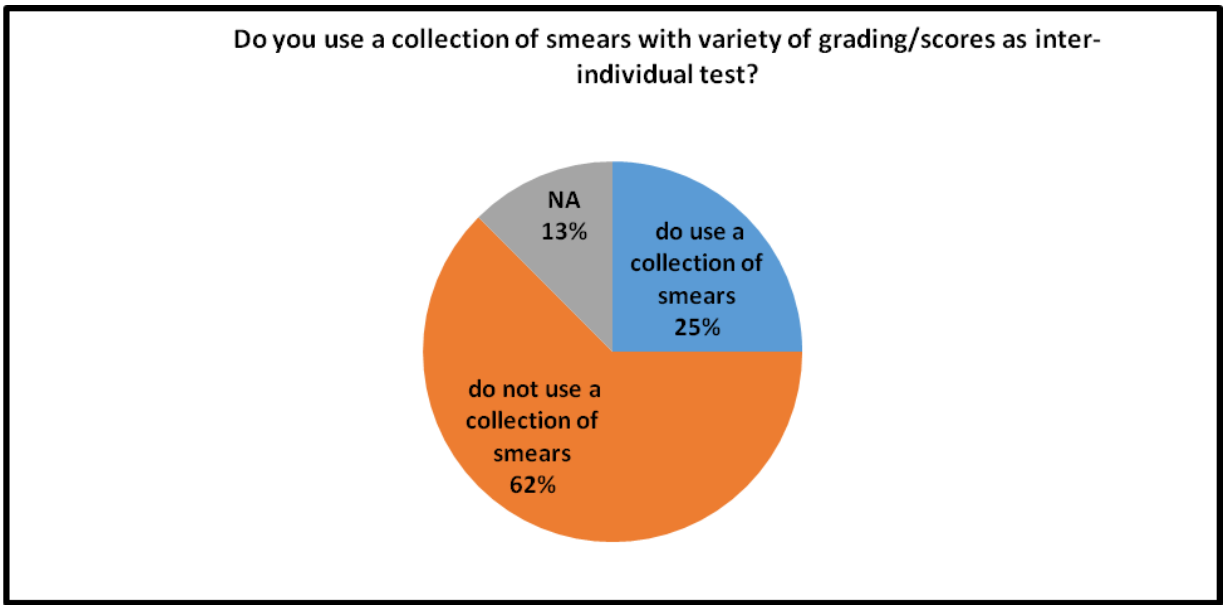
3.3. *<u>Competence testing (inter-individual testing)</u>*:

According to ISO 15189: this should happen on a periodic basis.

Survey results:







After analysis of the survey, the frequency of inter-individual proficiency testing is extremely variable. The types of samples that are used are mostly diverse and it is usually done with a single slide, not with a collection of slides.

4. Diagnostic performance of Gram stain on each type of primary clinical samples:

4.1. *Respiratory samples for respiratory infections (pneumonia):*

Sputum samples, endotracheal aspirates (EA) and bronchoalveolar lavage (BAL) are the most common specimens submitted for diagnosis of lower respiratory tract infections.

Direct Gram stain and culture can indicate the causative organism with variable sensitivities according to the causative agent.

In a meta-analysis (21 studies) examining Gram stain of respiratory specimens (BAL and EA) for the diagnosis of ventilator-associated pneumonia, the pooled sensitivity was 79% and specificity 75%. Negative predictive value of Gram stain for diagnosis of VAP was 91% (prevalence of 20%–30%). The positive predictive value was 40%. Pooled kappa for correlation with culture results was 0.42 for Gram-positive organisms and 0.34 for Gram-negative organisms **(25).**

The negative predictive value of Gram stain on EA was also high (92.8%) for *Staphylococcus aureus* in patients with VAP **(26).**

A prospective, observational, cohort study from Israel (114 patients) showed that the sensitivity of Gram stain of EA compared with culture was 90.47% for Gram-positive cocci, 69.6% for Gram-negative rods, and 50% for sterile cultures. Specificity was 82.5%, 77.8%, and 79%, respectively. Negative predictive value was high for Gram-positive cocci (97%) and sterile cultures (96%) but low for Gram-negative rods (20%). *Acinetobacter baumanii* (45%) and *Pseudomonas aeruginosa* (38 %) were the prevailing isolates **(27).**

In 105 patients with Pneumococcal pneumonia proven by blood culture, only 31% of them had a positive Gram stained smear with Gram positive cocci suggestive for *Streptococcus pneumoniae*. After exclusion of inadequate sputum samples and patients with previous antibiotic treatment of >24 hour, the sensitivity of Gram stain was much higher (57%). The sensitivity of the cultures was also higher after exclusion of those two criteria **(7).**

The diagnostic performance of Gram stained sputum samples was also evaluated in a prospective observational study from Japan on hospitalized patients (478 patients with pneumonia).The sensitivity and specificity of sputum Gram stain were 62.5% and 91.5% for *Streptococcus pneumoniae*, 60.9% and 95.1% for *Haemophilus influenza*, 68.2% and 96.1% for *Moraxella catarrhalis*, 39.5% and 98.2% for *Klebsiella pneumoniae*, 22.2% and 99.8% for *Pseudomonas aeruginosa*, 9.1% and 100% for *Staphylococcus aureus* **(28).**

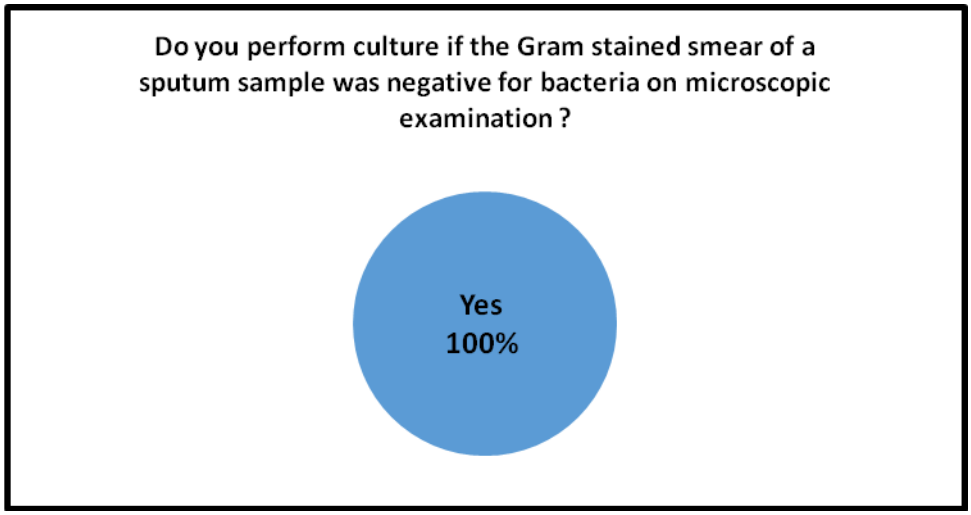
In contrast to what the first three studies had shown, a retrospective study over 131 ICU patients in Japan had evaluated the role of Gram stain of endotracheal aspirate to predict the causative bacteria in VAP patients. The NPV for Gram negative rods (88, 9%) was higher than the NPV for Gram positive cocci (86, 6%) **(29).**

According to IDSA 2018, Gram stain for sputum samples is important for the purpose of screening for acceptability of samples.

There are different combinations and cutoffs of the minimum number of epithelial cells and/or polymorphonuclear cells per low power field, which can be used to evaluate the quality of sputum samples. None of these parameters can be considered to be clearly superior. The following parameters are for poor quality sputum samples:

1. >10 plate-like epithelial cells per LPF.
2. <25 polymorphonuclear cells per LPF.
3. >10 plate-like epithelial cells and <25 polymorphonuclear cells per LPF.
4. >25 plate-like epithelial cells per LPF.

According to Leber et al.,2016 and IDSA 2018, inadequate sputum sample should not be accepted for culture. Such sample can be accepted if it is impossible to recollect specimens. It should also be mentioned in the patient report that the specimen was not acceptable but was processed at the specific request of physician, with name on the test report. Bronchial aspiration and bronchoalveolar lavage are not allowed to be rejected even if they were contaminated with epithelial cells. In contrast to what all the six laboratories do, it is also mentioned that cultures should not be performed if the Gram stained smear of the sputum sample was negative for bacteria.



The reporting of a potentially pathogenic organism in a non-representative sputum sample could be misleading, particularly in cases of clinical pneumonitis (IDSA 2018).

It is also important to mention that there is a documented inter individual variability (subjectivity) in the quality assessment of respiratory specimens in addition to the variability in the followed criteria for rejection of sputum samples **(30).**

Gram stain for sputum of cystic fibrosis patients should be only performed if explicitly requested. The rejection criteria should not be applied for sputum samples from cystic fibrosis patient. Although 40% of these samples would be rejected according to Gram stain rejection criteria, >90% of the cultures of such specimens will grow potential pathogens **(31).**

Other rejection criteria include: duplicate specimens on the same day unless the initial sample was inadequate or post-bronchoscopy.

As **conclusion**: The processing of respiratory samples varies widely in practice from what is stated in the guidelines. Sensitivity of lower respiratory tract

samples (EA and BAL) is higher than that of sputum. Pre antibiotic therapy and contamination of sputum with epithelial cells decrease the sensitivity of the Gram stain. There is a controversy in literature over the diagnostic performance of Gram stain for lower respiratory tract samples especially for Gram negative rods. The high negative predictive value of Gram stain for Gram positive cocci suggests that diagnosis of pneumonia due to Gram positive cocci is unlikely with negative Gram stain. Kappa statistics suggest that there is a poor correlation between Gram stain and the bacterial recovery from cultures. Antibiotic therapy for pneumonia should not be narrowed according to Gram stain results **(25).** Gram stain is mentioned in IDSA guidelines for screening for acceptance of sputum samples and for its moderate to high negative predictive value.

4.2. *Genital samples*:

4.2. A. *Genital samples for bacterial vaginosis*:

The diagnosis of bacterial vaginosis is based on the presence of Amsel criteria or Nugent score. At least 3 of the 4 Amsel criteria should be present:

- Homogeneous, grayish-white vaginal discharge
- Elevated pH (>4, 5)
- Fishy odor
- The presence of 20% clue cells by microscopic examination of the epithelium (wet mount). Microscopy is needed for such evaluation.

The first three findings (grayish-white vaginal discharge, elevated pH > 4, 5, fishy odor) are sometimes also present in patients with Trichomoniasis. The sensitivity of the clinical criteria to detect bacterial vaginosis is 90%, the specificity 77%. Lower sensitivity is recorded in pregnant women (62%) **(32).**

Gram stain of vaginal discharge is the gold standard for the diagnosis of bacterial vaginosis. By Gram stain-based Nugent score, a total score of 7 to 10 is indicative of bacterial vaginosis infection.

A multicenter study has shown that the sensitivity and specificity of the Gram stain compared with the Amsel criteria were 89 and 83%, respectively. The sensitivity and specificity of Amsel criteria compared to Gram stain as the standard are 70% and 94% respectively **(33**).

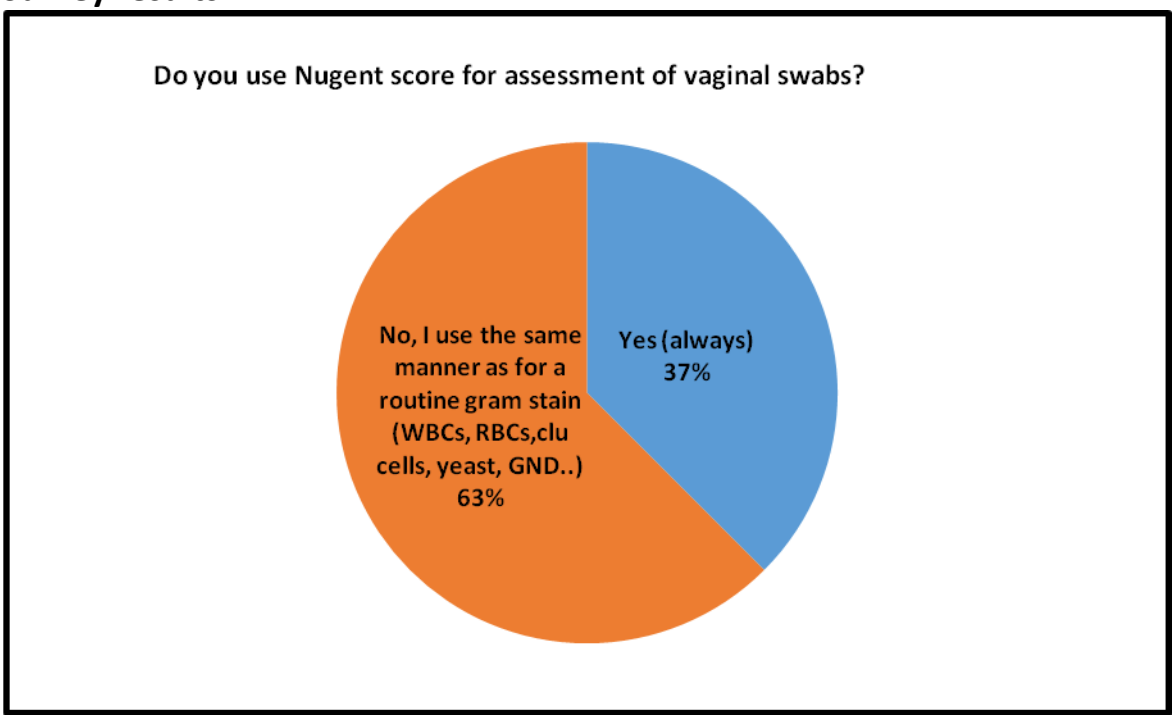
Amsel criteria and Gram stain based Nugent score are the two most commonly accepted methods for the diagnosis of bacterial vaginosis **(34).** Beside Gram stain-based Nugent score, there are also Gram stain-based Hay/Ison criteria. This can be used as an alternative to Nugent score in busy hospitals **(35).**

