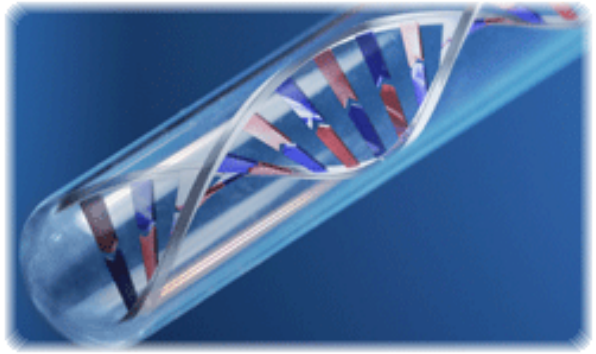


Role of Molecular biology, Histopathology in leprosy

Dr. Swapna. M

Asst. Director (Microbiology)

Contents

1. Molecular biology
    - PCR, sequencing
    - Real time PCR
2. Histopathology
    - Spectrum-classification

Molecular biology

1. Conventional PCR
    - Diagnosis
    - Antimicrobial resistance detection
2. Real Time PCR
    - Quantification
    - Viability assay
    - HRM assay

Diagnosis

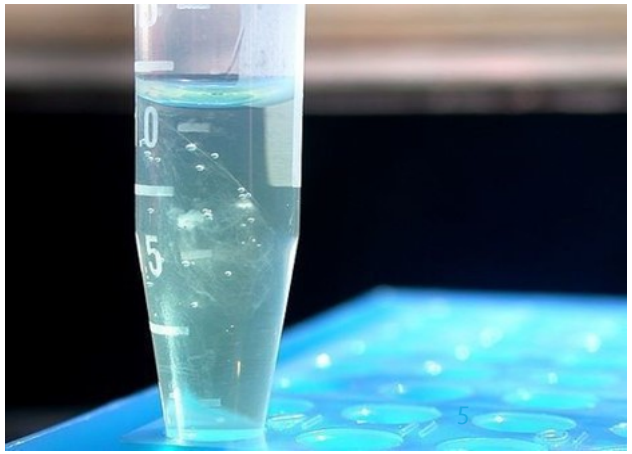
Specimens:

- Slit Skin Smear
- Skin biopsy tissue
- Nerve biopsy tissue

Step 1

Extraction of DNA from tissue specimen

Mince of Tissue with TE buffer
Add Lysozyme, incubate
Add Proteinase K-SDS
Add NaCl
Add CTAB
Add Choloroform:Isoamyl alcohol
Centrifuge at 10000 rpm at 4 C
Remove supernatant
Add Isoprophyl acohol
Centrifuge at 10000 rpm at 4 C
Discard supernatant
Wash with 70% alcohol
Centrifuge at 10000 rpm at 4 C
Dry the pellet at 45 C for 15 min
Add TE buffer, keep it overnight at 4 C
Store at -20 C



Step 2

Preparation for PCR

Add Millipore water, reaction buffer, dNTPs, set of primers, Taq polymerase, extracted DNA in eppendorf and place in thermocycler

Step 3

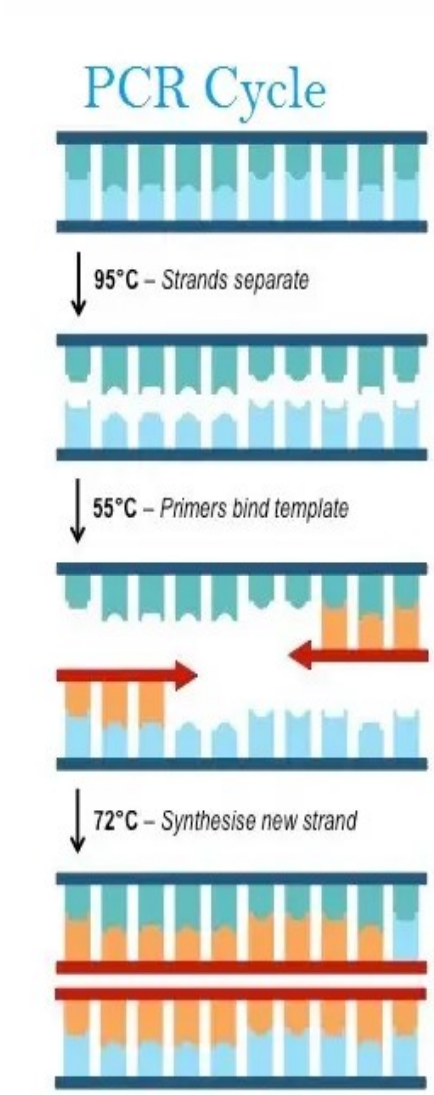
PCR

Programme thermocycler for 40 cycles with initial denaturation: 95 C (5 min), denaturation: 94 C (30 sec), annealing: 58 C (30 sec), extension: 72 C (1 min) and final extension: 72 C (10 min)

Primers

Gene of interest	Primer sequence	Base pair
RLEP gene M. leprae	5′- TGCATGTCATGGCCTTGAGG-3′ 5′-CACCGATACCAGCGGCAGAA -3′	129bp *(Donoghue HD et al, 2001)*

Thermocycler


PCR Cycle
95°C – Strands separate
55°C – Primers bind template
72°C – Synthesise new strand

Step 4

Gel electrophoresis

Use 1.5% agarose gel, TBE buffer and ethidium bromide

Step 5

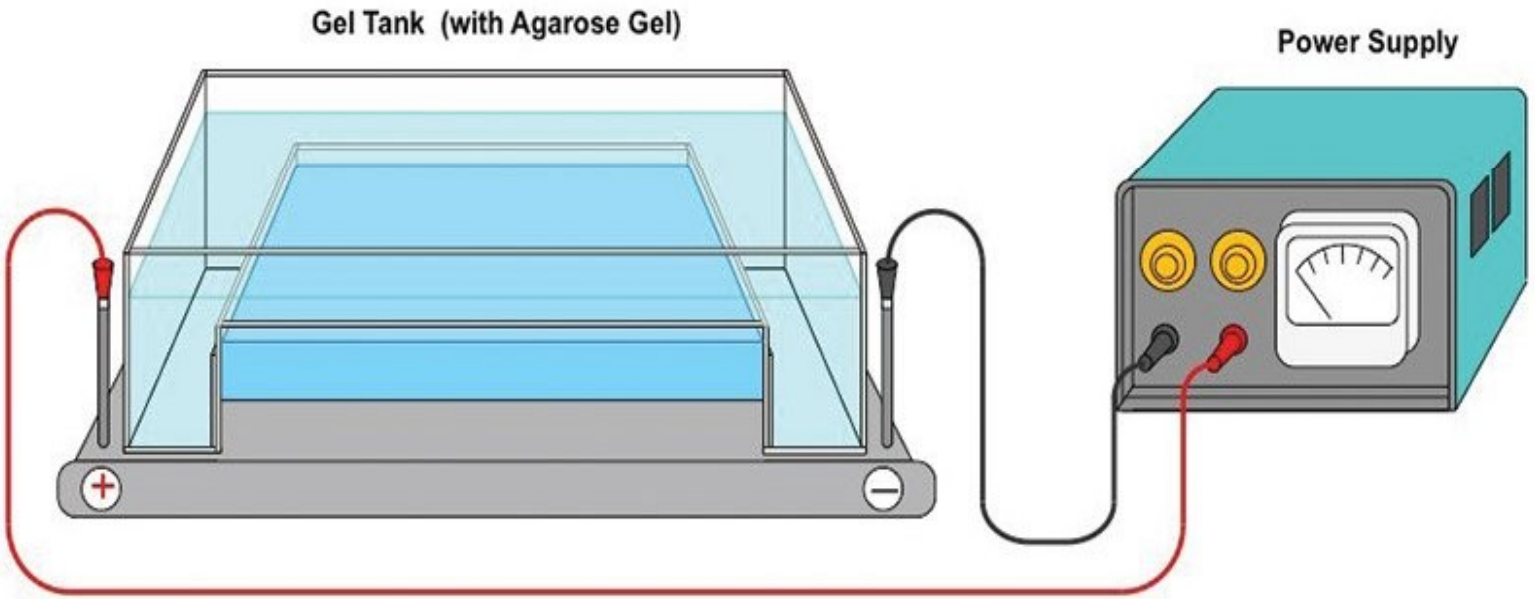
Documentation

Place the agarose in gel documentation system under UV light connected to digital camera for documentation

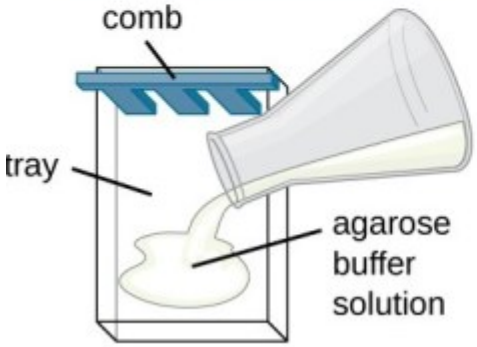
Step 6

Interpretation : presence of absence of 129bp compared with DNA ladder

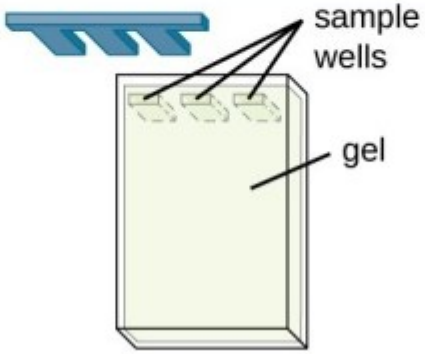
Agarose Gel Electrophoresis



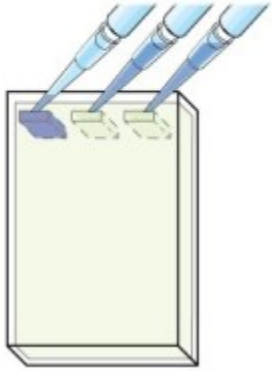
1 An agarose and buffer solution is poured into a plastic tray. A comb is placed into the tray on one end.

