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# OPTIMIZATION OF BACTERIOLOGICAL DIAGNOSIS OF TUBERCULOSIS: CONTRIBUTION OF CENTRIFUGATION AND CULTURE ON MIDDLEBROOK 7H9

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## ABSTRACT

**Introduction:** The bacteriological diagnosis of tuberculosis is mainly based on the isolation and identification of the responsible mycobacteria. However, the slow growth of mycobacteria remains a real obstacle for studies interested in this disease. Microscopic examination isthe initial step and often the only possibility in many developing countries to make the diagnosis. Faced with this problematic, this work focused on evaluating the contribution ofcentrifugation of the pathological product on the positivity of the microscopic examination and to make a comparison of the cultures of mycobacteria in Löwenstein Jensen (LJ) and in Middlebrook 7H9 (MB7H9) for the bacteriological diagnosis of tuberculosis.

**Methods:** Over a period of 6 months, all samples for diagnosis of tuberculosis, received at the Bacteriology-Virology-Hygiene laboratory of the IBN ROCHD University Hospital center in Casablanca were included in our work. Middlebrook 7H9 was prepared at our laboratory.

**Results:** On 613 clinical samples analyzed. The study showed that smear microscopy after centrifugation showed a better yield with a specificity of 100%; it increased the sensitivity of direct examination, allowing a gain of 50%, with a VPP of 100% and a VPN of 97, 56%. The MB7H9 liquid also gave a better yield with a very short growth time compared to the solid LJ.

**Conclusion:** The implementation of MB7H9 culture technique requires well-adapted structures and its systematic use in bacteriological diagnosis remains dependent on financial resources.

**Keywords:** Diagnosis – Centrifugation - Microscopic examination - Tuberculosis, MiddleBrook7H9 - Löwenstein Jensen.

## INTRODUCTION

Tuberculosis is a major global public health problem. According to the World Health Organization in 2019 there were 10 million new cases of the disease with 1.4 million deaths; the highest number of new cases was recorded in the South Asia Region East, with 44% new cases, followed by the African Region, with 24% new cases, and the Western Pacific with 18% [1]. Tuberculosis is one of the top 10 causes of death worldwide. In Morocco, nearly 30,000 cases are recorded each year, which includes new cases and relapse cases. Theincidence rate is around 87 cases per 100,000 inhabitants, half of which is pulmonary tuberculosis [2].

The objectives of this work are as follows: To determine the effect of centrifugation

of the pathological product on the positivity of the microscopic examination in the bacteriological diagnosis of tuberculosis by comparing the results of the microscopic examination before and after concentration of the pathological product, in order to improve the sensitivity of the direct examination to obtain more precise results in a shorter time (a few hours). Compare the two mycobacteria culture techniques: the standard reference technique on solid medium (Löwenstein-Jensen) and the rapid technique on liquid medium (Middlebrook 7H9).

## MATERIALS AND METHODS

### 1. Materials

**Specimens**

The samples for research of BK have two different origins, pulmonary (sputum, gastric tubing, LBA) and extra-pulmonary (urine, abscess, skin lesions, effusion fluid, stool)

**Patients**

The patients are either hospitalized in the various clinical services of the CHU Ibn-Rochd Casablanca, or outpatients, on prescription from the clinician for research of mycobacteria.

**Analysis materials**

Oven set at 37°C (HERAEUS) with a capacity of 750 L, two vertical laminar flow hosts, refrigerator at 4°C, two optical microscopes, centrifuge (speed of 4000 rpm), autoclave, heating plates and bunsen burners.

**Reagent**

Antiseptic (Trisodium Phosphate), 5% sulfuric acid (H2SO4), Fuchsin solution, Gabett's dye, colored indicator (Bromothymol blue), methanol, rubbing alcohol, immersion oil, OADC, PANTA, culture medium solid (Löwenstein-Jensen) and liquid culture medium (Middlebrook 7H9).

### 2. Contribution of centrifugation in the bacteriological diagnosis of tuberculosis

The bacteriological analysis in search of BK without centrifugation includes the reception of the samples, the realization of the smear, its fixation which is done by methyl alcohol, and the Ziehl Neelsen staining. On the other hand, the bacteriological analysis in search of BK with centrifugation includes centrifugation for 20 minutes at 4000 rpm of the sample after decontamination, and production of smears.