	Lactobacilli morphotypes	Gardnerella morphotypes
Normal	Many	Few

Intermediate	Equal amount	Equal amount
BV	Few	Many

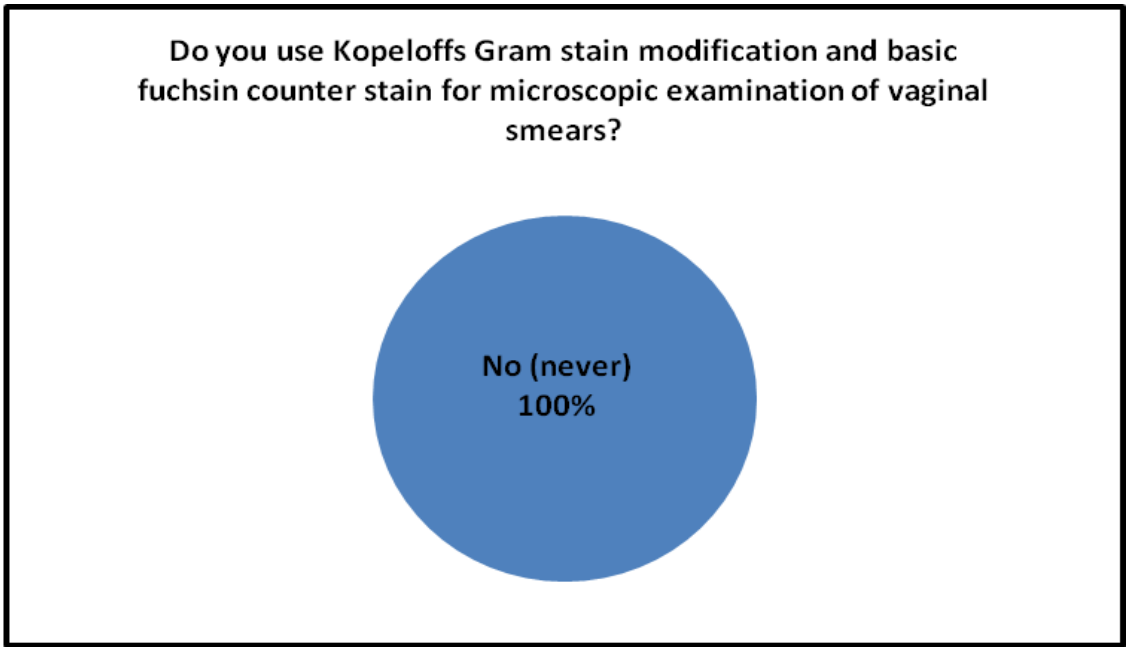
Table 7: Hay/Ison criteria

Survey results:



Some authors have suggested to use the Kopeloff modification of the Gram stain. The Kopeloff stain minimizes over-decolorizing of Gram-positive bacteria and enhances the visibility of Gram-negative organisms. *Gardnerella* tends to appear Gram-positive using the Kopeloff stain. This helps to differentiate *Gardnerella f*rom *Bacteroides* morphotypes. The Nugent score resolved this issue by combining these into a single Gram-negative or Gram-variable rod morphotype. One study has compared Gram with Kopeloff stain in the diagnosis of bacterial vaginosis in pregnancy. Gram staining gave significantly higher (more abnormal) Nugent scores than Kopeloff staining (29% cases more). Inter-rater agreement of the Nugent score for Kopeloff staining was significantly better than the Nugent score for Gram staining (agreement=74% versus 63%) **(36).**

Survey results:



Vaginal culture has no role in the diagnosis of bacterial vaginosis. Although cultures for *Gardnerella vaginalis* are positive in almost all women with symptomatic infection, the organism is detected in 6% to 50% of healthy asymptomatic women; thus, its presence alone, no matter how identified, is not diagnostic.

Other tests include:

1) DNA hybridization probe (Affirm VPIII) to detect *Gardnerella vaginalis* at high concentration in less than one hour. This can be helpful in the diagnosis when microscopic examination is not available. Good sensitivity and specificity have been reported when this test used in combination with other criteria such as vaginal pH (>4.5). There is also a possibility of overdiagnosis **(37).**
2) Chromogenic diagnostic test BVBlue (POCT), which detect the presence of elevated sialidase enzyme activity in vaginal fluid samples. This enzyme is produced by bacterial pathogens associated with bacterial vaginosis including *Gardnerella*, *Bacteroides*, *Prevotella*, and *Mobiluncus*. The reported sensitivity is 88%-94% and specificity 91%-98% in comparison with Amsel and Nugent criteria respectively **(38-35).**
3) Molecular test that assays the vaginal microbiome for the diagnosis of bacterial vaginosis, vaginal candidiasis, and Trichomonas (BD MAX Vaginal Panel has been approved by the US FDA for use) **(39-40).**
4) Under investigation: quantitative polymerase chain reaction (PCR) based on molecular quantification of bacterial vaginosis associated bacteria. These tests have good sensitivity and specificity but they are expensive and of questionable advantage **(41).**
5) Under investigation: a urine test that uses fluorescence in situ hybridization (FISH) to identify the bacterial vaginosis biofilm on desquamated vaginal epithelial cells in urine sediment **(42).**

According to IDSA 2018, Gram stain and recently available microbiome-based assays (vaginal panel) are more specific than culture and probe testing for *Gardnerella vaginalis.*

4.2. B.Other genital samples (urethra/cervix/genital ulcer):

According to IDSA 2018, Gram stain is unnecessary for the diagnosis of cervicitis. The presence of leucocytes in a cervical swab has a limited value since a specific diagnosis requires identification of an organism (chlamydia or Gonorrhea) **(43).**

According to IDSA 2018, Gram stain has a role in the diagnosis of chancroid, granuloma inguinale and (Gonorrhea in males).

Gonorrhea urethritis can be diagnosed by microscopic examination of the urethral swab. A swab should be inserted gently at least 2 cm into the urethra and rotated 360 degrees. The presence of ≥2 WBC (American guidelines) >5wbc (European guidelines) or intracellular Gram negative diplococci on microscopic examination of urethral discharge confirms gonococcal urethritis. The sensitivity is about 38% (cut off 2 WBCs) and the specificity 79% **(44)**.

Chancroid is mostly diagnosed clinically and after exclusion of the other causes of genital ulcers such as *Treponema pallidum* or *Herpes simplex*. Gram stain of the exudate from chancroid ulcer may show Gram negative rods. Gram stain sensitivity for this purpose is low (5% to 63%) and specificity (51% to 99%) **(45-46-125).**

Haemophilus ducreyi can grow on a special culture media after one to 2 days incubation or sometimes after 10 days incubation. These special culture media are not widely available. The reported sensitivity of these special culture media are 60% to 80%. Sensitivity of PCR test is (95%) **(47-48)**.

Conclusion: The presence of 3/4 of the Amsel clinical criteria, provide sufficient evidence for a clinical diagnosis of bacterial vaginosis.
Gram stain is the gold standard for the diagnosis of bacterial vaginosis. Cultures are not recommended. Other diagnostic methods are expensive and not widely used.
Very low diagnostic performance of Gram stain on other types of genital samples (male urethra and genital ulcers). Gram stain examination for cervical swabs is not necessary.

4.3. Wounds:

The majority of open and chronic wounds are polymicrobial. Superficial wounds and surgical incisions are usually monomicrobial. The organisms that are most frequently isolated from surgical site infections are *Staphylococcus aureus*, *coagulase-negative staphylococci, Enterococcus spp., and Escherichia coli* **(49).** Composition of the microbiota of chronic wounds has been analyzed in 2963 patient by 16s rDNA pyrosequencing by Wolcott AD and his group in 2016 **(50).**

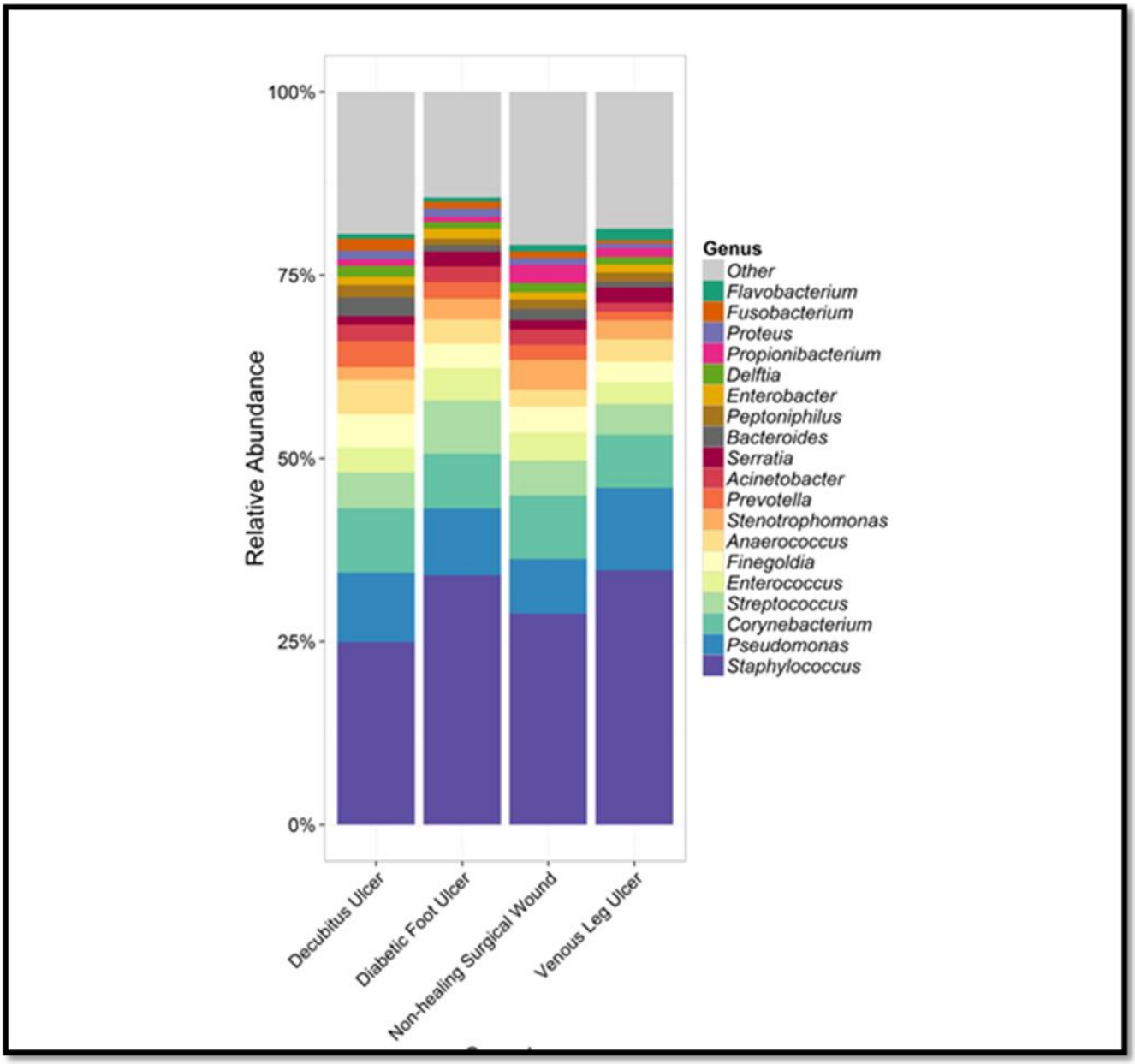


Figure 4: The relative abundance of the top 20 bacterial species by wound type. This figure shows for the top 20 species the percentage of amplicons assigned to a species vs. the total number of amplicons identified for each wound type (Wolcott RD et al.)[50]