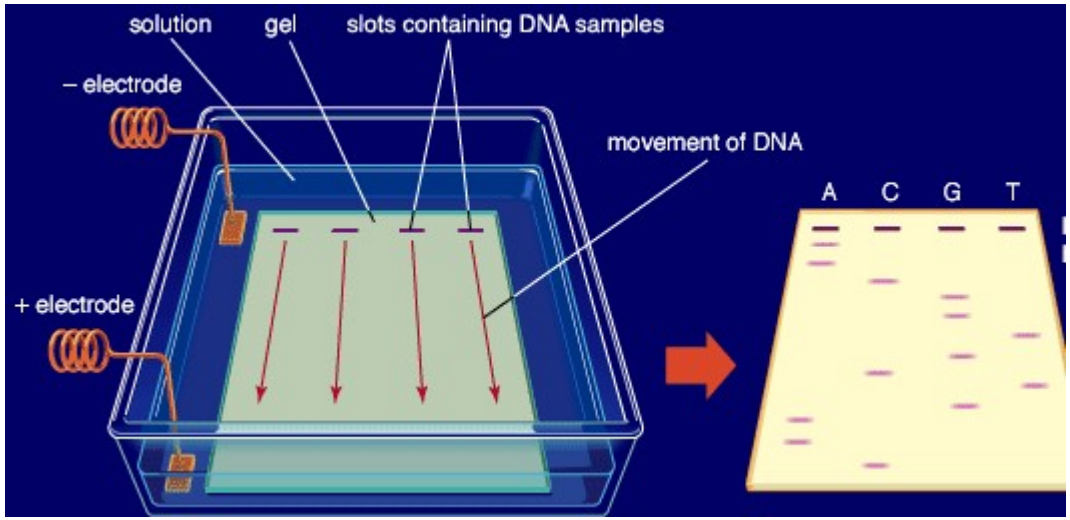


2 The agarose polymerizes into a gel as it cools. The comb is removed from the gel to form wells for samples.



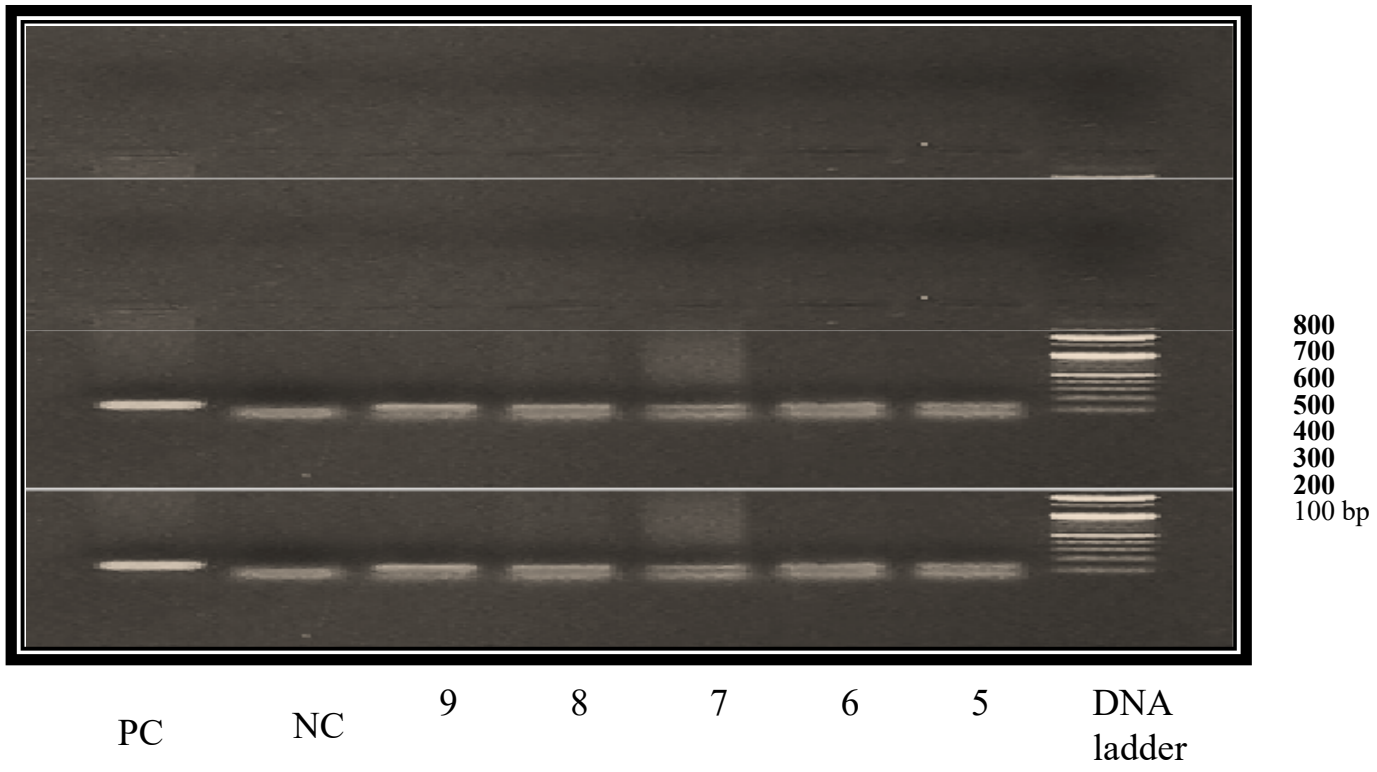
3 DNA samples colored with a tracking dye are pipetted into the wells.





Gel documentation

Gel Pic (129bp)

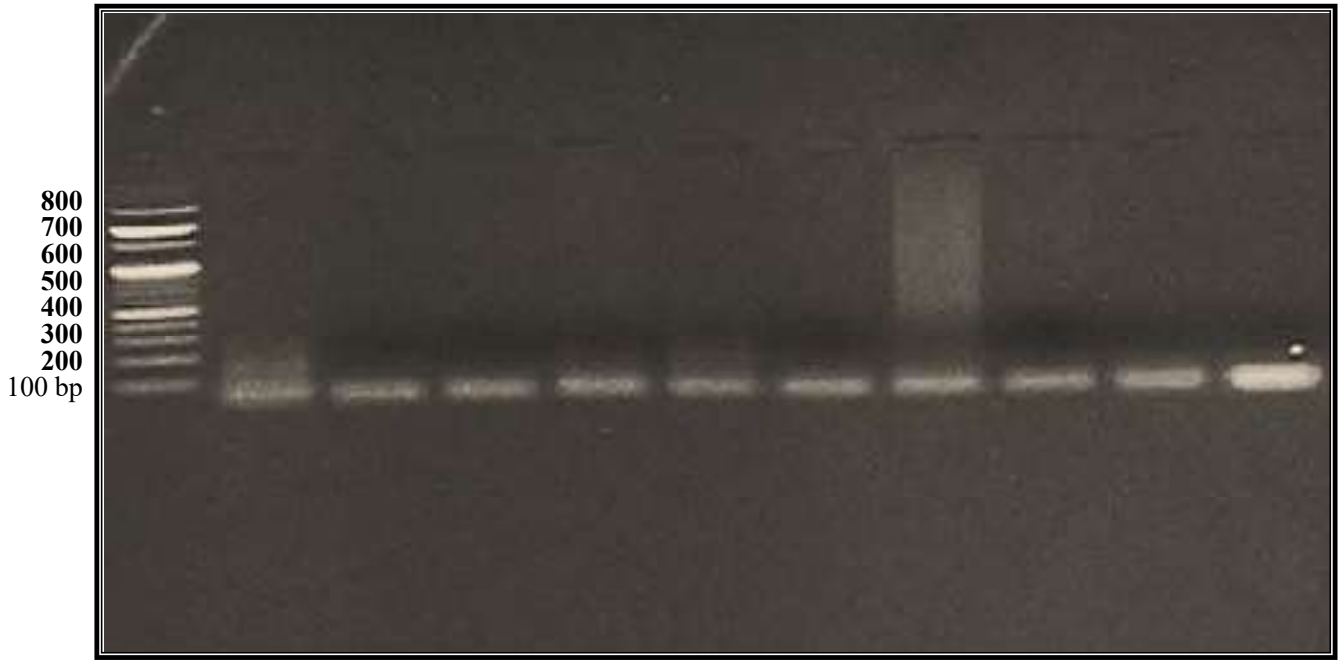


Primers

Gene of interest	Primer sequence	Base pair
16S r RNA gene M. lepromatosis	5'- GTCTCTTAATACTTAAACCTATTAA-3' 5'-CCACAAGACATGCGCCTTGAAG-3'	142bp *(Han XY et al 2014*

Initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 40 sec and initial extension at 72°C for 30 sec and final extension at 72°C for 10 min. Electrophoresis on 1.5 % agarose gel

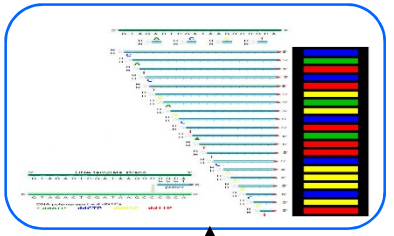
Gel pic *(142bp)*



AMR detection

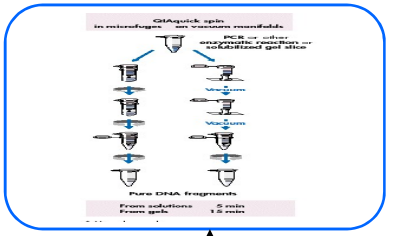
DNA extraction

PCR



DNA Sequencing

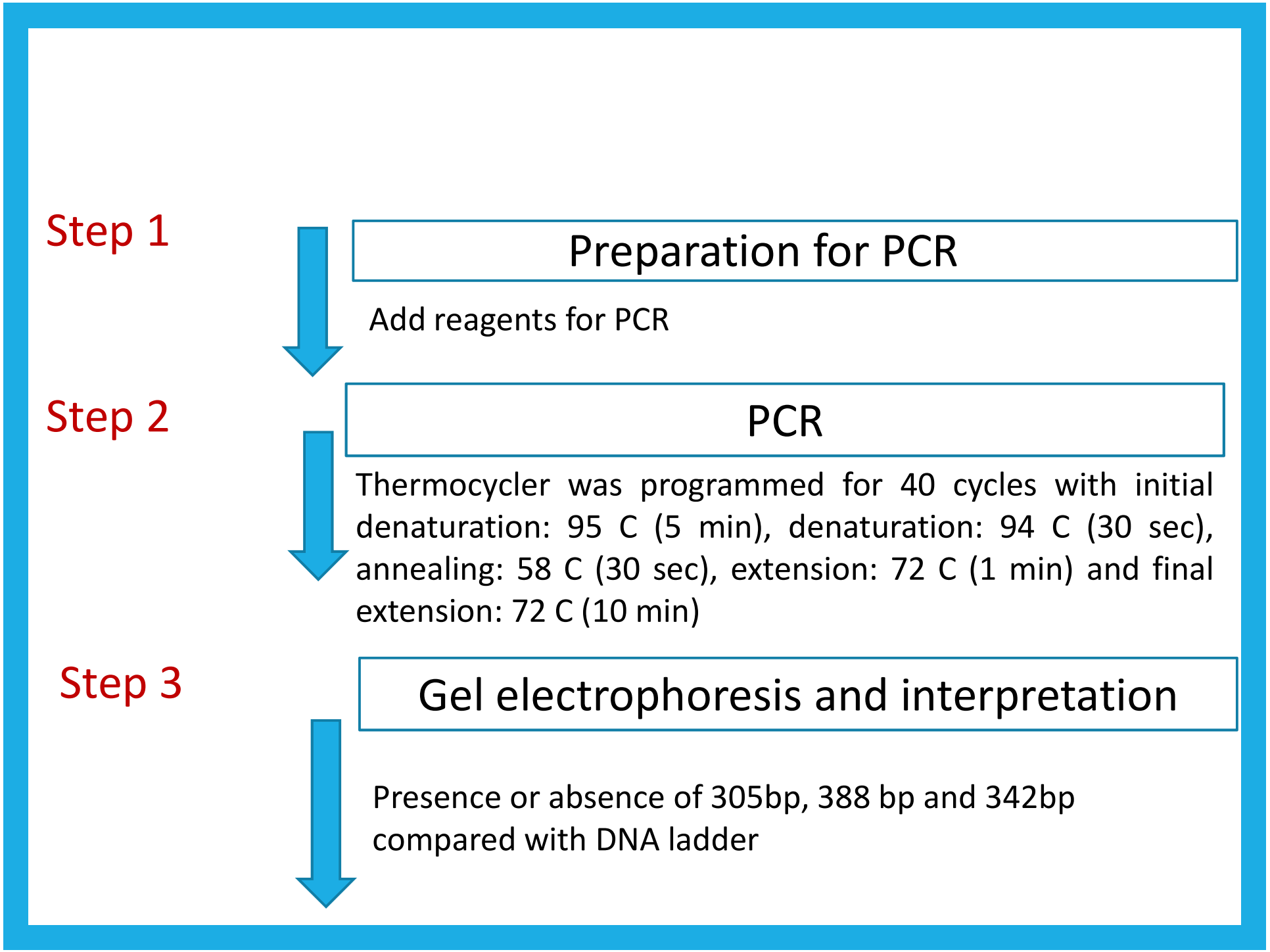
Purification



Electrophoresis & documentation

Primers

Gene of interest	Primer sequence
folP1	5’-GCTTCTCGTGCCGAAGCGCTCG-3’ 5’-CCATCGCGGGATCTGCTCGCCCA-3’
rpoB	5’ -GACGCTGATCAATATCCGT-3’ 5’ -ACGGTGTTTC GATGAACCCG-3’
gyrA	5’- ATGACTGATATCACGCTGCCA-3’ 5’ -ATAACGCATCGCTG CCGGTGG-3’