### 3. Comparison of culture media for mycobacteria

The culture of the samples includes decontamination, fluidification and homogenization which are done by 10% trisodium phosphate, which is brought into contact at equal volume with the sample in a conical 45 ml centrifugation tube with stopper. Contact is maintained throughout the night. Samples that are usually sterile (CSF, pleural, pericardial, joint fluid, bone marrow, blood) are inoculated into culture media without prior treatment or after centrifugation. Afterwards, neutralization is carried out with 50% sulfuric acid in the presenceof a colored indicator which is Bromothymol blue until an olive yellow color is obtained while respecting aseptic conditions. The culture on solid medium LJ is done on 2 tubes, whichare placed in the oven at 37°C on trays in an inclined position not aimed at the bottom to let the seeding liquid evaporate for 48 hours, because the medium must be dry. but not dried out so that mycobacteria can grow on it. The first examination of the cultures is done after 48 hours after inoculation to detect contaminated samples. Crops are sorted weekly for up to 8 weeks. The positive culture of colonies of M. tuberculosis is rarely recognized before 21st to 28th days of incubation, it has a characteristic appearance “rough surface, beige cream color, in “cauliflower”. The culture is usually declared negative if after

8 weeks to 12 weeks of incubation there has been no development of colonies. On the other hand, the culture on Middlebrook 7H9 liquid medium includes first of all the preparation, the conservation, andthe control of the culture medium; the latter aims to detect a possible microbial contamination. The inoculation is done with the same centrifugation pellet used to inoculate the LJ medium. We inoculated 0.5 ml per tube of MB7H9 medium supplemented with 0.5 ml of OADC (nutrient supplement for enrichment) and made selective by adding 0.1 ml of a mixture of antibiotics containing the PANTA solution. The culture time of the MB7H9 medium is rapid: 8 to 30 days depending on the richness of the AFB. We check all suspected positive cultures by Ziehl Neelsen staining.

**4. Statistical analysis**

Raw data is entered in Excel, taking into account all the variables to be used (clinical information, direct examination, conventional culture, culture in liquid medium, incubation date and date of results). The quantitative variables were expressed as mean and standard deviation of the mean and the qualitative variables as number n and as a percentage. Statistical comparisons were performed using SPSS 20 software (IBM Inc., Chicago, USA). Qualitative variables were compared using the chi-square test or Fischer's exact test in the case of small numbers. Quantitative variables were compared using Student's t test. The significance threshold was retained for a $p < 0.05$. For the two studies carried out, we evaluated the statistical performance by calculating: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), false positives, false negatives, and YOUDEN index.

## RESULTS

**1. Patient characteristics**

Out of a total of 613 samples analyzed, the average age of these patients is 37 years and the sex ratio is 1.42.

**2. Characteristics of samples (Table 1)**

Of the 613 pathological products received, 344 are used to study the effect of centrifugation and 269 for the comparative study of culture media. Of these 613 samples, 512 samples received were of pulmonary origin (83.5%), and 71 samples were of extra-pulmonary origin (16.5%).

**Table 1 : Frequency of samples according to their sampling site**

| | Nature of samples | Number of samples | Percentage (%) |
|---|---|---|---|
| Pulmonary(84.6%) | Sputum | 199 | 57,8 |
| | Bronchial aspiration | 50 | 14,5 |
| | Gastric tube | 27 | 7,8 |
| | Broncho alveolar fluid | 5 | 1,5 |
| | Protected distal bronchial specimen | 10 | 2,9 |
| Extrapulmonary(15.4%) | Pleural fluid | 10 | 2,9 |
| | Pus | 11 | 3,2 |
| | Biopsy | 4 | 1,2 |
| | Urine | 15 | 4,4 |
| | Others | 13 | 3,7 |

**3. Comparison of centrifugation effect : Results of the microscopic examination before and after concentration of the pathological product (Table 2)**

Among the 344 samples studied, 320 are negative, and 16 are positive by the two microscopic methods, i.e. agreement for 336 smears (97.7%). A discrepancy was noted in 8 positive cases by microscopy after concentration and negative by direct smear microscopy (2.3%).