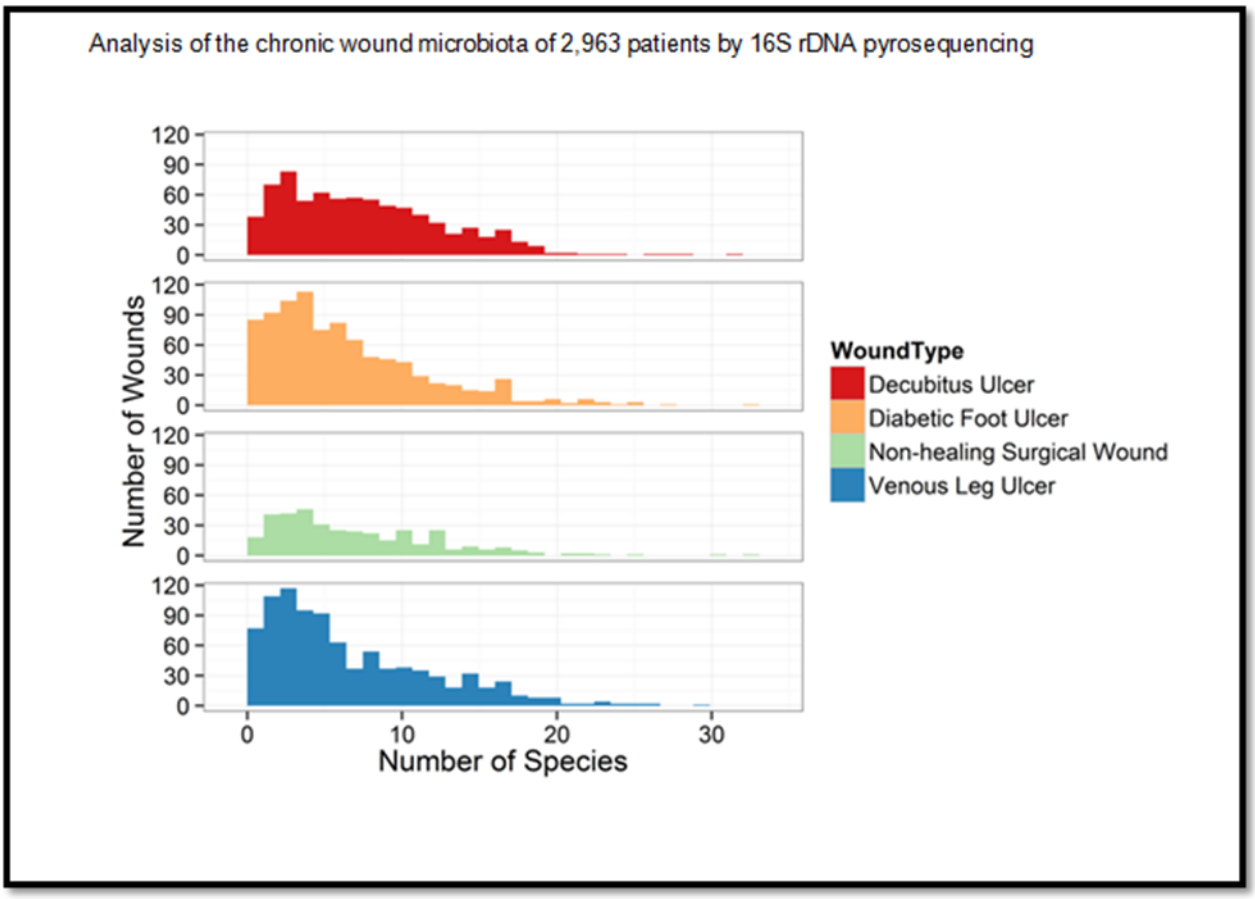


Figure 5: Number of reported species per wound for each wound type. Each wound type shows a Bell-shaped distribution for the number of microbes identified with the peak being from two to five species. Statistically, these graphs correlate quite closely (Wolcott RD et al.)[50].

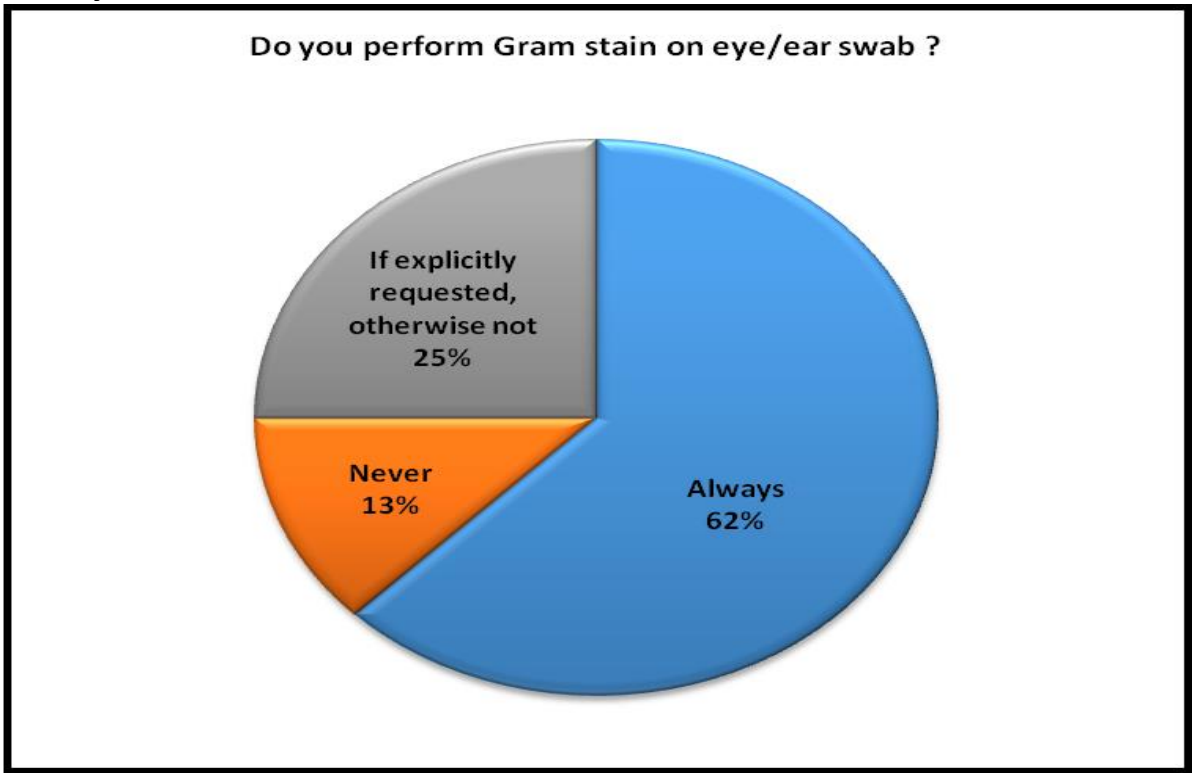
Dogs, Cats, also human bite wounds are mostly polymicrobial, aerobic (*Staphylococcus spp., Streptococcus spp*., and *Corynebacterium spp*.) and anaerobic(*Pasteurella spp*., *Bacteroides fragilis*, *Prevotella, Porphyromonas, Peptostreptococcus,* and *Fusobacterium spp.,* as well as *Veillonella pawula*) **(51).**

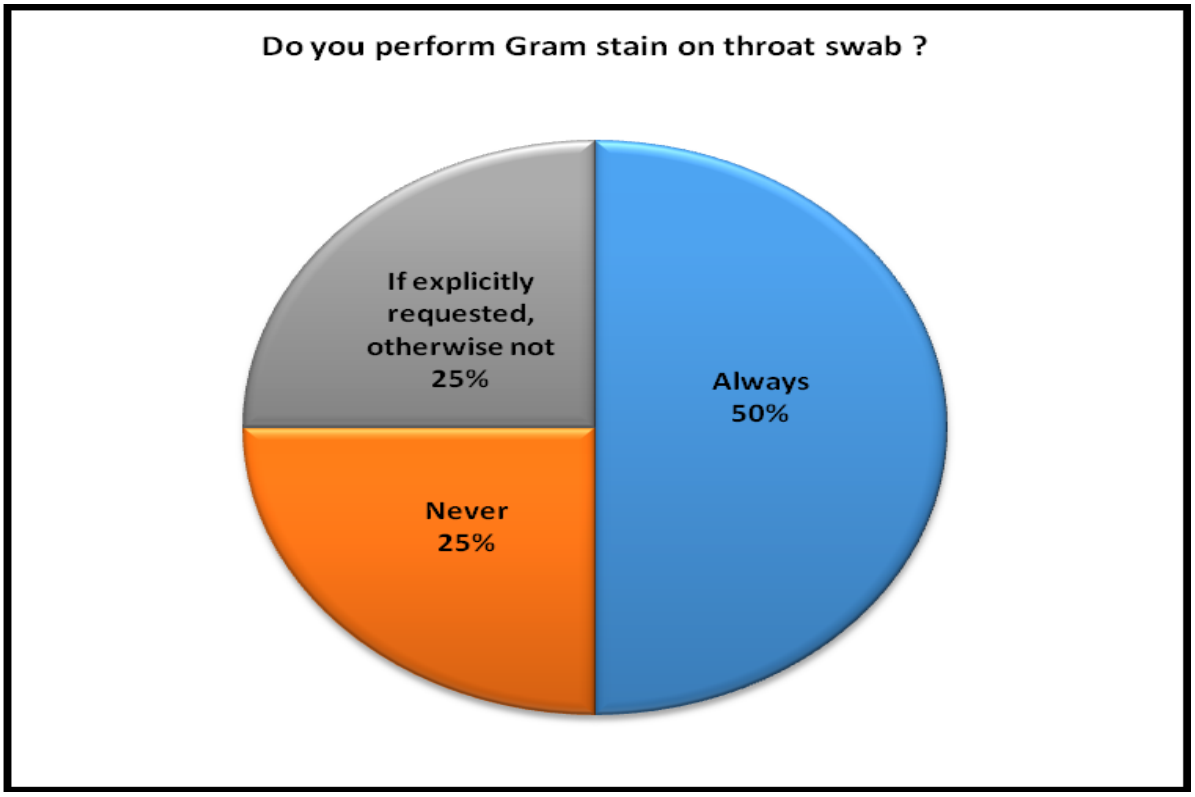
Ana Kaftandzieva and her group in Macedonia had compared wound culture results with Gram stained slides results (1970 specimens). Gram stain results have yielded a sensitivity of 38%, specificity of 90%, and positive predictive value of 83% and negative predictive value of 54% when used to predict positive culture results for bacterial wound infection **(52).**

In a prospective study from Canada, Kappa statistic for correlation wound Gram stain with culture was 0.32 in 375 burn wound specimens. The conclusion of this study was that Gram stain is not suitable for the microbiological analysis of burn wound surfaces **(53).**

4.4. Ear, mouth and nose swabs for upper respiratory tract infection:

Survey results:





Infections in the upper respiratory tract usually involve the ears, the mucus membranes lining the nose and throat above the epiglottis, and the sinuses. Most infections involving the nose and throat are caused by viruses. According to IDSA 2018, Gram stain is recommended by the laboratory diagnosis of the following infections:

- *Vincent angina (acute necrotizing ulcerative gingivitis/trench mouth):*
  Culture is not recommended for this indication, if attempted then the sample should be in anaerobic transport vial for the isolation of *Borrelia vincenii* and *fusiform* rods (anaerobes). In practice, Vincent angina does not occur frequently. It is usually a clinical diagnosis (systemic symptoms, pain, and ulcerated necrotic gingiva). It should be treated with systemic antimicrobials, such as metronidazole, amoxicillin-clavulanate, or clindamycin.

  The presence of Gram negative fusiform on Gram stain may support the diagnosis, but it does not have an additive therapeutic value.
  By reviewing the literature, very few case reports have been found **(**54-55). Further, no data are available about the diagnostic performance/clinical impact of Gram stain in this indication.

- *Peritonsillar cellulitis or abscess*: (Gram stain for biopsy or irrigation and aspiration of lesion; swab is not recommended).
  The diagnosis of peritonsillar abcess can be made clinically without laboratory or imaging studies. Ultrasound imaging is needed to distinguish peritonsillar abscess / cellulitis from retropharyngeal abscess or epiglottitis. Empiric therapy should include coverage for *Group a streptococcus, Staphylococcus aureus*, and respiratory anaerobes. Therapy should be continued for 14 days. No date are available about the diagnostic performance/clinical impact of Gram stain in this indication.

- *Epiglottitis and supraglottitis:* It is a clinical diagnosis, it does not require a specimen. A swab of epiglottis may be taken (only if necessary). In general, laboratory evaluation here should include:

  -Complete blood count with differential count
  -Blood culture
  -Epiglottal culture (the airway should be secured first).

  Treatment: maintenance airway and empiric antibiotic therapy.
  It should be a combination therapy with a third-generation cephalosporin (eg, ceftriaxone or cefotaxime) and an anti-staphylococcal agent (vancomycin or floxacillin according to the local prevalence of MRSA **(56).**

  No date are available about the diagnostic performance/clinical impact of Gram stain in this indication.

- *Otitis externa, otitis media, mastoiditis*:
  Otitis externa (Gram stain for scraping or fluid from external canal)
  Otitis externa rarely requires systemic antimicrobial therapy. Topical antibiotic therapy is usually enough. The choice of topical therapy

depends on the severity of external otitis. For patients with deeper tissue infection (extension beyond the external auditory canal), systemic in addition to topical antibiotics is recommended. Antibiotic coverage should include the most common pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.
Otomycosis can occur in approximately 9% of ear canal infections **(57).** *Aspergillus niger* and *Candida* are the most common organisms. It can usually be diagnosed by patient's history, otoscopic examination, and imaging studies. It occurs often in the setting of persistent otorrhea. Direct microscopy with potassium hydroxide (KOH), culture (mycology / bacterial), and histopathology are strongly recommended. Fungal cells can also be observed on Gram stain. *Aspergillus niger* and *Candida* are the most common organisms **(58-59).**
No date are available about the diagnostic performance/clinical impact of Gram stain in this indication.