Step 1
Preparation for PCR
Add reagents for PCR
Step 2
PCR
Thermocycler was programmed for 40 cycles with initial denaturation: 95 C (5 min), denaturation: 94 C (30 sec), annealing: 58 C (30 sec), extension: 72 C (1 min) and final extension: 72 C (10 min)
Step 3
Gel electrophoresis and interpretation
Presence or absence of 305bp, 388 bp and 342bp compared with DNA ladder

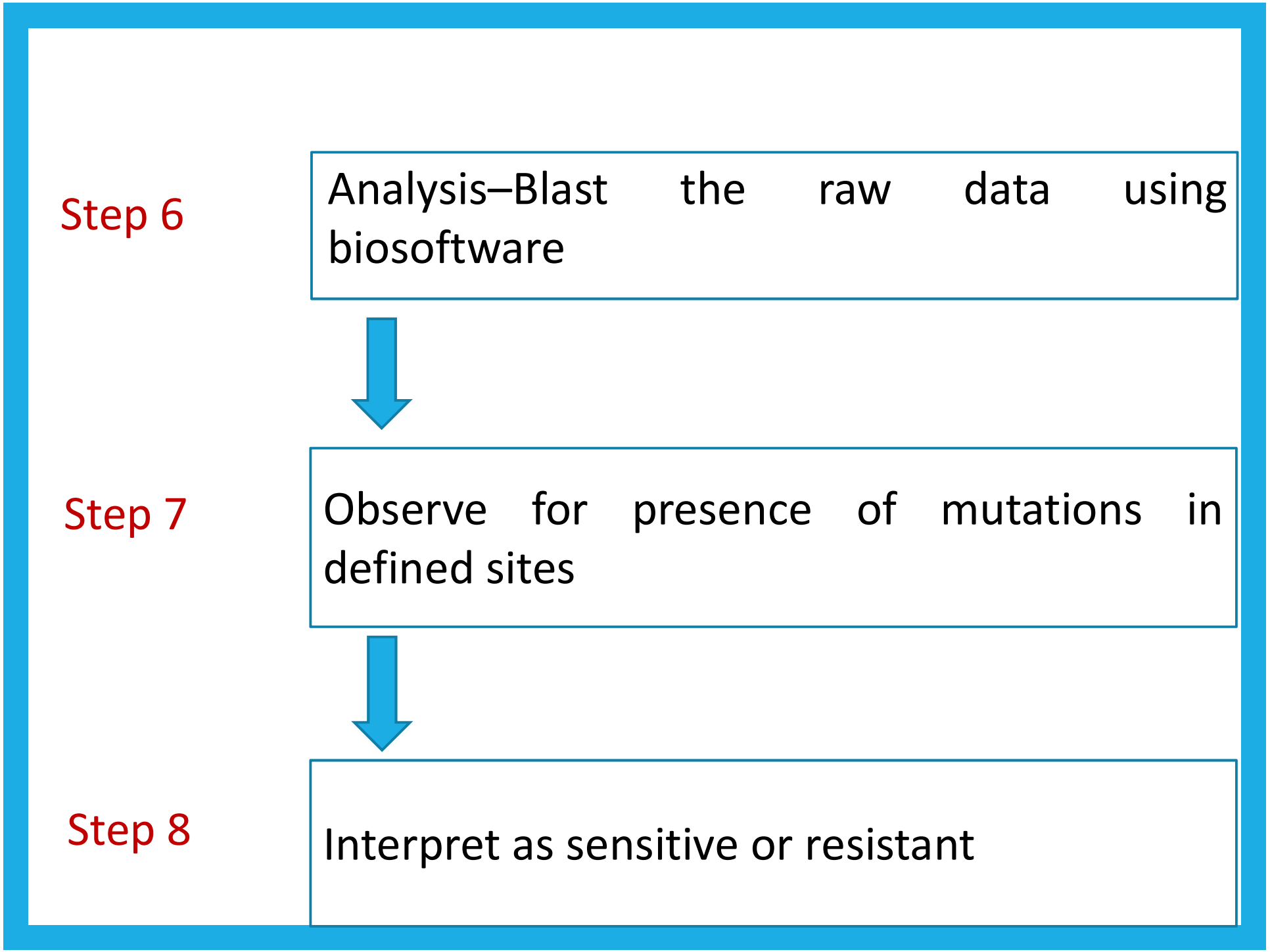
Step 4

Purification of amplified products

Add amplified product and binding solution to the column, wash with alcohol
Add elution buffer

Step 5

Sequencing

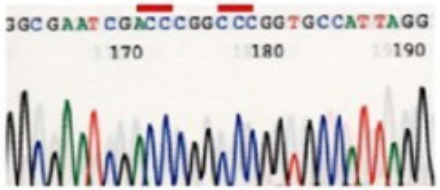

Step 6
Analysis–Blast the raw data using biosoftware
Step 7
Observe for presence of mutations in defined sites
Step 8
Interpret as sensitive or resistant

Drug resistance determining regions

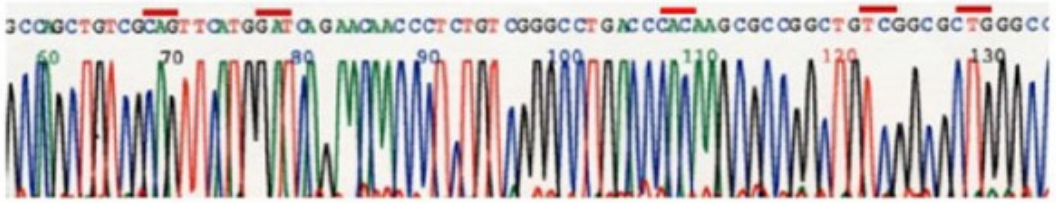
Antimicrobial agent	DRDR (position of codons)
Dapsone	53, 55
Rifampicin	407,410,420,425, 427,438,441,451,456,458
Ofloxacin	89,91,92,95

Sequence data

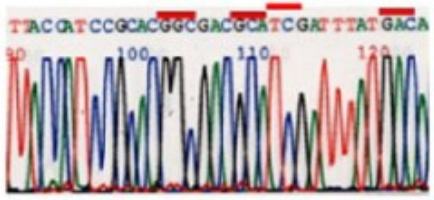
Dapsone: *folP1* threonine (ACC) at 53, proline (CCC) at 55



Rifampicin: *rp0B* Glutamine (CAG) at 438, aspartic acid (GAT) at 441,
Histidine (CAC) at 451, serine (TCG) at 456, leucine (CTG) at 458



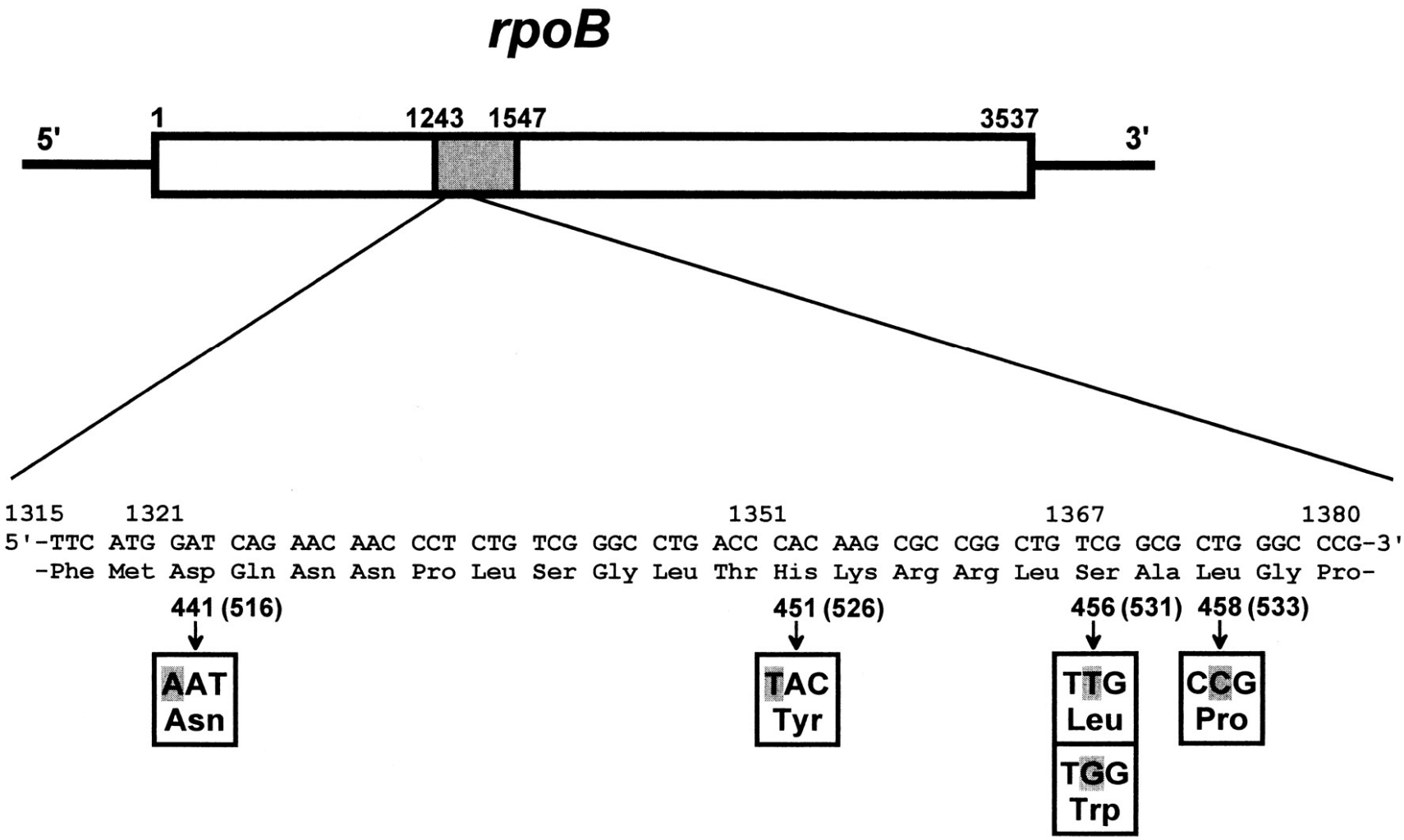
Quinolone: *gyrA* Glycine (GGC) at 89, alanine (GCA) at 91, serine (TCG) at 92,
aspartic acid (GAC) at 95