**Table 2: Results of the microscopic examination before and after centrifugation of theanalyzed samples.**

| **Microscopy afterconcentration**<br><br>**Direct microscopy** | **Negative** | **Positive** | **Total** |
|---|---|---|---|
| Negative | 320 | 8 | 328 |
| Positive | 0 | 16 | 16 |
| **Total** | 320 | 24 | 344 |

**4. Statistical expression of the results of the direct examination, before and aftercentrifugation (Table 3)**

The direct examination before centrifugation, presents a sensitivity of 66.67% compared to the direct examination after centrifugation, and consequently this last technique has a yield which makes it possible to recover 50% of the smears positive to the direct examination, passing from 16/24 to 24/24 with a specificity of 100%. However, this technique can present false negatives since the VPN is 97.56%. The YOUDEN index for this test is 0.67.

**Table 3: Informational index of the microscopic examination results before centrifugation compared to the microscopic examination after centrifugation.**

| | | | |
|---|---|---|---|
| **Sensibility** | 66,67% | **Negative predictive value** | 97,56% |
| **Specificity** | 100% | **False negative** | 2,44% |
| **Positive predictive value** | 100% | **False positive** | 0% |
| **YOUDEN Index** | 0,67 | | |

**5. Yield of the direct examination according to the nature of the samples (Figure 1)**

Out of a total of 344 samples, 291 samples were of pulmonary origin: the yield of the direct examination before centrifugation is 15/291 positive samples, i.e. 5.15%. Centrifugation allowed us to recover 22/291 positive smears, i.e. 7.56%, and on 53 samples of extra-pulmonary origin: the yield of the direct examination before centrifugation is 1/53, i.e. 1.88%, centrifugation allowed us to recover 2/53 positive smears; i.e. a rate of 3.77%. The total yield of the samples before centrifugation is 16/344 samples (4.65%), it increased after centrifugation to 24/344 samples (7%), i.e. a gain of 50%.

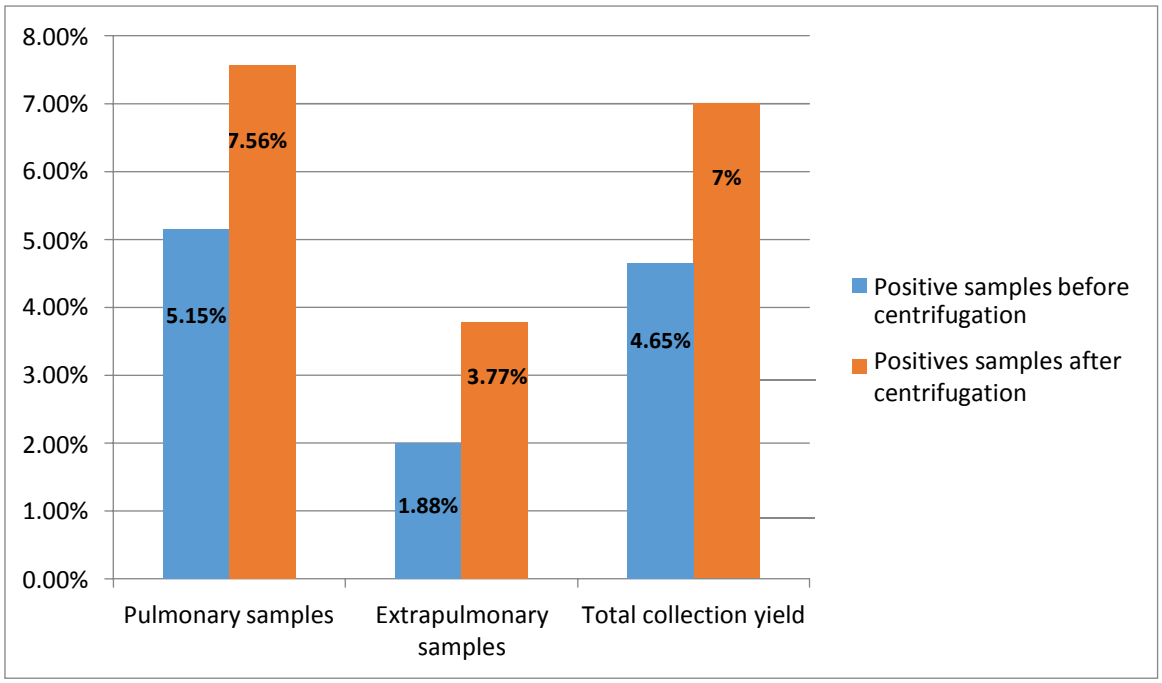



**Figure 1: Percentage and total yield of positive samples before and after centrifugation**

## 6. Comparison of LJ and MB7H9 culture media

### Culture on LJ solid medium

Culture on solid medium is positive for 22 samples, or 8%, negative for 236 samples, or 87.5% and contaminated for 11 samples, or 4.5%.