Otitis media/chronic supportive otitis media: Gram stain is indicated for tympanocentesis fluid or mini-tipped swab of fluid draining from the middle ear cavity in patients with myringotomy tubes or otorrhea (IDSA 2018, Leber et al., 2016).
Most cases of otitis media can be diagnosed clinically and treated without Gram stain or culture support.
In a study from 1978, Gram-stained smears were obtained from 108 ears, in 54 ears (50%), the smears showed bacterial species not found in the culture and in 47 ears (44%), the culture revealed bacteria not seen in the smears. Further, all cultured diplococci (4 Veillonellae and 8 Neisseriae) failed to stain **(60).**
Bacteria were seen 17% of the Gram stained smears of sterile effusion from patients with persistent otitis media **(61).**
In a study from Istanbul (Oğuz F. et al.), 92 middle ear effusion samples diagnosed with AOM were analyzed. Two samples showed bacteria on Gram-stained smear of sterile effusion. Sensitivity of 96% and specificity of 100% have been reported **(62).**
In a study from Israel, 145 samples were obtained by tympanocentesis. WBC counts were higher in the middle ear fluid of patients with culture-positive AOM than in those with culture-negative AOM and in those with AOM caused by *S. pneumoniae* **(63)**
No date are available about the clinical impact of Gram stain in this indication.

Mastoiditis: Gram stain is indicated for middle ear fluid obtained by tympanocentesis or biopsy of mastoid tissue; swabs are not recommended.

Antibiotics treatment of mastoiditis presenting as a complication of chronic otitis should include coverage for *Staphylococcus aureus*, *Pseudomonas*, and enteric Gram-negative rods, as well as *Streptococcus pneumoniae* and *Haemophilus influenza*. If patients do not respond to conservative therapy with IV antibiotics, surgical intervention is warranted.
No date are available about the diagnostic performance/clinical impact of Gram stain in this indication.

- ***Sinusitis:*** Swabs are not recommended for collecting sinus specimens since an aspirate is much more productive of the true etiologic agent(s). The endoscopically obtained swabs (specimen of choice) can recover bacterial pathogens but rarely detect the causative fungi.
  No date are available about the clinical impact of Gram stain in this indication.

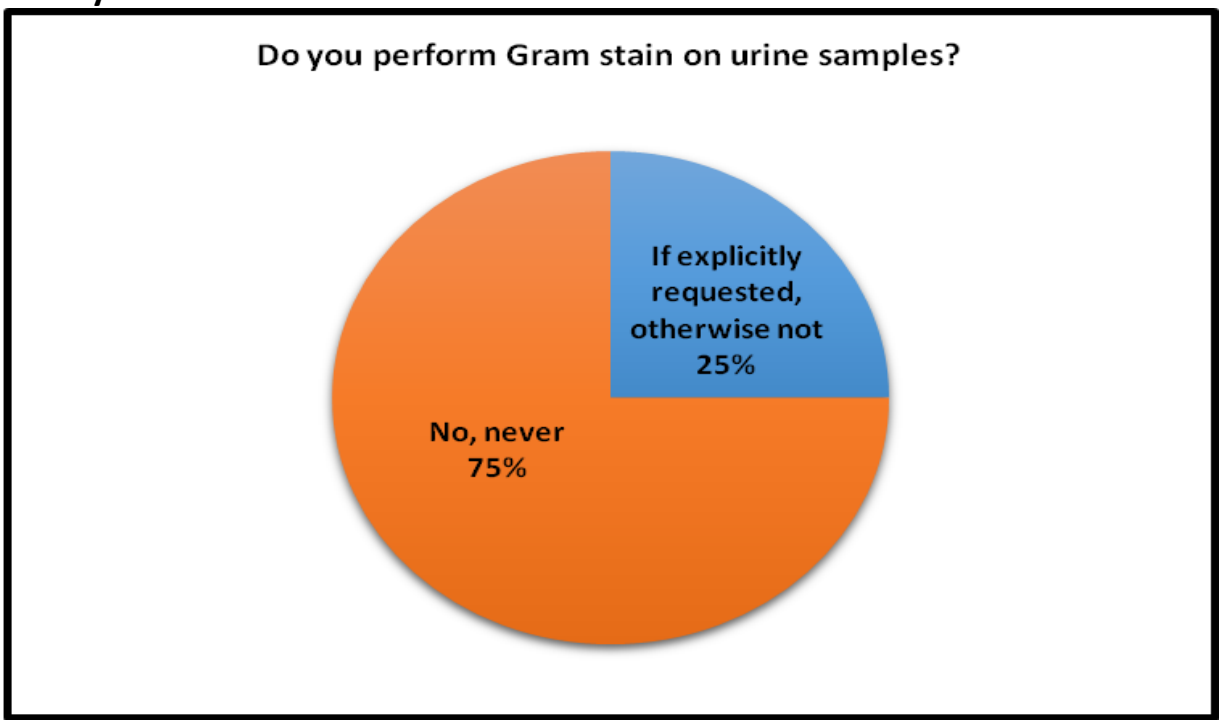
4.5. Eye swabs in patients with ocular infection:

The most commonly collected eye specimens are from the conjunctiva. According to IDSA 2018 and Leber et al. 2016, Gram stain is useful in the diagnosis of conjunctivitis. It is also useful in the diagnosis of keratitis and endophthalmitis (inner eye specimens).
2 swabs per eye are recommended; a paired specimen from the uninfected eye can be used as a "control" to assist in culture or Gram stain interpretation.
The intention here is to check for the presence of polymorphonuclear cells and Gram negative diplococci. High polymorphonuclear count suggest for bacterial infection and mononuclear cells suggest for viral conjunctivitis.
Neisseria gonorrhoeae conjunctivitis is uncommon but it can cause serious complications and hence topical antibiotics alone are not sufficient.
The rest what you see on Gram stained smear can be indigenous conjunctival microbiota or skin microbiota.
According to literature, the detection of Gram-negative diplococci (*Neisseria gonorrhoeae)* in Gram stain of conjunctival secretion smear is extremely useful for good therapeutic outcome and also to start prophylaxis for close contacts **(64).**
In a review of 84 cases of Primary meningococcal conjunctivitis (Barquet N et al.), Gram stain of conjunctival exudate disclosed Gram-negative diplococci in all cases. Culture of the conjunctival exudate yielded *N. gonorrhoea* in all cases **(65).** In general, NAAT is preferred for the diagnosis of Neisseria/Chlamydia infections because of their increased sensitivity and shorter turnaround time.
For keratitis, a retrospective analysis of comparative data from India (1092 patients with keratitis) has shown the limited value of Gram stain in therapeutic decisions for bacterial keratitis. Gram stain sensitivity was

36% in early and 40.9% in advanced keratitis cases; however, the specificity was higher in both groups (84.9% and 87.1%, respectively) **(66).** In general, recommendations for the laboratory diagnosis of ocular infections are often based on studies where only small numbers of clinical specimens were examined so the evidence base for many recommendations is limited. Frequent pretreatment with topical antibacterial agents complicates laboratory diagnosis of both bacterial conjunctivitis and keratitis (IDSA, 2018).
Appropriate choices for treatment of bacterial conjunctivitis include erythromycin ophthalmic ointment or trimethoprim-polymyxin drops. Fluoroquinolones are the preferred agent in contact lens users due to the high incidence of *pseudomonas* infection.

4.6. Urine samples:

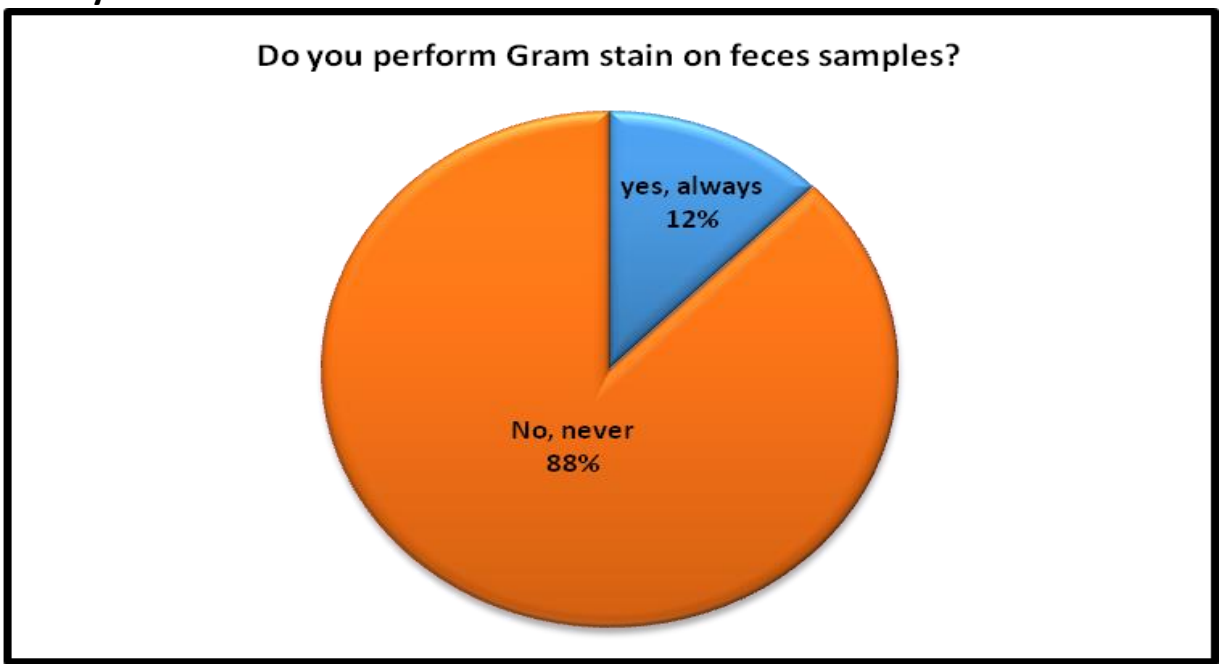
Survey results:



According to IDSA 2018, the Gram stain is not the appropriate method to detect polymorphonuclear cells in urine, but it can be ordered as an option for detection of high numbers of Gram-negative rods when a patient is suspected of suffering from urosepsis. The Gram stain urine test will be positive only if the concentration of bacteria in the urine is $\geqslant 10^5$ cfu/mL; so infections with lower bacterial concentrations may not be detected. Gram stain is too insensitive to be used to identify infected patients, particularly those patients with small numbers of pathogens. This was the conclusion of Murray PR et al. after screening of 500 randomly selected fresh urine specimens in 1987 **(67).**

4.7. Faces samples for the infection of gastrointestinal tract:

Survey results:



Laboratory tests are not routinely warranted for most patients with acute diarrhea. Blood cultures should be obtained in patients with high fever or who appear systemically ill. The specimen of choice to diagnose diarrheal illness is the diarrheal stool, not a formed stool or a swab. An exception is made for pediatrics where a swab is acceptable.
Most laboratories detect routinely: *Salmonella, Shigella, Campylobacter and Yersinia* by using culture or non-culture technique (NAAT).
Culture and multiplex NAATs for stool pathogens are very sensitive. When a stool sample is positive for a stool pathogen, the sample should be cultured to recover the isolate for susceptibility testing.
The intention from direct Gram smear for stool samples is to detect WBC and bacteria. According to literature, the sensitivity of WBC in stool is 50% to 60% for gastroenteritis and 14% for *clostridium difficile* colitis (68-69-70). No study has compared the relative number of leukocytes found with each type of infection.
By reviewing the IDSA Guidelines, Gram stain for stool samples has not been mentioned by laboratory diagnosis for gastrointestinal infection. According to Leber et al. 2016, Gram stain on stool samples is rarely performed but it can be helpful in selected cases such as *Campylobacter.*

4.8. Body fluids:

4.8. A. CSF in patient with central nervous system infection:

Cerebrospinal fluid evaluation is an important aspect in the diagnosis of CNS infections. It should be collected prior to initiating antimicrobial therapy. The diagnostic procedure for meningitis involves the following:

- Gram stain
- Aerobic bacterial culture (+/- anaerobic)
- Fungal culture
- +/- India Ink stain
- Molecular testing
- Bacterial antigen testing on CSF
- Blood cultures

A positive Gram stain may be helpful in the identification of the causative microorganism before the result of the culture is available such as in *pneumococcal* or *meningococcal* meningitis. Negative Gram stain does not exclude the diagnosis of meningitis because of the documented low sensitivity of Gram stain (60%–80% without antibiotic treatment and much lower 40%–60% in patients who have received antibiotic treatment) **(1)**

The sensitivity of Gram stain in diagnosing CNS infection varies depending on the organism and population being tested (neonates, children, adults and elderly) **(71).**

Pathogen	Sensitivity (%)[a]			
	Blood culture	CSF Gram stain	Latex agglutination test[b]	PCR
Haemophilus influenzae	25-90	25-65	78-100	72-92
Streptococcus pneumoniae	60-90	69-93	59-100	61-100
Neisseria meningitidis	40-60	30-89	22-93	88-94
Listeria monocytogenes	10-75	10-35	NA	NA
Streptococcus agalactiae	80-85	80-90	NA	NA
Streptococcus	60-65	66-73	NA	NA