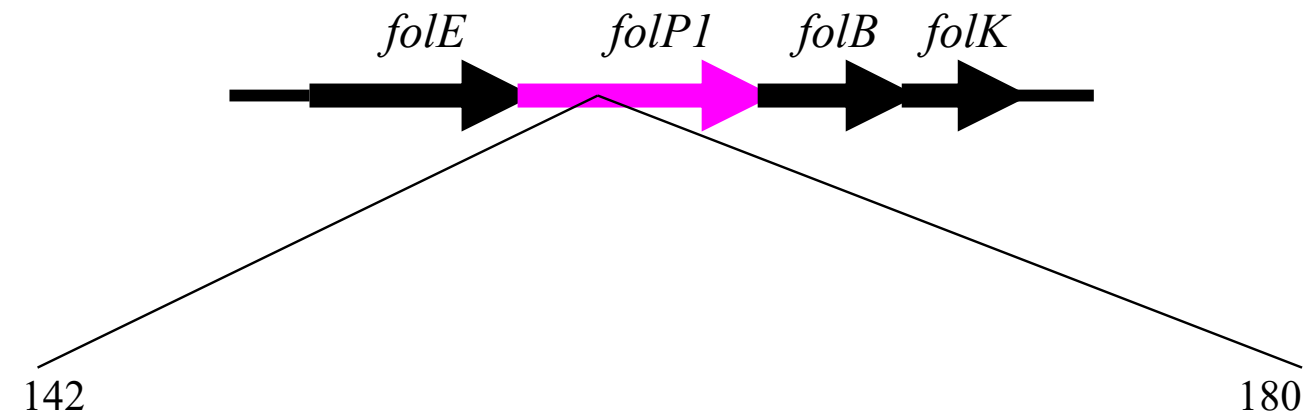
Sequence analysis

```
Query: 7   catcgttgacgatccgtgcgccgctttgcagcgccgcccgtgcaacatcagcgcgcgtag 66
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 319 catcgttgacgatccgtgcgccgctttgcagcgccgcccgtgcaacatcagcgcgcgtag 260


Query: 67  tatcgatacttactgtaatcccctgtgctgcaagttcttttacgacaggaacgatacgag 126
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 259 tatcgatacttactgtaatcccctgtgctgcaagttcttttacgacaggaacgatacgag 200

Query: 127 agagttcaactcgaggatcggtcctaatggcaccgcgccgggtcgattcgccaccgacgt 186
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 199 agagttcaactcgaggatcggtcctaatggcaccgggccgggtcgattcgccaccgacgt 140

Query: 187 cgacaatcgccgcgccttccgcgaccattgccaggccgtgctggacagcatcgtcaggat 246
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 139 cgacaatcgccgcgccttccgcgaccattgccaggccgtgctggacagcatcgtcaggat 80

Query: 247 caaggtaacgtccgccatctgagaacgaattgtcagtcacgttcaaaaccccaataacct 306
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 79  caaggtaacgtccgccatctgagaacgaattgtcagtgacgttcaaaaccccaataacct 20

Query: 307 gcactggcgccaaactcac 325
           ||||||||||||||||||
Sbjct: 19  gcactggcgccaaactcac 1
```

rpoB gene


rpoB
5'
1
1243
1547
3537
3'
1315
1321
1351
1367
1380
5'-TTC ATG GAT CAG AAC AAC CCT CTG TCG GGC CTG ACC CAC AAG CGC CGG CTG TCG GCG CTG GGC CCG-3'
-Phe Met Asp Gln Asn Asn Pro Leu Ser Gly Leu Thr His Lys Arg Arg Leu Ser Ala Leu Gly Pro-
441 (516)
451 (526)
456 (531)
458 (533)
AAT
Asn
TAC
Tyr
TTG
Leu
TGG
Trp
CCG
Pro

folP1 gene



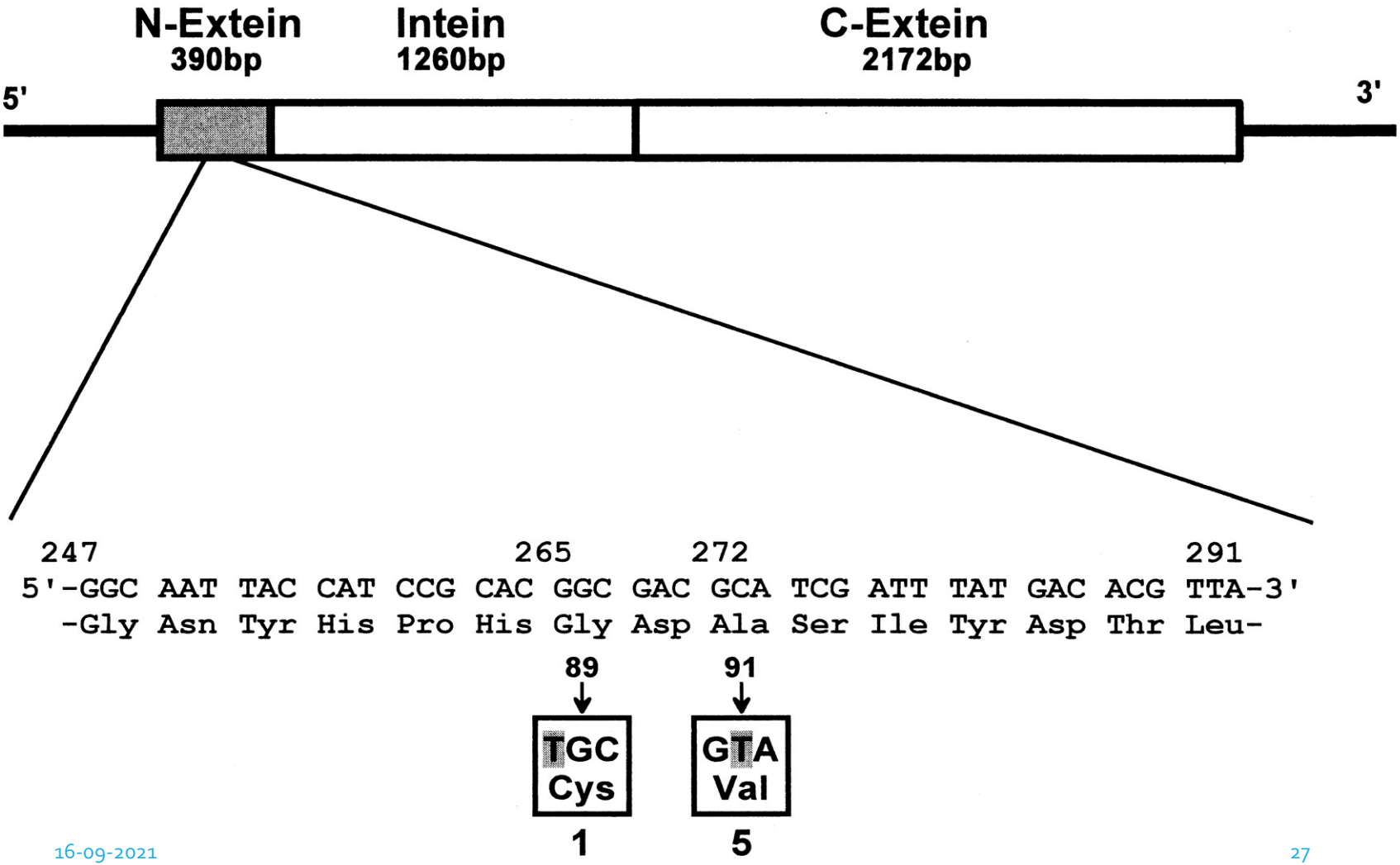
5’ - GTC GGT GGC GAA TCG ACC CGC CCC GGT GCC ATT AGG ACC - 3’

- Val Gly Gly Glu Ser Thr Arg Pro Gly Ala Ile Arg Thr -

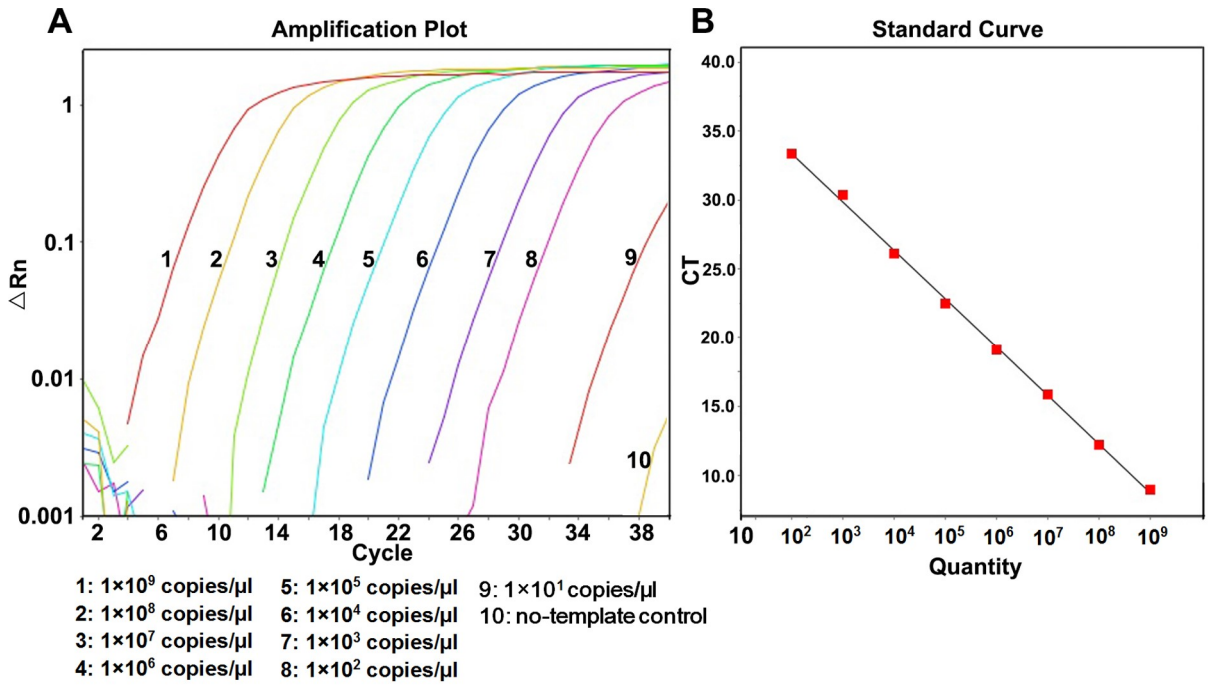
53 55

GGC	AGG	CTC	CGC
Gly	Arg	Leu	Arg

gyrA gene


N-Extein
390bp
Intein
1260bp
C-Extein
2172bp
5'
3'
247
265
272
291
5'-GGC AAT TAC CAT CCG CAC GGC GAC GCA TCG ATT TAT GAC ACG TTA-3'
-Gly Asn Tyr His Pro His Gly Asp Ala Ser Ile Tyr Asp Thr Leu-
89
91
TGC
Cys
GTA
Val
1
5