### Culture on MB 7H9 liquid medium

The culture on liquid medium is positive for 32 samples (12%), negative for 197 samples (73%) and contaminated for 40 samples (15%).

## 7. Statistical expression of the results of the culture on solid medium LJ compared to liquid medium MB7H9 (Table 4)

Culture on solid medium has a sensitivity of 68.75%, compared to culture on liquid medium, and consequently the latter technique made it possible to recover approximately 45.5% of positive cultures, with a specificity of 100%. Moreover, despite this high specificity, this technique has a false negative rate of 4.83%. The YOUDEN index has a positive value (0.69).

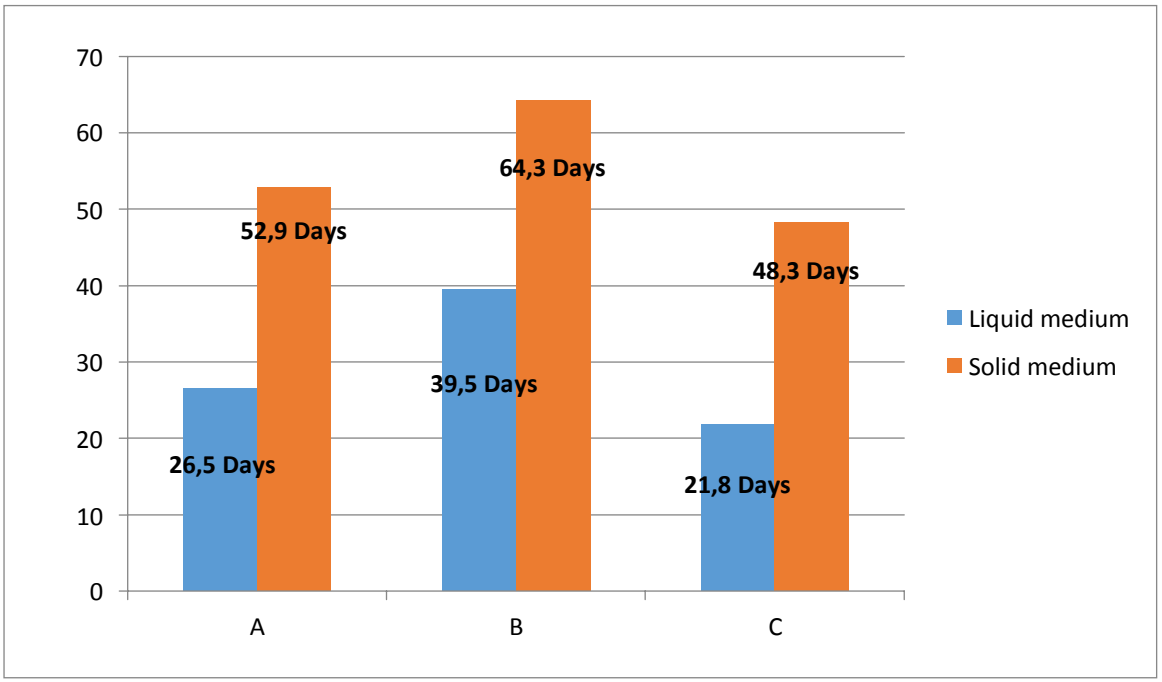
**Table 4 : Culture on solid medium LJ compared to liquid medium MB7H9**

| | | | |
|---|---|---|---|
| **Sensibility** | 68,75% | **Negative predictive value** | 95,17% |
| **Specificity** | 100% | **False negative (FN)** | 4,83% |
| **Positive predictive value** | 100% | **False positive (FP)** | 0% |
| **YOUDEN Index** | 0.69 | | |

**8. Growth rate of micobactéria (Figure 2)**

By focusing on the average time of revelation of positive cultures in relation to the date of culturing the samples, we find that the growth rate of mycobacteria on liquid medium is 26.5 days, whereas it is 52 .9 days on solid medium, therefore a difference of 26 days, this difference is statistically significant (p <0.05).