Table 8: sensitivity of Gram stain in diagnosing CNS infection varies depending on the organism (Brouwer MC et al.)[72]

A German prospective study in 2004 showed that Gram stain had a sensitivity of 80% and specificity of 97%. They had evaluated 652 cases of community-acquired acute bacterial meningitis (51% due to *Streptococcus pneumoniae*, 37% *Neisseria meningitidis*, 4% *listeria*, 8% other **(72).** In 524 of 652 cases was the Gram stain positive. There was no difference in Gram stain results between those who had previously received antimicrobial therapy and those who had not **(73).** It was not mentioned how long (> or

<24 h) or how many antibiotic doses got these patients before CSF puncture.
F. Tissot et al. had assessed the impact of overnight positive and negative CSF Gram stain on the empirical antibiotic therapy in patients with community-acquired meningitis in a retrospective analysis **(5).**

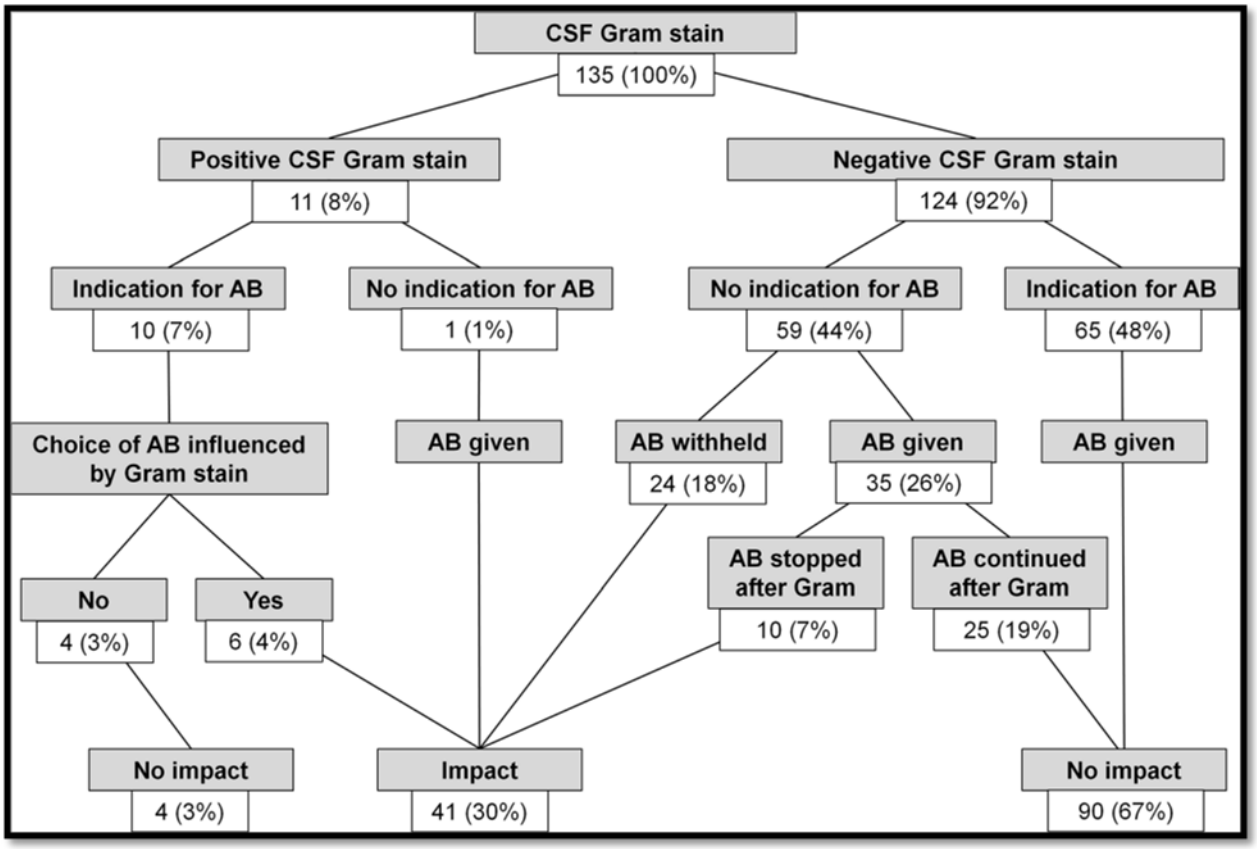


Figure 6: Effect of 24/24 CSF Gram stain (F. Tissot et al.)[5]

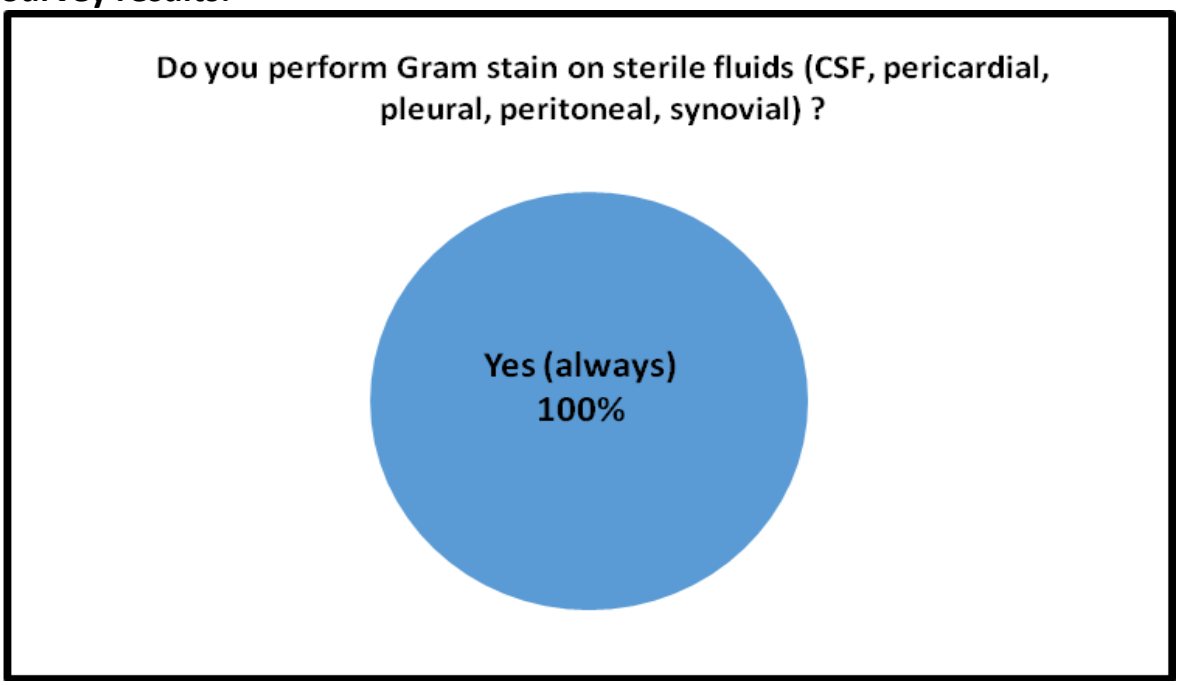
Apparently, a positive CSF Gram stain had a little impact (5%). A negative CSF Gram stain had more impact (25%) on the overnight antibiotic management of suspected CNS infections
The clinicians depend mostly on the severity of the clinical illness and results of CSF analysis (WBC, glucose, protein and LDH). The clinician will almost never depend on the result of CSF Gram stain to simplify therapy in patients suspected to have bacterial meningitis. According to the figure above, the broad-spectrum antimicrobial therapy will be continued whether Gram stain result is positive or negative (until CSF culture results are available). Gram stain may help to stop unnecessary antibiotics.

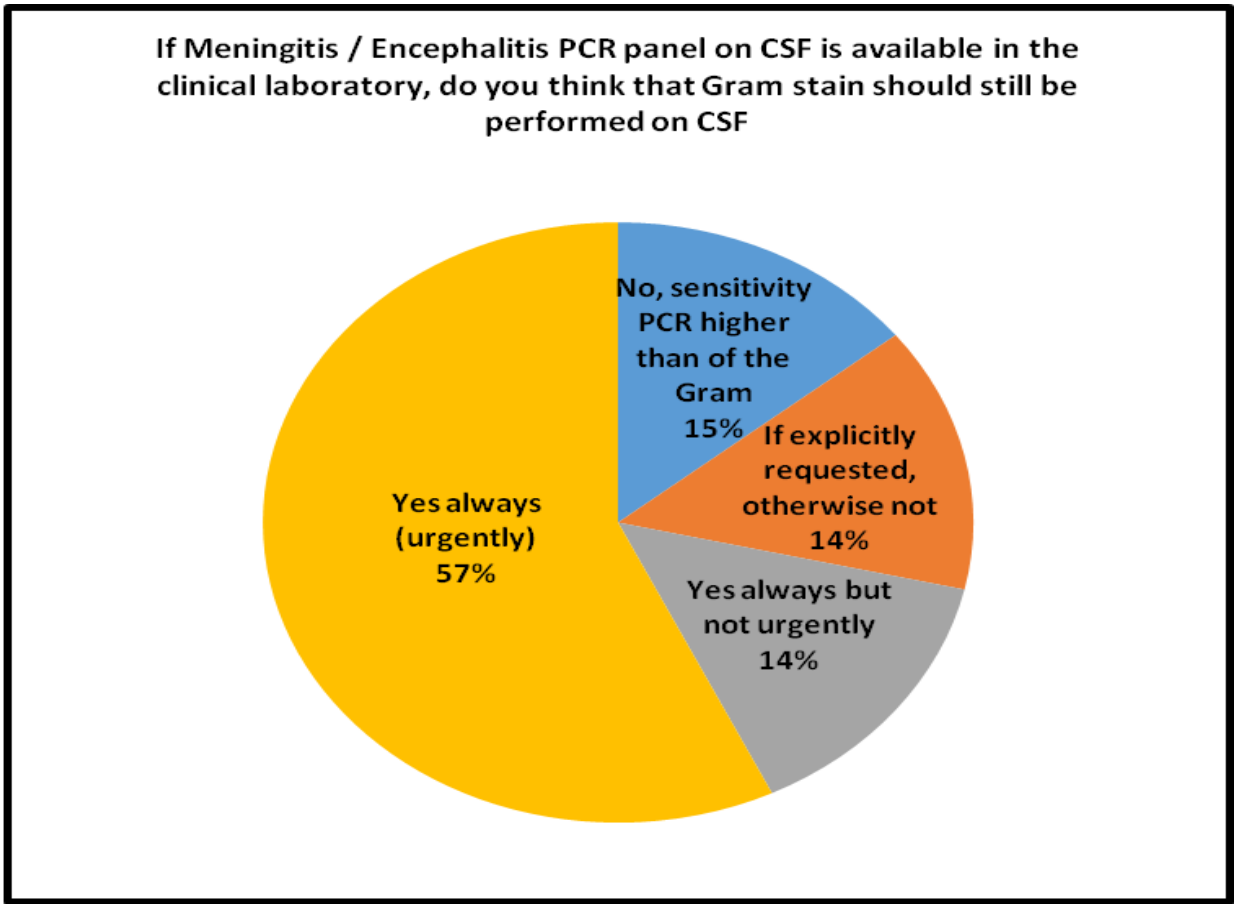
The use of latex agglutination test is not recommended for such indication. According to IDSA, 2018: It may have some value in patient with negative Gram stain and negative culture due to therapy after 48 hours incubation. It may be reserved for such cases only **(74-75).**
The multiplex FilmArray meningitis/encephalitis panel (BioFire) can detect 14 (viral-bacterial and fungal) pathogens causing meningitis and encephalitis. It is approved by the U.S. Food and Drug Administration

(FDA) in October 2015. These tests are highly sensitive and specific, and lead to increase pathogens that can be identified. These tests may be used as alternative for overnight Gram stain but not as alternative for culture because multiplex panels cannot detect all causes of CNS infections. False positive and false negative results can also occur **(76-77-78).**
The benefits of such molecular test (in comparison with Gram stain) are the following:

- It does not depend on bacterial load
- It is not affected by antibiotic exposure
- It does not depend on experience of the examiner in Gram stain interpretation.
- Superior to Gram stain in detection of co-infection of CSF

Survey results:





4.8. B Pleural fluid for empyema:

Different pathogenic mechanisms are involved in the creation of pleural effusion. It can be exudate (empyema, tuberculous pleurisy, others) or transudate (organ failure, malignancy, others). When thoracentesis is considered, pleural fluid will usually be analyzed for the following:

-Cell count and differential count.
-LDH and proteins (Light's criteria)
-PH, glucose.
-Gram and acid fast bacilli stain.
-Culture.

Exudate plural fluid may clear spontaneously with treatment of the pneumonia or may need surgical drainage (empyema).

According to the British and American thoracic society 2010, pleural fluid that is frankly purulent or that has a pH < 7.2 (in the appropriate clinical setting), or organisms on Gram stain or culture, is an indication for formal intercostal drainage.