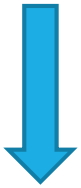
Quantification



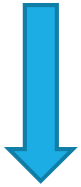
Viability assay

RNA are less stable than DNA

r RNA, m RNA



c DNA



PCR

qPCR-HRM analysis

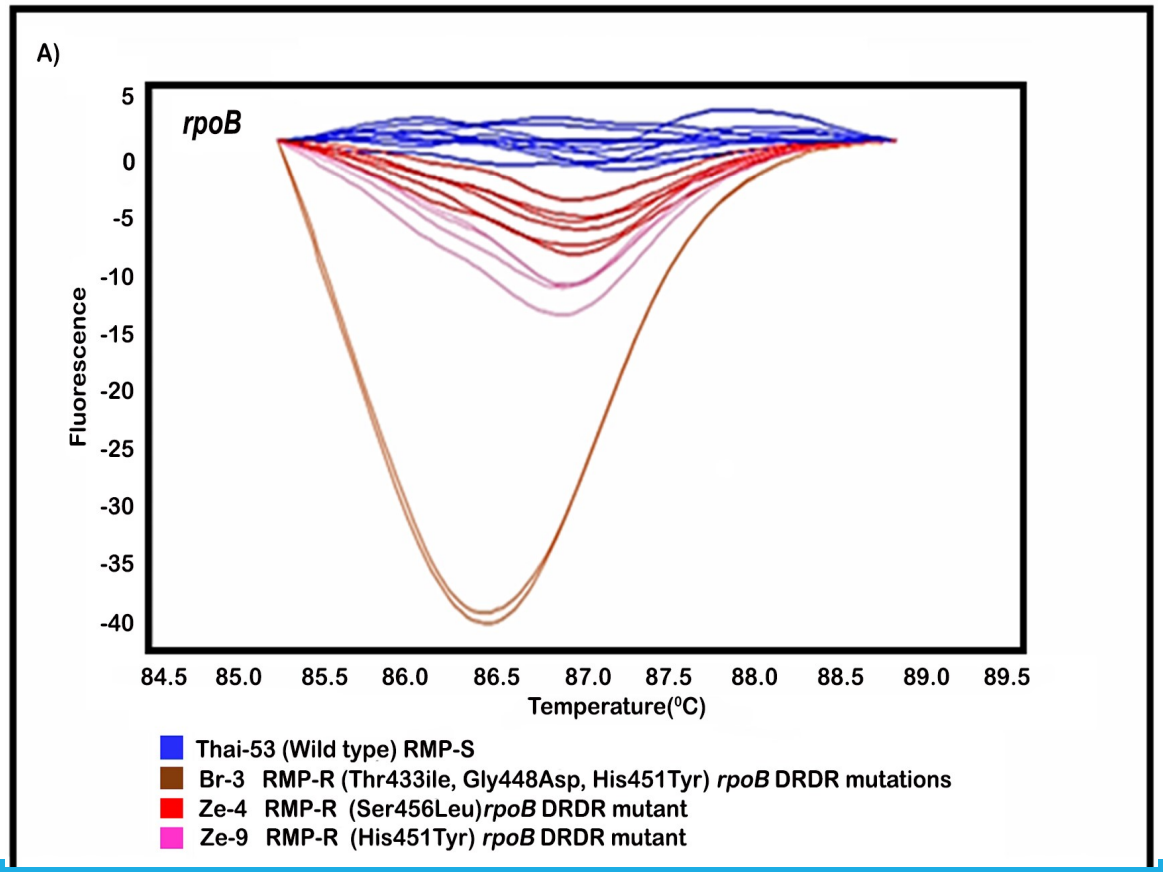
Amplification of the DRDRs of *folP1*, *rpoB* and *gyrA*

Amplicons subjected to high temperature

Each strand has different melting point due to specific ATCG sequence

Wild-type (drug-susceptible) and variant (drug-resistant) DRDR DNA fragments generate distinct melting curves analysed using HRM software

HRM curve

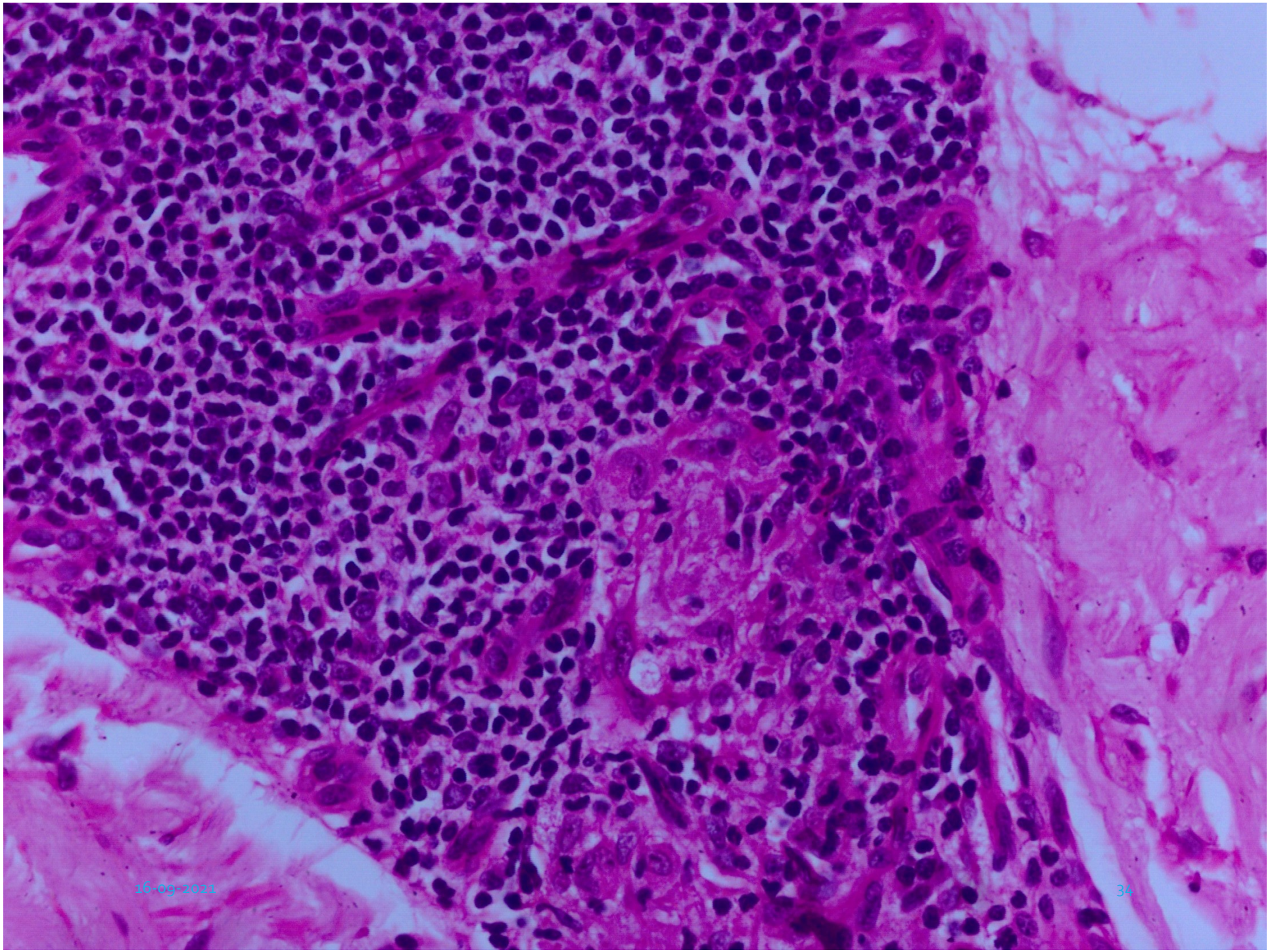

A)
rpoB
Fluorescence
5
0
-5
-10
-15
-20
-25
-30
-35
-40
84.5
85.0
85.5
86.0
86.5
87.0
87.5
88.0
88.5
89.0
89.5
Temperature(⁰C)
Thai-53 (Wild type) RMP-S
Br-3 RMP-R (Thr433ile, Gly448Asp, His451Tyr) rpoB DRDR mutations
Ze-4 RMP-R (Ser456Leu)rpoB DRDR mutant
Ze-9 RMP-R (His451Tyr) rpoB DRDR mutant