For samples with a negative direct examination, the growth rate of mycobacteria on liquid medium is 39.5 days, whereas it is 64.3 days on solid medium, with a difference of 24 days. For samples with a positive direct examination, the growth rate of mycobacteria on liquid medium is 21.8 days, whereas it is 48.3 days on solid medium; therefore a difference of 26.5 days. These differences are statistically significant (p <0.05).




**A: Average growth rate of mycobacteria.**
**B: Average rate of growth of microscopy-negative mycobacteria.**
**C: Average rate of growth of microscopy-positive mycobacteria.**

**Figure 2: Average growth rate of mycobacteria on the two LJ and MB7H9 media for positive and negative microscopy samples on direct examination.**

## DISCUSSION

In this study, the average age of the patients is 37 years old, and the sex ratio is 1.42; 50% of the cases studied were between 30 and 40 years old, 25% were between 40 and 50 years old. These results show that 75% of requests for bacteriological diagnosis of tuberculosis in this series relate to patients aged between 30 and 50 years, it is in this age group where people are active and that tuberculosis occurs with a relatively high frequency. In Congo, according to a study reported by Ngama et al. in 2014 [3], the age of patients with tuberculosis was between 21 and 40 years old. These results are consistent with ours, since 50% of the cases studied were between 30 and 40 years old.

**1. Contribution of centrifugation in the bacteriological diagnosis of tuberculosis**

The comparison of the results of the smears carried out before and after centrifugation showed that the samples which were considered positive at 1+ before centrifugation were confirmed after centrifugation at 2+, or at 3+ or even at 4+, and those which were considered positive at 2+, were confirmed positive after centrifugation at 3+ or 4+. Thus microscopic examination after centrifugation quantitatively modified the bacillary density detected on direct examination, which improved the sensitivity of the latter, and increased the percentage of positive fields found on smears after concentration as reported in many studies [4, 5].

The direct examination before centrifugation, presents a sensitivity of 66.67% compared to the direct examination after centrifugation, and consequently this last technique made it possible to recover 50% of the positive smears with the direct examination. A study reported by Cattamanchi. A et al. in San Francisco in the USA [6], revealed that bacilloscopy before centrifugation had a sensitivity of 64%. According to the same study, a gain in sensitivity of 13% was noted after centrifugation.

These results are in agreement with those found in this series for bacilloscopy before centrifugation. Thus bacilloscopy after centrifugation in our series, allowed us a significantly higher gain in sensitivity. The specificity of this technique is 100%. The YOUDEN index calculated on this test was 0.67; which makes it possible to say that the direct examination after centrifugation is more effective compared to this examination where centrifugation was not practiced.

According to the results found for the yield of the direct examination according to the natureof the samples in this study, it turns out that the samples of pulmonary origin are more frequently positive on microscopic examination, compared to those of extra-pulmonary origin. The concentration of the samples made it possible to increase the positivity of the smears carried out, with a direct effect on the improvement of the sensitivity of the microscopic examination. According to a study carried out in Madagascar by Rakotoson et al. in 2013, the authors analyzed different types of samples for the bacteriological diagnosis of tuberculosis, 94.7% of the samples analyzed were sputum and the positivity rate was also the highest for these samples, with 6.7% of cases [7]. These results are consistent with our data.

The performance of the direct examination after centrifugation allowed a gain of 50% compared to the direct examination before centrifugation. But this does not prevent bacilloscopy after centrifugation from having drawbacks, such as: The increased risk of contamination which could be due to the centrifugation of the tubes for sputum homogenization, because when opening the spittoons or tubes, micro-aerosols are created and are projected up to more than 60 cm from the handling point, thus favoring contamination by inhalation of these particles. In case of doubt on the result of the smear reading, or when it comes to paucibacillary products, it is not possible to redo a smear, since the pellet will be neutralized and inoculated afterwards.