A study from 1991 has evaluated the utility of these criteria in three-year experience of three Rochester, NY, hospitals on 133 patients undergoing thoracentesis for putative para-pneumonic effusions. The sensitivity of a positive Gram stain was 18%. The

patients that are included were only those patients whom believed that the effusion on the basis of a bacterial pneumonia **(79).**

Test Result	Surgery Eventual	Surgery None	Predictive Values	Surgery Eventual	Surgery None	Predictive Values
A. Positive Gram Stain p>0.05*				B. Glucose <40 mg/dl p>0.10*		
	(n = 17)	(n = 49)		(n = 20)	(n = 38)	
Positive	3	2	60%	5	5	50%
Negative	14	47	77%	15	33	69%
Sensitivity	18%			25%		
Specificity		96%			87%	
C. pH <7.00 p<0.005*				D. LDH>1,000 IU p<0.01*		
	(n = 16)	(n = 38)		(n = 17)	(n = 49)	
Positive	7	3	70%	9	9	50%
Negative	9	35	80%	8	40	83%
Sensitivity	44%			53%		
Specificity		92%			82%	

Table 9: Operating Characteristics of Pleural Fluid Criteria for Patients not Undergoing Immediate chest tube drainage of para-pneumonic effusions (Poe RH et al.)[80]

In a retrospective review on pleural fluid samples from 525 patients undergoing diagnostic thoracenteses for pleural effusions of unknown cause. From all Gram stained smears performed, 2.5% was positive. This showed the low yield of Gram stained smears especially in the outpatient setting and in patients with free-flowing effusions (not infectious) **(80).**

4.8. C Pericardial fluid in patients with pericarditis:

Pericarditis may be idiopathic or after cardiac surgery. Other causes include: tuberculosis, connective tissue disease, trauma, malignancy and others.

The following tests are usually performed on the pericardial fluid:

-Cytology.

-Gram stain and bacterial culture

-Polymerase chain reaction may be performed in cases with special context.
In most of the cases of pericarditis or myocarditis, the causative agent remains unknown. The incidence of bacterial pericarditis accounts for less than 1% of pericarditis **(81).** It is caused mostly by Gram-positive cocci (*Staphylococcus aureus, Streptococcus pneumoniae*). Other causes also have been discussed in the literature.
With the exception for some case reports, no data are available about Gram stain diagnostic performance and its clinical impact in pericarditis patients.

4.8. D. Peritoneal fluid in patients with ascites:
Paracentesis is usually done for evaluating patients with ascites for peritonitis. The diagnosis of spontaneous bacterial peritonitis SBP depends on the increased peritoneal absolute neutrophil count greater than 250 cells/mm.
A retrospective review of all peritoneal fluid analyses in a 3-year period in a 3 urban hospitals have shown the low sensitivity of Gram stain 10%, specificity of 97.5%, positive predictive value of 48% and negative predictive value of 81.3% in the detection of spontaneous bacterial peritonitis **(82).**
The very low sensitivity of Gram stain for spontaneous bacterial peritonitis has also been confirmed (9%) in another small study of 31 samples, all from patients with SBP **(83).**
Third-generation cephalosporin antibiotics is usually the empiric therapy for SBP. SBP due to *Listeria monocytogenes* has also been discussed in some case reports **(84).**
The presence of Gram-positive rods on Gram stain can cause confusion. It is mostly a contaminant such as *diphtheroids*, but it could be also *Listeria monocytogenes.* However, a positive Gram stain result would be beneficial if it is correctly identified.

Conclusion: in spite of the low yields of Gram stain on pleural fluid, pericardial and ascitic fluid, Gram stain is still recommended according to the guidelines and it is almost always performed on all body fluids.

4.8. E. Synovial fluid for septic arthritis and prosthetic joint infection:
Gram stain is used as a screening tool to detect septic arthritis. It is performed on every joint aspiration. For the diagnosis of septic arthritis, synovial fluid should be submitted for the following:

- Gram stain
- Culture (aerobic +anaerobic)

- Possibly fungal and mycobacterial culture
- WBC telling and differentiation in synovial fluid
- Crystal analysis

According to IDSA 2018, leucocytes count of >50,000 is suggestive for septic arthritis, leucocytes count lower than 50000 do not exclude the diagnosis.
According to Clinical Microbiology Procedures Handbook 2016, Gram stain for body fluids including synovial, pericardial, pleural should be done and interpret immediately. Selective media should be inoculated if mixed microorganisms are seen on Gram. Culture results should be correlated with results of direct Gram stain. If only body fluid in blood cultures are received, then Gram stain order is cancelled. Culture results should be correlated with the results of direct Gram stain.
We have searched in literature about the value of Gram stain on synovial fluids and possible alternative. Joshua T. Bram et al 2018, had the clinical relevance of Gram stain evaluated in 302 paediatric septic arthritis in a case control study. Gram stain sensitivity was 40% and much lower for Gram negative organisms and specificity was 97% for the diagnosis of septic arthritis **(85).**
The low sensitivity of Gram stain of native joint septic arthritis has also been shown in studies on adult population. The sensitivity was between 30% to 70%, and specificity up to 100% **(86-87-88-89-90)**.

	Gram's stain sensitivity	Culture sensitivity
Non-gonococcal arthritis	50–70%	75–95%
Gonococcal arthritis	10–25%	10–50%
Tuberculous arthritis	20%	79%

Table 10: Sensitivities of Synovial Fluid Gram's Stain and Culture in Infectious Arthritis (Brannan SR et al.)[91]

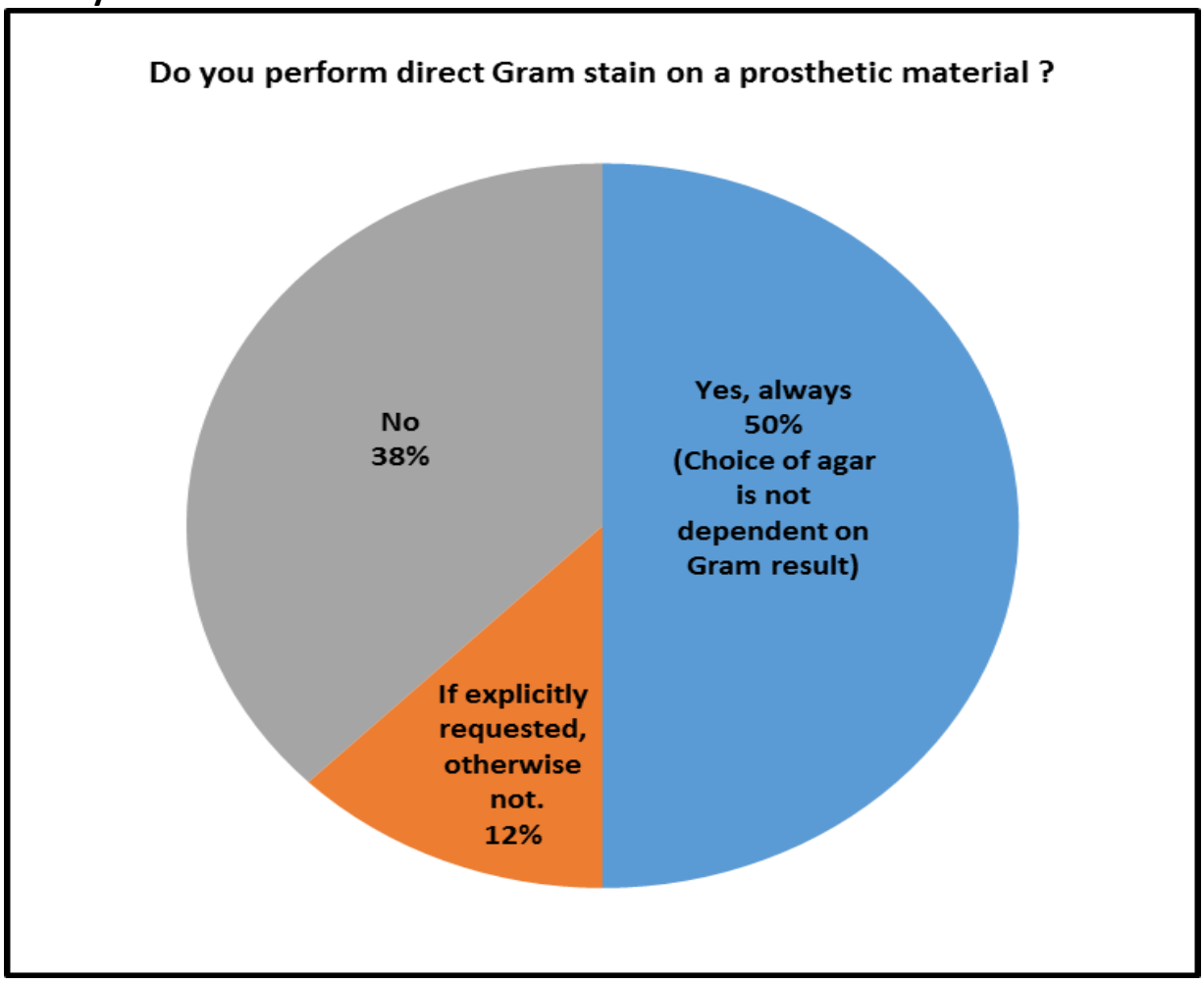
Few studies had evaluated patients with coexistence septic and gouty arthritis **(92-93).** These 2 studies emphasize the importance of thorough evaluation of the aspirated synovial fluid.
Other studies had focused on patient with prosthetic joint infections PJI. They showed the poor sensitivity 7%-27% and negative predictive value 57%-89% **(94-95).**
In a meta analysis, the pooled sensitivity and specificity for the detection of PJI using Gram stain were 19% and 100% respectively **(96).**
Intraoperative Gram staining was negative in 169 cases revision arthroplasty, sensitivity 0% for detecting infection **(97).**

For the diagnosis of septic arthritis, synovial fluid should be submitted for Gram stain, and culture (aerobic and anaerobic). If synovial fluid studies are negative, biopsy of the synovium may be required. Gram stains are not recommended for the diagnosis of PJI. Two or more intraoperative cultures or a combination of preoperative aspiration and intraoperative cultures that yield the same organism is considered definitive evidence of PJI. A single positive tissue or synovial fluid culture, especially for organisms that may be contaminants (eg, coagulase-negative *staphylococci*, *Corynebacterium acnes*), should not be considered as evidence of definite PJI (IDSA, 2018).

4.9. Divers:

4.9. A. Prosthetic joint material:

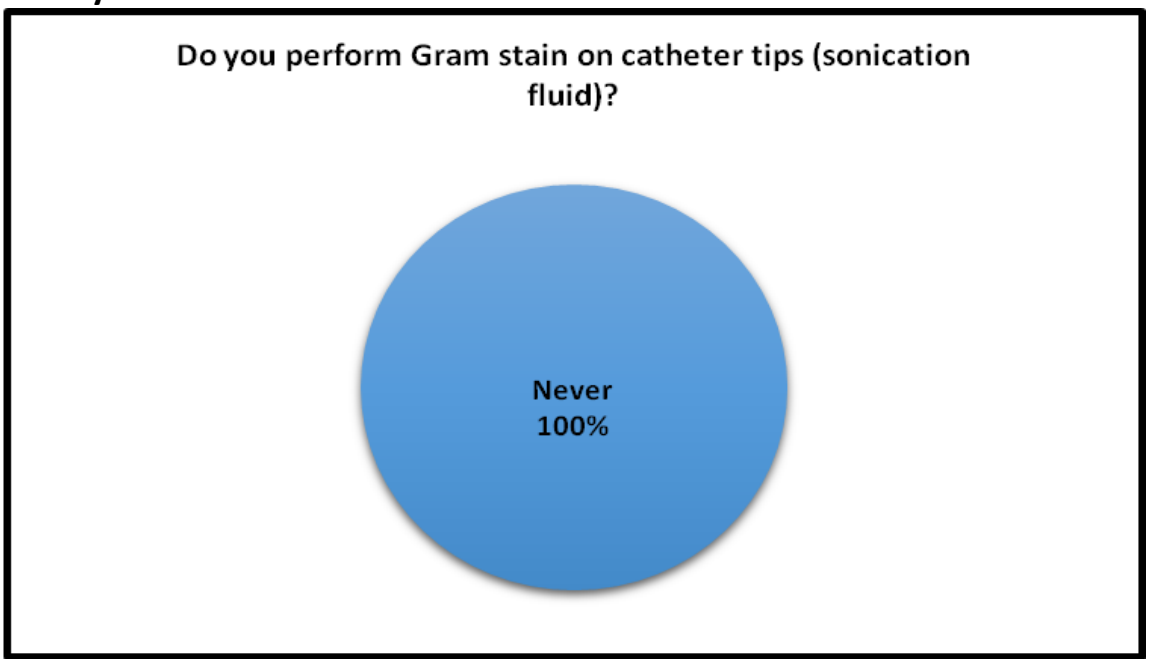
Survey results:



No data are available about the diagnostic performance of prosthesis Gram stain in patients with prosthetic joint infection.