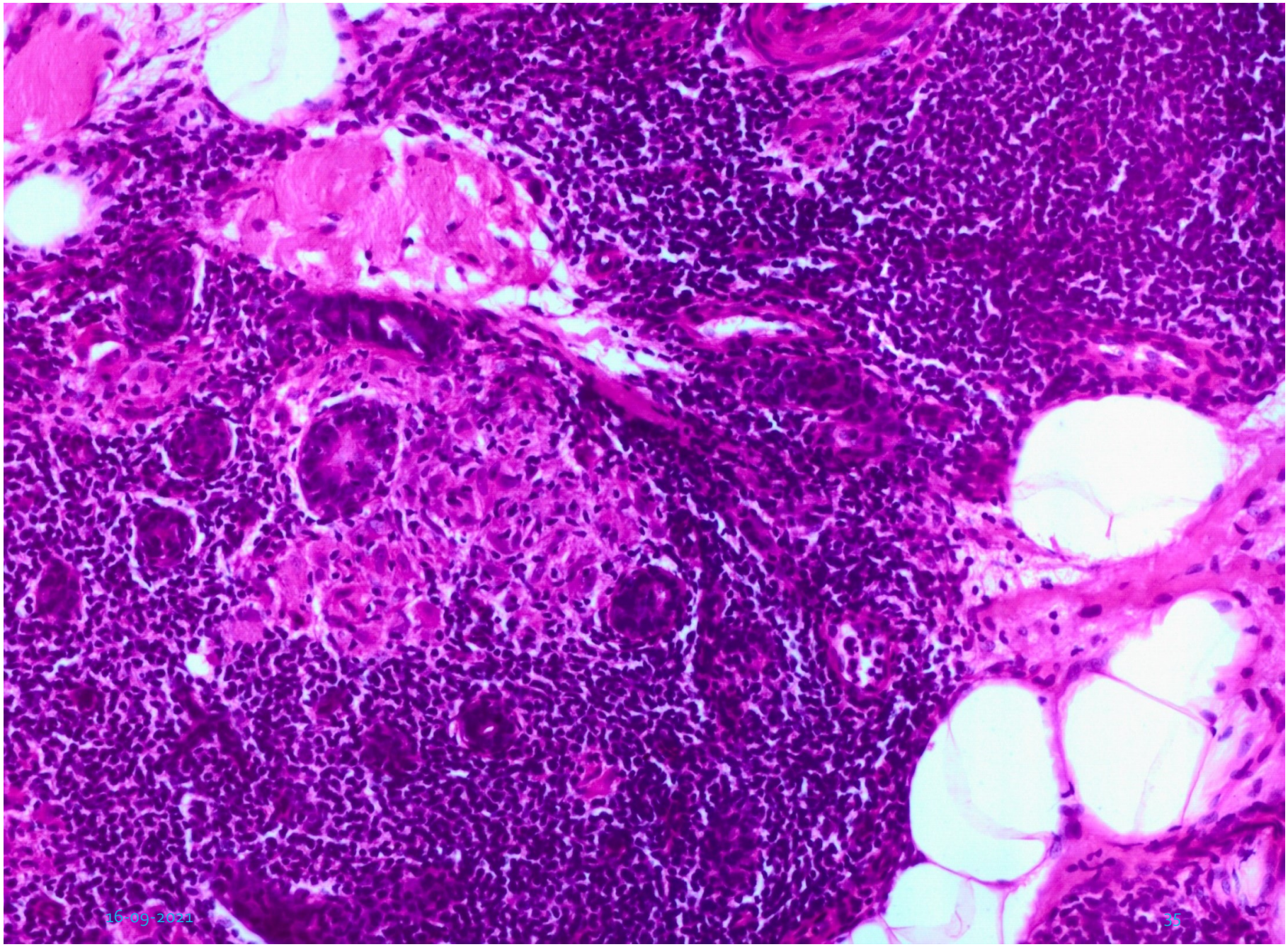
Histopathology

- Useful for classification and follow up
- Ridley Jopling classification-TT, BT, BB, BL, LL
- Hematoxylin and Eosin stain
- Fite-Faraco stain

Tuberculoid type

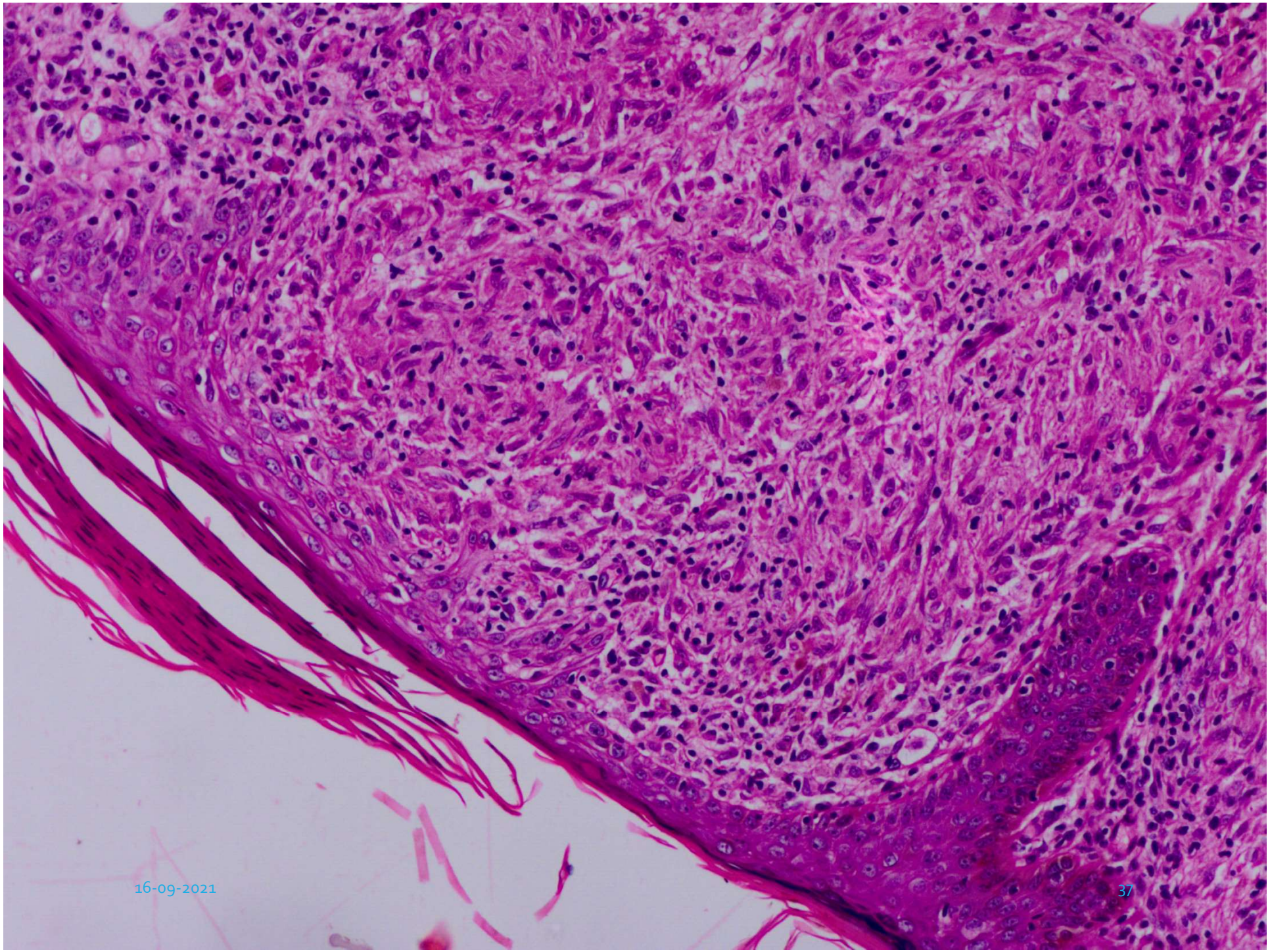
- Well formed granulomas with many lymphocytes and few epitheloid cells
- Reflects the good CMI response to *M. leprae*
- Partly destroyed dermal nerves
- Absence of caseation