**2. Comparison of culture media for mycobacteria**

We can explain the positivity of the cultures of negative samples on direct examination by the fact that 104 bacilli/ml to 105 bacilli/ml of pathological product are needed for the direct examination to be positive [8]. Liquid medium recovered approximately 45.5% of positive cultures, increasing from 8% to 12%. The recovery

rate of positive results on liquid medium shows a statistically significant difference compared to that of culture on solid medium ($p<0.05$).

Concerning the effect of the direct examination on the positivity of the culture : Our results make it possible to say that the rate of positivity of the cultures of mycobacteria is influenced by the positivity of the results of the direct examination. They are in agreement with several works in the literature, in particular that of Chihota et al in England **[9]**, the authors also found an increase in the rate of positive cultures with positive microscopy, compared to those with negative microscopy.

In order to improve the culture yield of mycobacteria, several liquid media have recently been developed. This is the case of Middlebrook 7H9, BacT/ALERT MP®, Bactec 12B® or the manual mycobacterial growth indicator tube (MGIT) **[10, 11].**

According to a study carried out in France by M. Soulier et al. [12], the sensitivity of the LJ was 51.7%. In our study, the sensitivity was 68.75%, our results allowed better detection of BK on LJ medium compared to this study. Battaglioli et al have also reported in a study carried out in Belgium and Peru [10], that MB7H9 and MGIT liquid culture media have a positivity rate of 21% and 24% respectively, and are endowed with a sensitivity ranging from 76% to 85%. The positivity rate of our results on the liquid medium is low compared to these data from the literature, this can be explained by several factors: The poor quality of the samples: which are rather salivary in nature and not real sputum; the patient has already been on treatment, this decreases the likelihood of having a positive culture.

Regarding the growth rate of mycobacteria on the two media 7H9 and LJ, and by calculating the average time to obtain the culture, we found that mycobacteria are characterized by a much faster growth rate on liquid medium than that found on solid medium, thus allowing a significant time saving, on average of 26.4 days. This average growth time obtained with our cultures on liquid medium is within the range reported by most studies, which is 14 to 27 days [11]. This delay is influenced by the results of the direct examination, the samples revealed positive on the direct examination allowed a gain of 26.5 days, and the samples revealed negative on the direct examination allowed a gain of 24.8 days. This difference is statistically significant ($p<0.05$). According to the literature, for samples presenting a negative direct examination, this period of positivity has an average of 25 days for liquid media and more than 30 days for solid media **[10, 11, 13].**

### 3. Contamination of culture media

The inoculation of samples on liquid medium presents a greater risk of contamination compared to inoculation on solid medium. This is due to the nature of the liquid medium which is rich which therefore promotes contamination despite the addition of PANTA (antibiotic mixture) to Middlebrook 7H9.

Concerning the cost of the culture media used: If the LJ medium is the medium most used in the majority of mycobacteria laboratories, it is because it is a medium which gives good satisfaction in terms of culture yield and especially from a cost point of view. In our work, a tube of LJ cost approximately 12.00DH, and consequently the cost price of the culture per sample is around 24.00DH, since 2 tubes are inoculated regularly for each sample.

When we introduced the MB7H9 medium, the cost increased considerably since the cost price of a tube of MB7H9 offered in

our laboratory with the addition of OADC and PANTA cameto approximately 24.00 MDH and consequently the culture of 'a sample looking for mycobacteria on MB7H9 medium was multiplied by 2, which amounted to approximately 50.00 MDH.

## CONCLUSION

A reliable classic bacteriological diagnosis of tuberculosis is very important for the management and without delay of patients. At the end of this study, we have contributed to showing the effectiveness of centrifugation to improve the performance of direct examination, despite some drawbacks such as the increased risk of contamination. In addition, the performance of the Middlebrook 7H9 liquid medium compared to the Löwenstein Jensen solid medium resulted in a gain in yield and a considerable time to positivity despite some drawbacks such as a contamination rate and a higher cost compared to the solid medium.

## POTENTIAL CONFLICT OF INTEREST

None declared.

## AUTHORS CONTRIBUTION

All authors have contributed to the conduct of this work. All authors also declare that they have read and approved the final version of the manuscript.

## ETHICAL CONSIDERATION

All the data has been collected anonymously following patient confidentiality.

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