4.9. B. <u>Catheter tip:</u>

Survey results:



Gram stain for catheter tips is not part of the standard protocol (Leber et al.,2016/IDSA 2018). The first report of using direct Gram stain for diagnosing catheter related infection was in 1985 from Cooper **(98).**

De catheter was stained by suspending it in Petri dishes containing the appropriate stains and then examined under microscope. The reported sensitivity was 100%, specificity 96%, PPV 83%, NPV 100%. In order to avoid overuse of reagents, impression smears was used by Collignon and his group. This method was able to detect 3 patients with catheter-related bacteremia (including one patient that was negative by semi-quantitative culture), with negative Gram stains in all five patients where another source was identified. **(99).**

In a prospective study from Guembe M et al. in 2012, the validity values of impression Gram smears for the prediction of positive long term catheter cultures have been studied. The results were as follows: a sensitivity of 35.9% to 54.5%, a specificity of 100% to 94.2%, a PPV 42%-100% and a NPV of 81.5% to 96.3% **(100).** In a recenter study from Guemba et al. 2015, the Gram stain sensitivity was lower (29.5%) in the prediction of catheter colonization and catheter sepsis. In spite of the small sample size (14 cases with sepsis), it has shown a high specificity and a high NPV (96%) for ruling out catheter related blood stream infection.

According to Guembe M et al., if the Gram staining is performed exhaustively (impression smears) and the slides are examined by a highly trained technician for relatively 3-5 minutes per sample (20

oil-immersion fields should be screened), this would reveal good results. His conclusion was: such method is impossible to be implemented in a busy laboratory **(101).**
The utility of a cytocentrifuge-prepared Gram stain of sonication broth as a rapid test for the accurate diagnosis of catheter-related infection was also evaluated by Kelly M et al. in 1996 (405 catheters in total). The conclusion of this prospective study was that Cytospin Gram stain on catheter sonication fluid does not correlate with the presence of catheter sepsis **(102).**

4.10. Biopsies:
Biopsies are usually sent in sterile containers. Gram stain can be done directly on primary samples (ground material) and on the broth culture of the biopsy (TSB/BHI/THIO).
According to Leber et al., 2016 and IDSA 2018, all tissue biopsies should be examined by Gram stain. This can include the following:

- Aortic aneurysm contents
- Brain
- Bone biopsies
- Lung biopsies

No evidence are available about the diagnostic performance/clinical impact of Gram stain on these biopsies (mentioned above).
For tissue ulcers: one study from Tanzania, over the Gram stain of ulcer biopsies from diabetic foots in limited laboratory services (not applicable) **(103).**

For other biopsies:

- Heart valves are submitted from patients with infective endocarditis undergoing valve replacement.

In a retrospective review for 480 patients. Valves were seldom culture positive after receipt of 50% of standard antimicrobial therapy, but microbiology Gram stain were positive for >60% of patients who were still receiving antibiotic treatment. The microbiology Gram stain was more likely to be positive than histopathology Gram stain (74% vs. 63%; *P* <.0001) **(104).**
In another small study (25 patients), 24% of patients had organisms seen on vegetation Gram stain but not cultured **(105).**

Variable	No. of episodes	Microbiological findings		Histopathological findings	
		No. of positive Gram stain results/ total no. of samples stained (%)	No. of positive culture results/ total no. of cultures (%)	No. of samples with organisms present/no. examined (%)	No. of samples with no organisms found but acute inflammation present[a]/no. examined (%)
Proportion of standard duration antibiotic treatment completed at time of operation					
≤25%	106	88/100 (88)	76/106 (72)	51/63 (81)	9/63 (14)
26%–50%	113	85/101 (84)	40/108 (37)	50/70 (71)	17/70 (24)
51%–75%	57	37/50 (74)	7/54 (13)	21/39 (54)	12/39 (31)
76%–100%	41	21/34 (62)	2/38 (5)	18/36 (50)	11/36 (31)
>100% but still receiving treatment	61	28/43 (65)	5/53 (9)	29/53 (55)	11/53 (21)
Patient stopped treatment					
≤1 month before operation	22	7/15 (47)	0/19 (0)	9/20 (45)	4/20 (20)
>1 month but <6 months before operation	33	4/18 (22)	1/22 (5)	6/25 (24)	9/25 (36)
Negative blood culture[b]	63	31/52 (60)	13/60 (22)	26/48 (54)	11/48 (23)
Incubating endocarditis present[c]	10	7/8 (88)	9/9 (100)	4/6 (67)	2/6 (33)

Table 11: Gram stain, culture, and histopathological findings for 506 episodes of infective endocarditis that required removal or resection of heart valves (Morris AJ et al.)[104].

Apparently, nonviable bacteria persist for weeks to months in sterilized vegetations, and also acute inflammation may persist for weeks to months after microbiological cure. This means that culture results should be the index of whether the surgery has been performed in an infected field or not, because it may take months for dead bacteria in a vegetation to be removed by phagocytosis and/or bacterial cell lysis **(104-106-107).**

- Observations over biopsies:

Some times when authors do a comment on the detection of organisms, it is unclear whether they are referring to the microbiology Gram stain or to the histopathology Gram stain, or both.
Most evidence in literature searched the value of Gram stain on biopsies without mentioning of it is direct or indirect Gram stain.

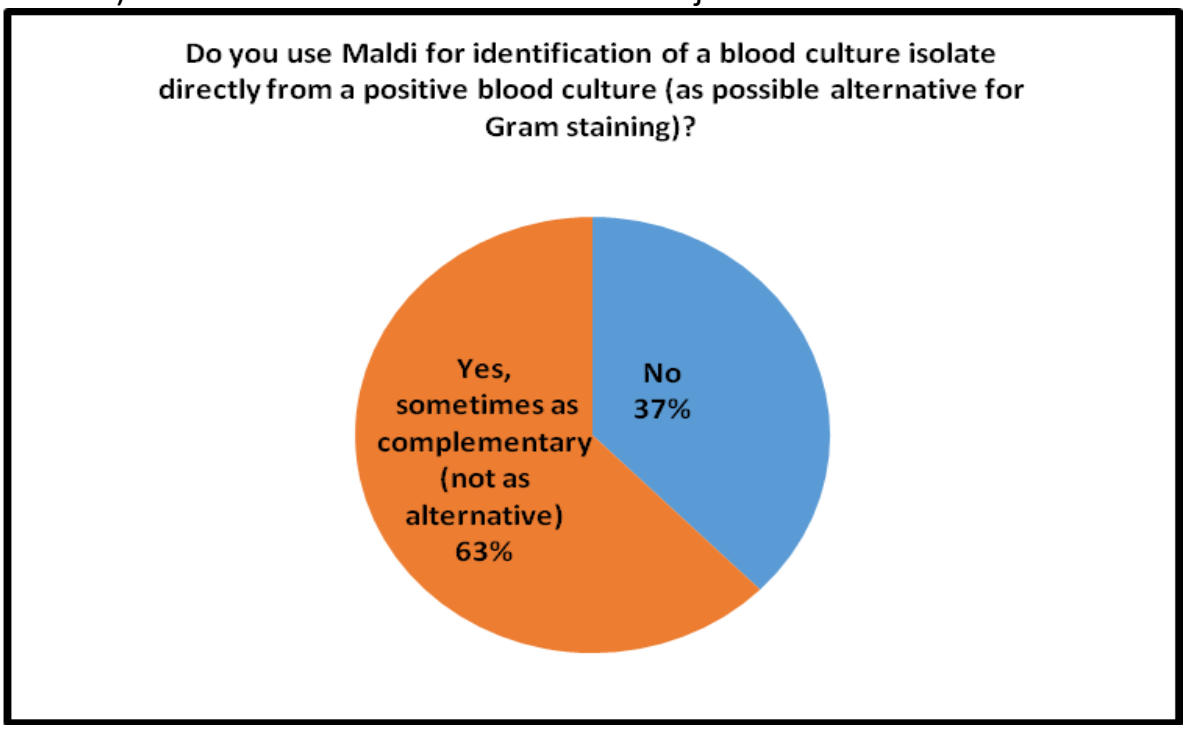
4.2. Indirect Gram stain on positive subcultures:

4.2. A. Blood cultures:

The diagnosis of bloodstream infections (BSIs) is one of the most critical functions of clinical microbiology laboratories. Blood cultures containing bacteria or yeasts are flagged as positive by an automated continuous-monitoring blood culture system. The flagged blood culture bottles are removed and the Gram staining is performed. The results of the Gram stain determine the type of solid media used to subculture the microorganisms, and from which identification and the antimicrobial susceptibility tests are performed.
Categorizing bacteria as Gram positive or Gram negative and to cocci or bacilli can guide the choice of the empirical antimicrobial therapy.
Identification of bacteria and fungi from agar culture (to the species level) by using Maldi-TOF or Vitek or other instrument is the most important information here. It helps to differentiate pathogens from contaminants

(important for Gram-positive organisms) and for tailoring antimicrobial therapy to the intrinsic resistance of certain pathogens (important for Gram-negative organisms). This often takes 24 hour or sometimes longer. Timo Hautala and his group have evaluated the results of Gram stain for 1901 cases of bloodstream infection. The conclusion was: the knowledge of Gram stain results and where the infection was occurred allow accurate choice of empirical antimicrobial therapy **(108).**
The Q-probes study of 65 institutions showed that Gram stain reporting for blood stream infection was usually correct **(23).**
We tried here to look for possible alternatives for Gram stain in diagnosing bacteremia. A recent study from India has evaluated the diagnostic performance of Gram stain and Acridine Orange stain on a total of 700 blood cultures. The sensitivity of Gram stain was 98% for single type culture and for mixed cultures 82%. The sensitivity of Acridine Orange stain is 100% for single and mixed cultures. Positive and Negative Predictive Value was 100% for each. The specificity of both the stains was 100%. Candida species and some Gram negative bacilli were missed by Gram stain and detected by Acridine orange stain **(109).** Acridine orange stain is a fluorochromatic dye which binds to nucleic acid of bacteria and other cells. By using this staining method, all bacteria and fungi look orange (are fluorescent) and the background looks green-yellow (non-fluorescent).
In literature, the usage of MAIDI-TOF for reliable identification of blood culture isolates directly from a positive blood culture has been extensively studied **(110-111-112-113).**
Eigner U et al and Stevenson et al were among the firsts who used MAIDI-TOF for direct identification of bacteria from positive blood culture in 2009-2010 **(114-115).** Many centers have developed in-house methods to optimize the bacterial recovery from the blood culture using the MAIDI-TOF **(116-117-118).**
Martiny D et al had compared an in house method with the commercial Sepsityper kit **(119).** Spanu T et al had evaluated the direct Maldi-TOF identification of candida species from the blood culture with good and reliable results **(120).**
The percentage of errors that found in the identification was almost similar in various studies, this depends on the bacteria being studied and methods that were used for the reference identification.
The usage of MAIDI-TOF for rapid identification will reduce the time needed for identification of bacteria in bloodstream infections. The results are mostly concordant to the genus level from the score of 1.7 for more than 95% of blood culture in comparison with conventional identification methods (Jorgensen, 2015).
There are still challanges ahead. These challanges are:

1) Interfering substances such as charcol when present or proteins from RBCs or WBCs. This usually depend on the ability of the lysis buffer to optimize the recovery.
2) Low organism numbers (contaminating bacteria, slowly growing bacteria)
3) Polymicrobial blood cultures
In most of the studies mentioned above, Gram stain and subcultures are assumed to be always needed for all positive blood cultures for the purpose of identification of polymicrobial blood culture and for performing susceptibility testing. This despite the implementation of MAIDI-TOF for direct identification bacteria.
4) The operating cost
5) Because of the long turnaround time for each direct identification on MAIDI-TOF, MAIDI-TOF was batched.
6) The identification score should be adjusted.



Next to MAIDI-TOF, there are also other alternative rapid methods for identification of bacteria without usage of Gram stain. These include molecular assays:

- Broad range nucleic acid amplification PCR which can detect a broad range of bacteria. This has a high financial cost and intensive labor requirements.
- Real time PCR which can detect specific pathogen in a short time
- Multiplex PCR which can detect relatively a broad range of bacteria

Additional methods such as immune chromatographic lateral flow assay, for example the BinaxNOW *Staphylococcus aureus* which detects *Staphylococcus aureus* -specific protein, in order to differentiate it from

other Gram-positive cocci with 97.6% sensitivity and 100% specificity (Jorgensen, 2015).

Conclusion: Up to this moment, there is no alternative to Gram stain to guide the choice of initial antibiotic therapy in patient with sepsis. The direct identification by using MALDI-TOF is promising and give mostly accurate results. It is considered as complementary (not as alternative) to Gram stain especially in mixed infections. The implementation of molecular methods in the workflow for such purpose is costly and limited to specific organisms. Polymicrobial infection may be missed even in broad range PCR. So you will often need an additional or confirmatory testing.