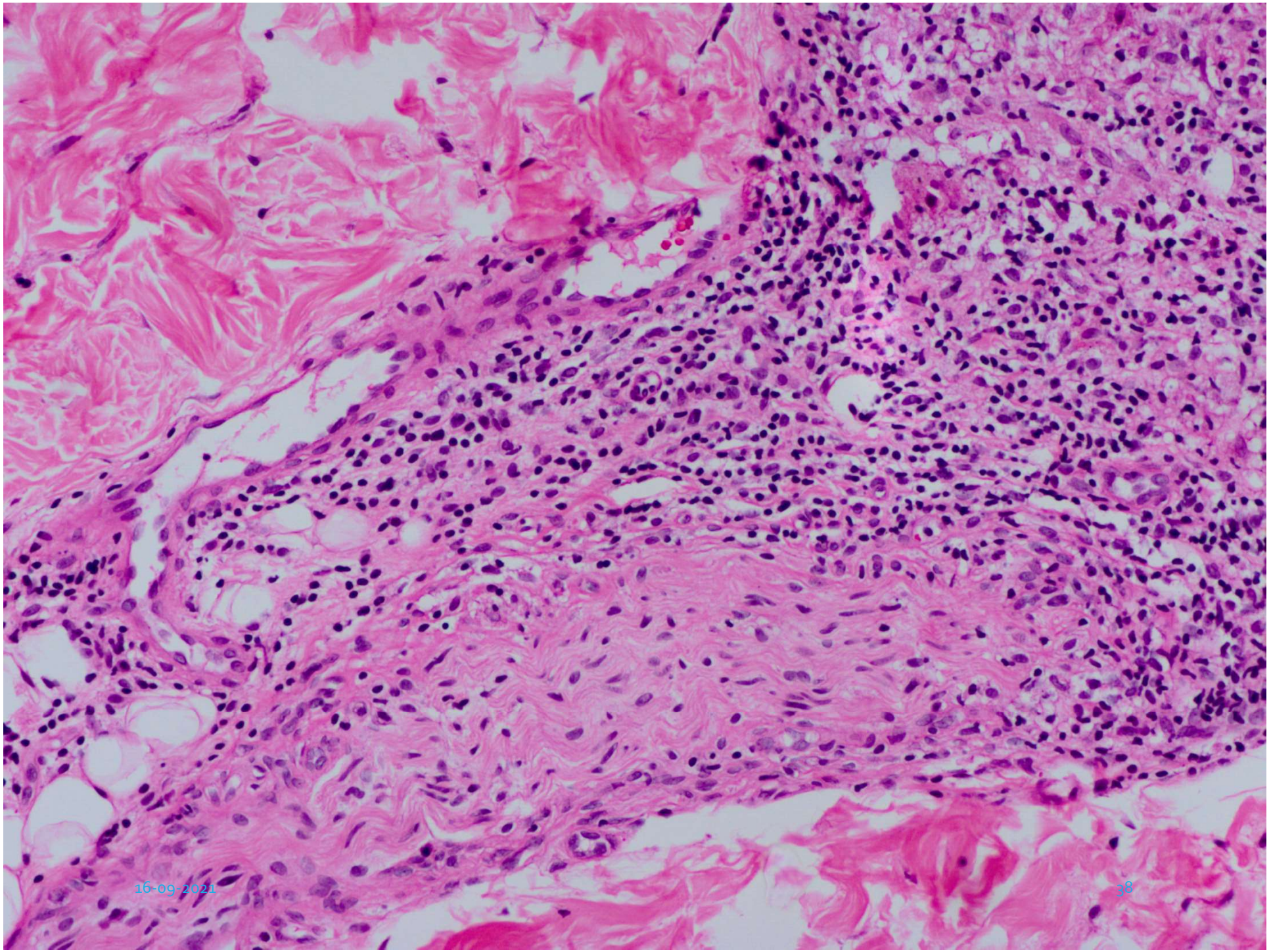




Borderline Tuberculoid type

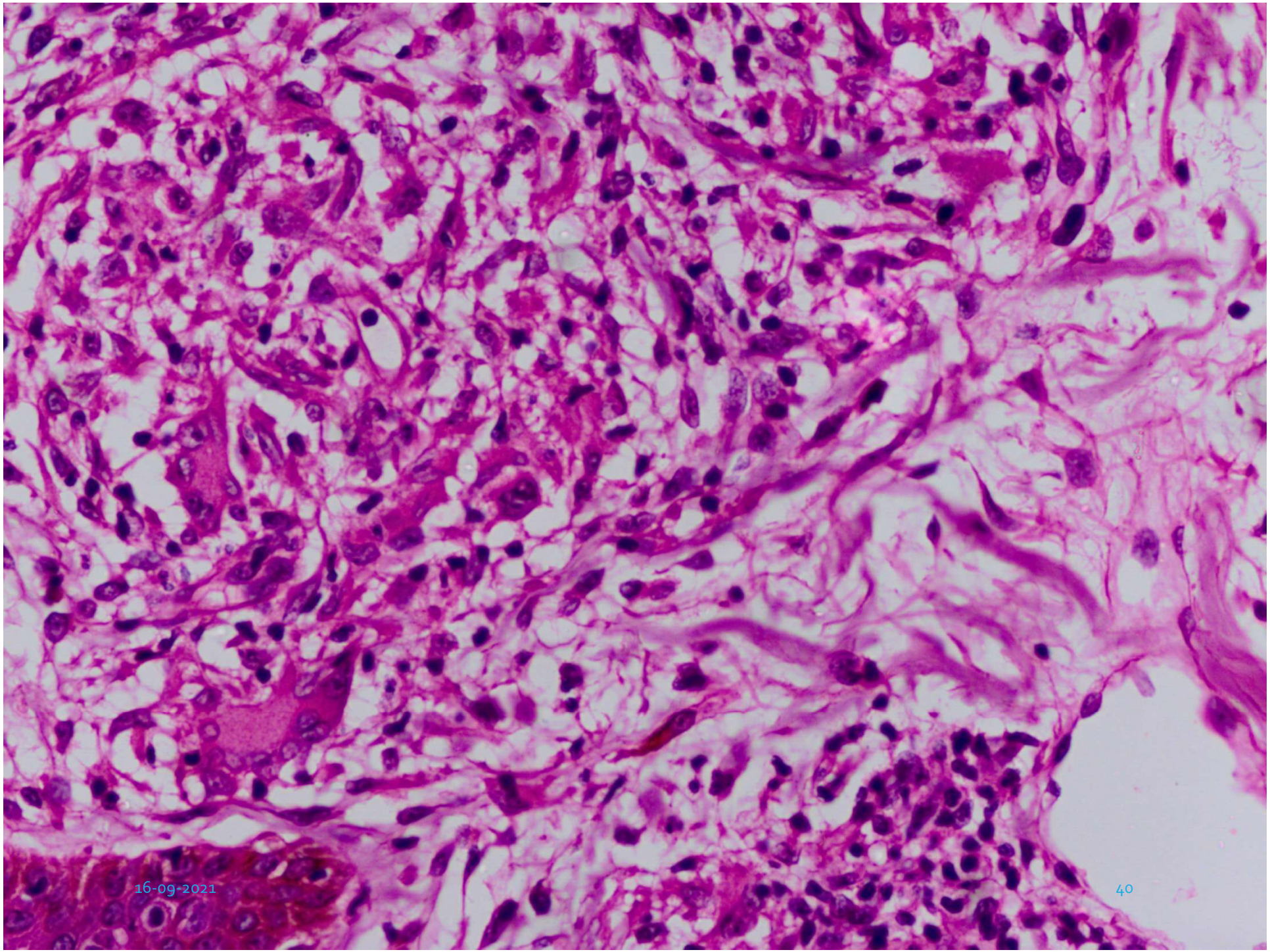
- Well to ill-defined granulomas with more epitheloid cells and less number of lymphocytes
- Dermal nerve changes
- In 20-25% cases M. leprae can be demonstrated in dermal nerves

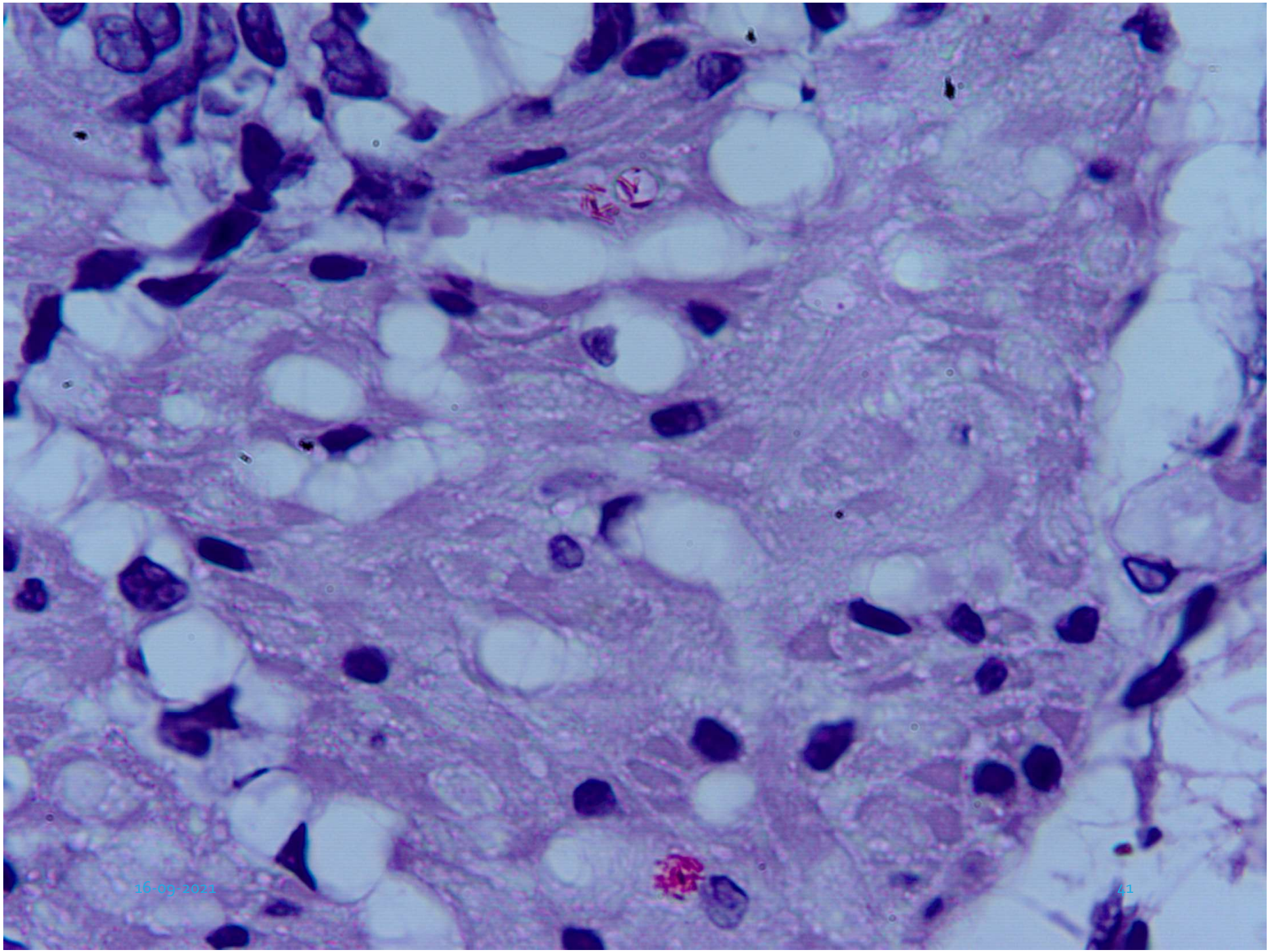




Borderline borderline type

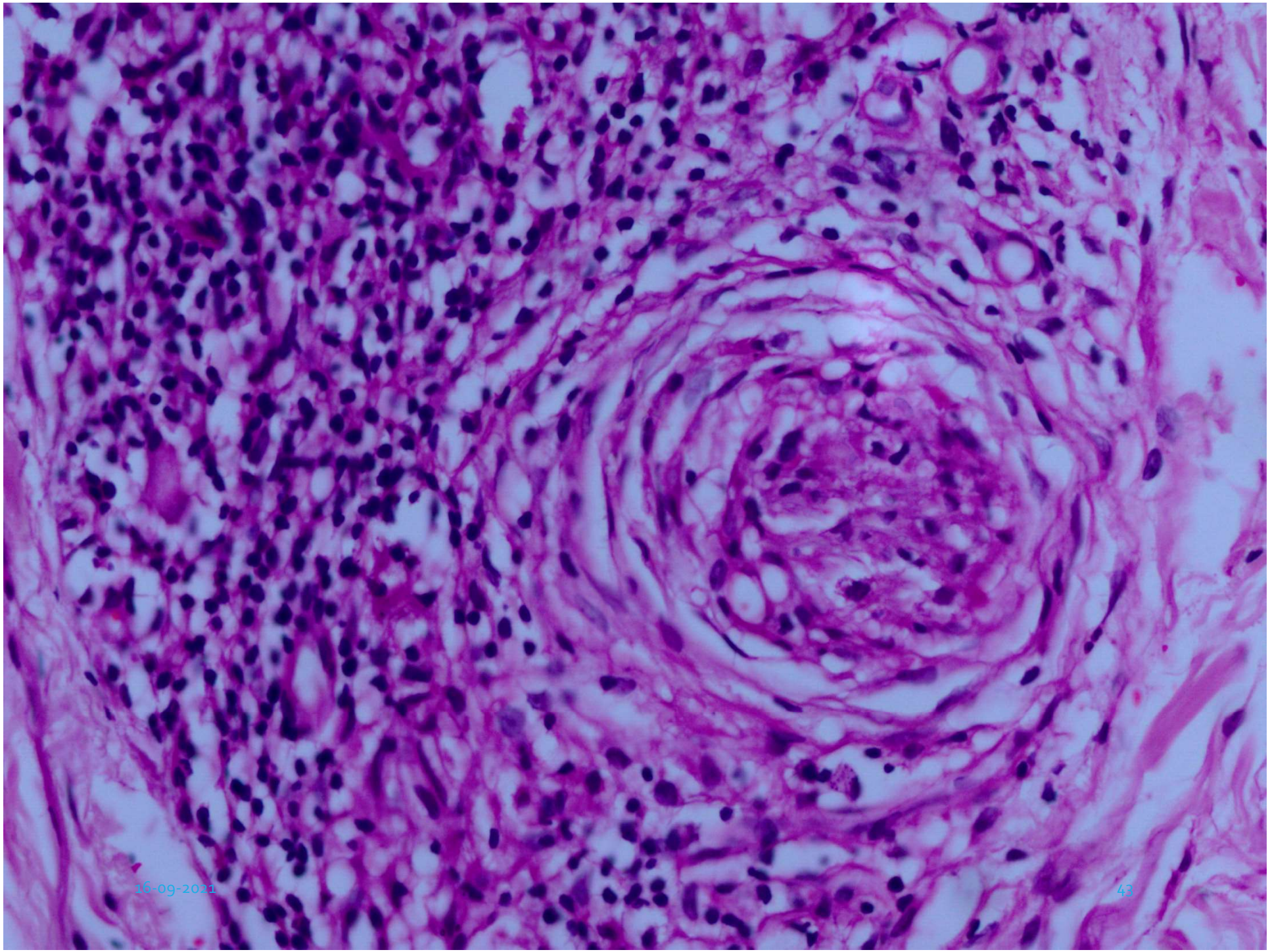
- Very unstable group
- Ill defined granulomas with uneven mixture of epitheloid cells, lymphocytes and macrophages and occasional small Langhan's giant cells
- *M. leprae* can be easily seen in macrophages