5. Clinical impact:

5.1. Clinical impact in patient with lower respiratory tract infection:

Streptococcus pneumonia is the most common pathogen in nearly all studies with community-acquired pneumonia in adults which required hospitalization. Other causative organisms include *Haemophilus influenzae, Staphylococcus aureus,* Gram-negative bacilli, *Moraxella catarrhalis, Strep. pyogenes* and others. According to American and British thoracic society 2009-2010 and European respiratory society 2011, the empirical treatment of hospitalized CAP should always cover *Streptococcus pneumoniae.* It should also cover the atypical causative agents by severe and very severe pneumonia. The treatment should also be directed against *Staphylococcus aureus* during epidemics of influenza. Usually antibiotic therapy will be started after taking samples and blood cultures. No clinician will narrow the empirical antibiotic therapy for a patient with established sever pneumonia on basis of Gram results **(25).**

5.2. Clinical impact on patients with bacterial vaginosis/ urethritis/genital ulcers:

Gram stain is the gold standard for the diagnosis of bacterial vaginosis. Cultures are not recommended. Amsel criteria alone may lead to underdiagnosis of BV. A treatment of bacterial vaginosis can be started based on the results of Gram stain (a symptomatic bacterial vaginosis). Treatment of a symptomatic bacterial vaginosis is recommended in females with gynecologic complications. It is reasonable to treat asymptomatic bacterial vaginosis prior to hysterectomy and before pregnancy termination to prevent post procedure infection. Reported reductions in postoperative infectious complications range from 10% to 75% **(121-122).**

American College of Obstetricians and Gynecologists (ACOG, 2017) and CDC recommend to not routinely screen and treat all pregnant women with asymptomatic bacterial vaginosis. There is insufficient evidence that screening and treatment of asymptomatic bacterial vaginosis in pregnant women will reduce the risk of preterm birth. There may be benefits to early screening and treatment of asymptomatic pregnant women who have a history of a previous preterm delivery, but there are insufficient data to recommend this as a routine practice
There is also insufficient evidence to make a conclusion regarding the screening for BV prior to intrauterine device insertion.
For cervicitis/ urethritis, NAAT on genital samples is wide available, reimbursed by RIZIV and very accurate in comparison with Gram stain. An additional advantage is that NAAT retains accuracy with patient-collected specimens (vaginal swab in women and urine in men).

Even if intracellular diplococci can be seen on Gram stain, NAAT is still indicated to confirm the presence of *Neisseria gonorrhoeae* and to exclude coinfection with *Chlamydia trachomatis.* There are reports of *Neisseria meningitidis* causing symptomatic urethritis and being initially mistaken for *Neisseria gonorrhoeae* on Gram stain **(123-124).**

For the diagnosis of Chancroid, most clinicians depend on clinical criteria to make the diagnosis. These criteria are:

- One or more painful genital ulcers with regional lymphadenopathy.
- Other causes like *Treponema pallidum* or *Herpes simplex* (which are more likely to oocur) should be excluded.

According to IDSA 2018, chancroid may be identified by Gram stain and culture but is not recommended to be performed unless by a laboratory experienced in this testing. Gram stain sensitivity for such diagnosis is low (5% to 63%) and specificity (51% to 99%) **(45-46-125).**
Culture and PCR are mostly not available. Empiric treatment with Macrolides is reasonable if the clinical manifestations and epidemiology are strongly suggestive for the diagnosis.

As conclusion: Gram stain has a clinical impact (diagnostic) only on patients with bacterial vaginosis and almost no clinical impact on patient's urethritis/cervicitis/genital ulcers.

5.3. Clinical impact on patients with wound infection:
All wounds are colonized with microbes, but not all wounds are infected. Acute wounds include lacerations, burns, postoperative surgical incision and others.
Chronic wounds include, but not limited to diabetic foot wounds, chronic orthopedic wounds, chronic abdominal wounds, decubitus ulcers,

malignancy-related wounds, and venous ulcers. Management of these wounds may include: cleansing/ debridement/drainage/antibiotic therapy/specific therapy, such as negative pressure.
Antibiotic therapy is indicated for wounds that appear clinically infected. All wounds are colonized with microbes and the Gram stain information would not be sufficient to guide the choice of antimicrobial therapy. Empiric antimicrobial therapy should be a broad-spectrum antibiotic with coverage of Gram-positive cocci from the skin (when needed) as well as the expected pathogens at the site of operation. Definitive antimicrobial treatment is guided by the clinical response of the patient and type bacteria in wound culture and their antimicrobial susceptibilities. However, wound swab cultures often reveal polymicrobial growth, making it difficult to distinguish colonization from true infection.

5.4. Clinical impact of eye/upper respiratory tract Gram stain swabs:
Although few studies over the diagnostic performance of Gram stain for *Neisseria* conjunctivitis. These studies have shown good sensitivity and specificity.
According to CDC, chemoprophylaxis should be administered as soon as possible (ideally within 24 hours after identification of the index patient). Conversely, chemoprophylaxis administered >14 days after onset of illness in the index patient is probably of limited or no value.
This means that Gram stain is important for therapy and prophylaxis in patients with hyper acute conjunctivitis in the absence of molecular technique.

Vincent angina is mostly diagnosed clinically. Positive Gram satin can support the diagnosis but it has no additive diagnostic or therapeutic value. Culture is not recommended for this indication.

Both Vincent angina and *Neisseria* conjunctivitis are not common diseases.

5.5. Clinical impact on patients with urinary tract infection:
Urine culture remains the gold standard for diagnosis.
According to IDSA 2018:

- It is not the appropriate method to detect polymorphonuclear cells in urine.
- It can be ordered as an option for detection of high numbers of Gram negative rods in suspected urosepsis.
- Infections with lower bacterial concentrations than 10^5 CFU/mL may not be detected.

312 pediatric patient, suspected with UTI have been evaluated in a prosective American study. Empirical therapy was prescribed before the urine Gram stain result was known in 40 (49%) patients and after in 42

(51%) patients. The antibiotics chosen did not differ between the two groups (P=0.81), nor did they differ for patients with Gram-negative rods on urine Gram stain compared to those with Gram-positive cocci (P=0.67) **(126**

5.6. Clinical impact of faces Gram stain on patients with gastrointestinal infections:

Treatment is not indicated in most cases of acute diarrhea since the illness is usually self-limited. Empiric antibiotic therapy (azithromycin) will be indicated when patients has a severe disease, with symptoms and signs suggestive for invasive bacterial infection, or at high risk for complications, irrespective for the result of Gram stain. Azithromycin is preferred for patients with fever or dysentery (bloody or mucoid diarrhea) and in other patients suspected to be at risk for a fluoroquinolone-resistant pathogen. It can also be given in suspected cases for cholera.

Specific circumstances such as severe diarrhea in the setting of prior antibiotic therapy, empiric treatment for *Clostredium difficile* is reasonable if the clinical suspision is high. The results of C*lostredium difficile* antigen/toxin tests are usually known on the same day.

5.7. Clinical impact of body fluids Gram stain:

5.7. A. Clinical impact of CSF Gram stain on patient with central nervous system infection:

It seems that the diagnosis of meningitis is a big challenge without the multiplex PCR. This might support the proposing of round-the-clock 24/24 CSF PCR instead of Gram stain (especially overnight). Both PCR and Gram stain may be needed in case of unusual or atypical microorganism (when meningitis occur in a particular context such as trauma). There are 2 case reports: the first one is posttraumatic meningitis with *Achromobacter xylosoxidans*. The *Achromobacter (*Gram negative rods) was observed in CSF Gram stain. The CSF bacterial culture was also positive. The second case report was *Mycobacterium tuberculosis* meningitis in 32 years old male in Japan. Abnormal finding was observed on Gram stained slide (Gram-positive bacilli that had been phagocytosed by neutrophils).This suggest the presence of mycobacterium which then had been detected by Ziehl-Neelsen staining of the CSF **(127-128).**

PCR applications (BioFire or in-house PCR) lead to more detected cases of meningitis, more targeted use of antibacterial and antiviral therapy especially in children.

Implementing the BioFire panel is costly. This has prevented the widespread use of this technology in the diagnosis of CSF infection, although Soucek DK et al suggest that cost savings through targeted therapy use were able to offset the increased cost **(129).**

5.7. Clinical impact of other body fluids Gram stain (pleural-pericardial-peritoneal):

Gram stain is considered as a routine in the evaluation of body fluid. The utility of this practice has not been adequately assessed.

The ultimate diagnosis of empyema (pleural fluid), SBP (ascitic fluid), and bacterial pericarditis (pericardial fluid) depends mostly on the analysis of the fluid. Fluid analysis usually reveals in such situations leukocytosis, high percentage of polymorphonuclear cells, low glucose, high protein, and elevated lactose dehydrogenase levels.

In practice, Gram stain result follows nearly always the cell count result. Gram stain may give a misleading information to the clinician. For example, a Gram-positive organism that turned out later to be a contaminant.

5.7. C. Clinical impact of synovial fluid on patients with periprosthetic joint infection or septic arthritis:

Gram stain is an unreliable tool for ruling out periprosthetic infection or septic arthritis because of the low sensitivity and low negative predictive value. Many studies and guidelines recommend against the use of Gram stain to diagnose periprosthetic joint infection.

IDSA 2018, IGGI, UpToDate, still recommend the usage of Gram stain for the diagnosis of septic arthritis or bursitis (high specificity). Gram stain result will guide the choice of empirical antibiotic therapy.

The synovial WBC and percentage of polymorphonuclear cells from arthrocentesis are required to assess the likelihood of septic arthritis before the Gram stain and culture test results are known **(130).**

5.8. Clinical impact of biopsies Gram stain:

No data are available about the clinical value of direct/indirect Gram stain for the following biopsies types:

- Aortic aneurysm contents
- Brain
- Bone biopsies
- Lung biopsies

For Heart valves, there is no diagnostic effect for positive microbiology Gram stain of valve material. Modified Duke Criteria include the positive histological Gram stain and not the microbiological Gram stain.

Valve material Gram stain has no therapeutic effect either. Dead bacteria remain visible on the Gram stain for a long period.

5.9. Clinical impact on patient with positive blood culture:

Gram stain on blood culture has a high diagnostic performance. It guide the choice of initial antibiotic therapy. A recent prospective study (135 patients) from Germany has shown the clinical benefit of an immediate reporting (24/24) of the Gram stain results, especially in patients with fungus in the blood culture **(131).**

6. Organizational impact:

No evidence are available about the effect of Gram stain on duration of hospitalization or on usage of staff/non-staff resources.

7. Financial impact:

In the microbiology laboratory of Imelda hospital, we spend:

- 1,914 euro a year on Gram stain reagents
- 112 euro per year on immersion oil
- 760 euro on glass slides
- 11000 euro + 13000 euro for 2 automated stainers.

After exclusion of urgent requests, Gram staining occurs in multiple moments during the day (5 to 6 times/per day). In each time, +/-7 slides are stained and then examined by a MLT. Forty five Gram stained smears is the average number of slides examined per day.

Assuming that the preparation, examination and registration of one Gram stained smear take one minute, then a MLT spends at least 45 minutes per day (1350 minutes per month→270 hour per year) on direct Gram stain. A MLT costs 48 euro per hour (ordinary work hours). This means 0, 8 euro per minute. At least 1080 euro per month or more→12.960 euro per year.

The cost of Gram stain is 2786 euro in consumables and 270 hours (costed at 12960 euro) of laboratory staff time per annum (the one-time cost+maintenance cost of Bunsen burners and Gram stain machines is not included).

To calculate how much exactly the RIZIV spend on Gram stain in year 2018.

We have ordered the frequency of these 3 codes in RIZIV maps for year 2018:

- 126184 B70: microscopic examination for pus, exudate, sputum, body fluids (punction), sperm with or without simple staining.

- 126836 B90: Microscopic examination for pus, exudate, sputum, body fluids (punction), sperm with double staining.

- 549555 B400: Microscopic examination for CSF with double staining.

It was about.....

We have also ordered a query for these codes in our information system in 2018. It appears that these codes include also the telling of crystals in synovial fluid, eosinophils in pleural fluids and some other parameters from sperm morphological examination. So, we cannot know exactly how much RIZIV has spent on Gram stain.
From a simple query in our LIS, we were able to calculate how many direct Gram stained smears were performed in our laboratory in 2018.

For 7.351 direct Gram smears:

- 369 (direct Gram stain on CSF)
  369 x 400 x 0,031254 = 4, 613
- 6,982 (direct Gram other than CSF)
  6,982 x 70 x 0, 031254= 15, 275
  6,982 x 90 x 0, 031254= 19,639

19,904 euro

19,904 euro is the RIZIV reimbursement for direct Gram stain in Imelda microbiology laboratory in 2018.

8. Final conclusion:

- The clinical utility of Gram stain for most of microbiological specimens is not worth the time or cost it requires. Gram stain can be considered as a valuable test in the following indications:
  - Direct :
    - Vaginal samples to detect asymptomatic bacterial vaginosis, which is important for female who will undergo a gynecological procedure.
    - Septic arthritis: according to IGGI/UpToDate, the initial choice of empiric antimicrobial therapy for septic arthritis is guided by the result of Gram stain.
    - CSF by suspected meningitis, if PCR M/E panel is not available or in special context.
    - *Neisseria* conjunctivitis or *Neisseria* urethritis in the absence of NAAT.
  - Indirect :
    - Positive blood culture, in order to guide the choice of empirical therapy.

COMMENTS

Gram stain is considered as a routine test in the evaluation of most microbiological smaples. The utility of this test has not been adequetly assesed.

TO DO/ACTIONS

- Discuss with the clinicians the possibility of cancelling Gram stain when not needed:
  - Non sterile samples :
    - Wounds
    - Genital other than vaginal samples for bacterial vaginosis.
    - Upper and lower respiratory tract samples
  - Synovial fluid/biopsy Gram stain in periprosthetic joint infection.
- Participation in INSTAND EQC
- Inter-individual testing more frequent.
- Reporting Gram stain results for BV in a score system in LIS.