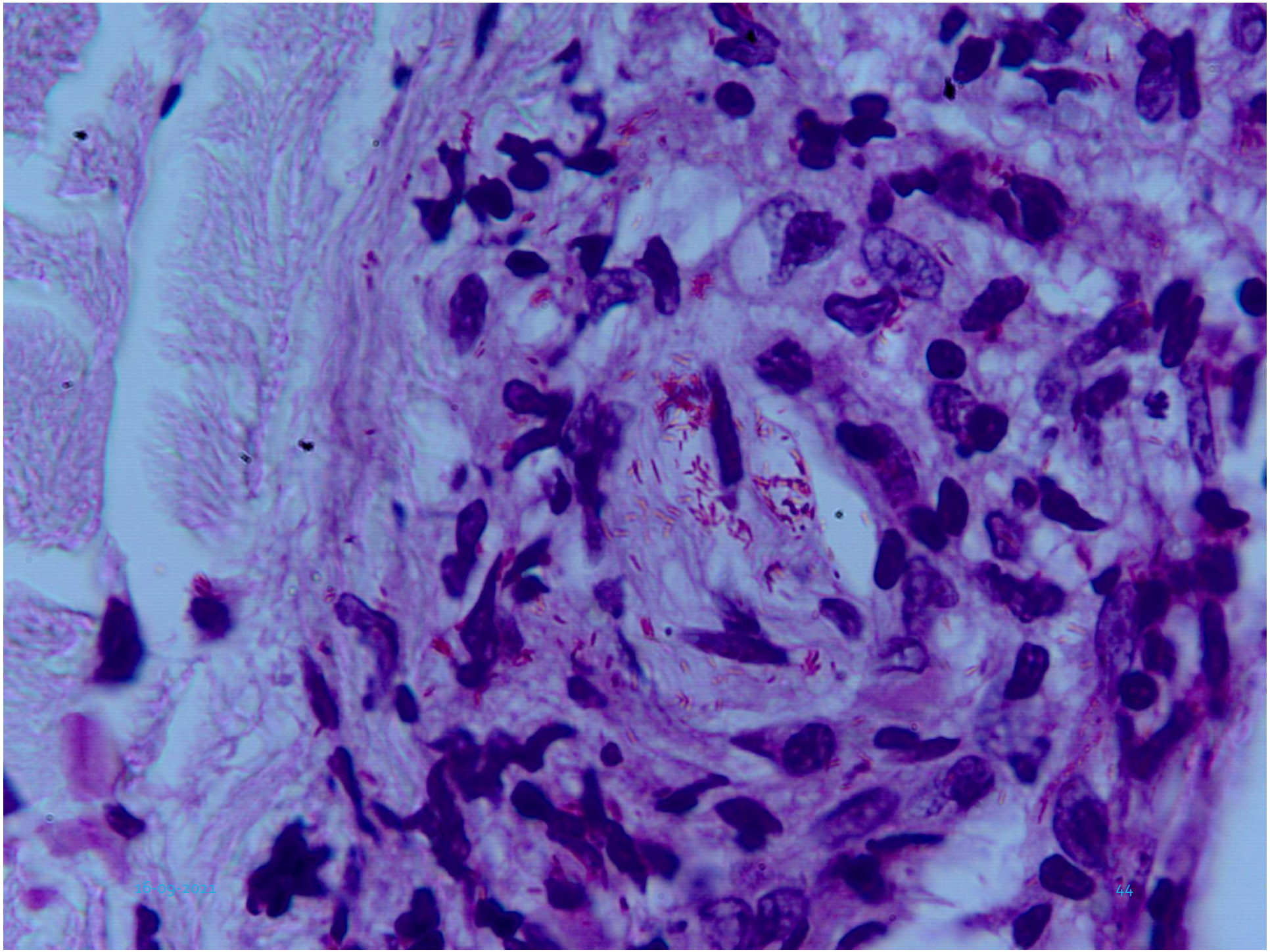




Borderline lepromatous type

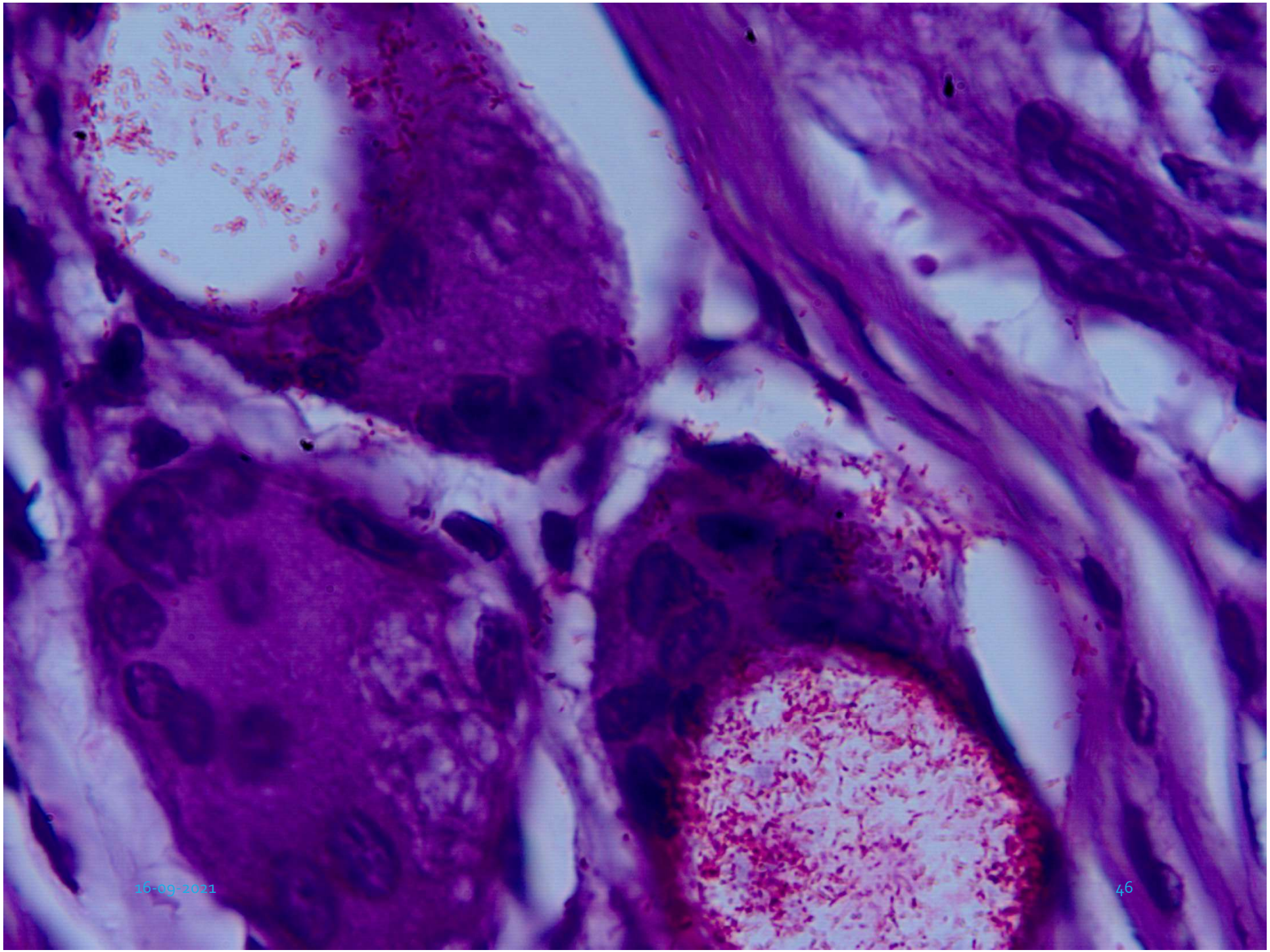
- Diffuse granulomatous inflammation consisting:
  - ✓Foamy macrophages
  - ✓Lymphocytes -less
  - ✓Very few epitheloid cells in some cases
- Dermal nerves show characteristic onion peal like epineurial thickening
- Many *M. leprae* will be seen

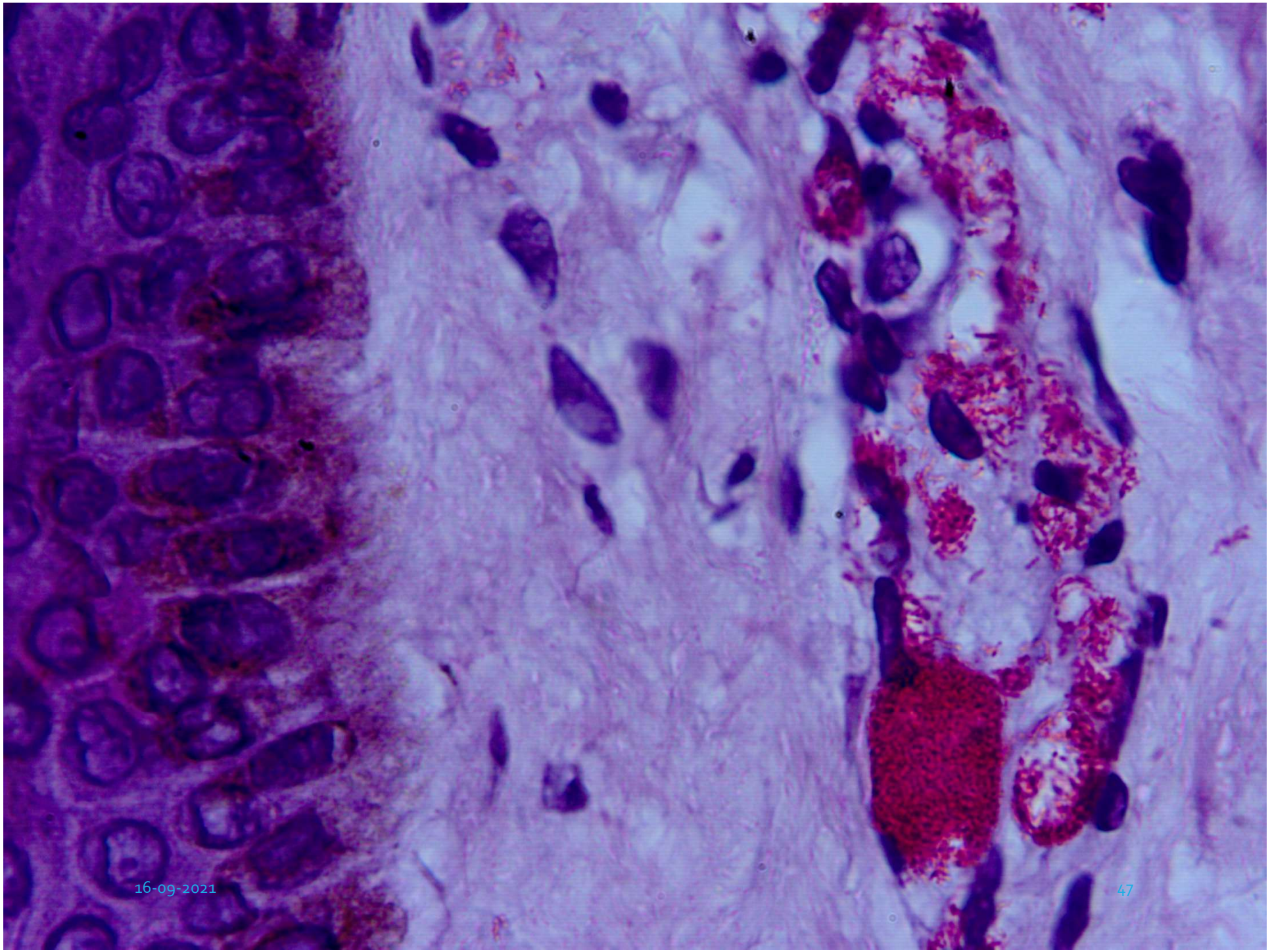


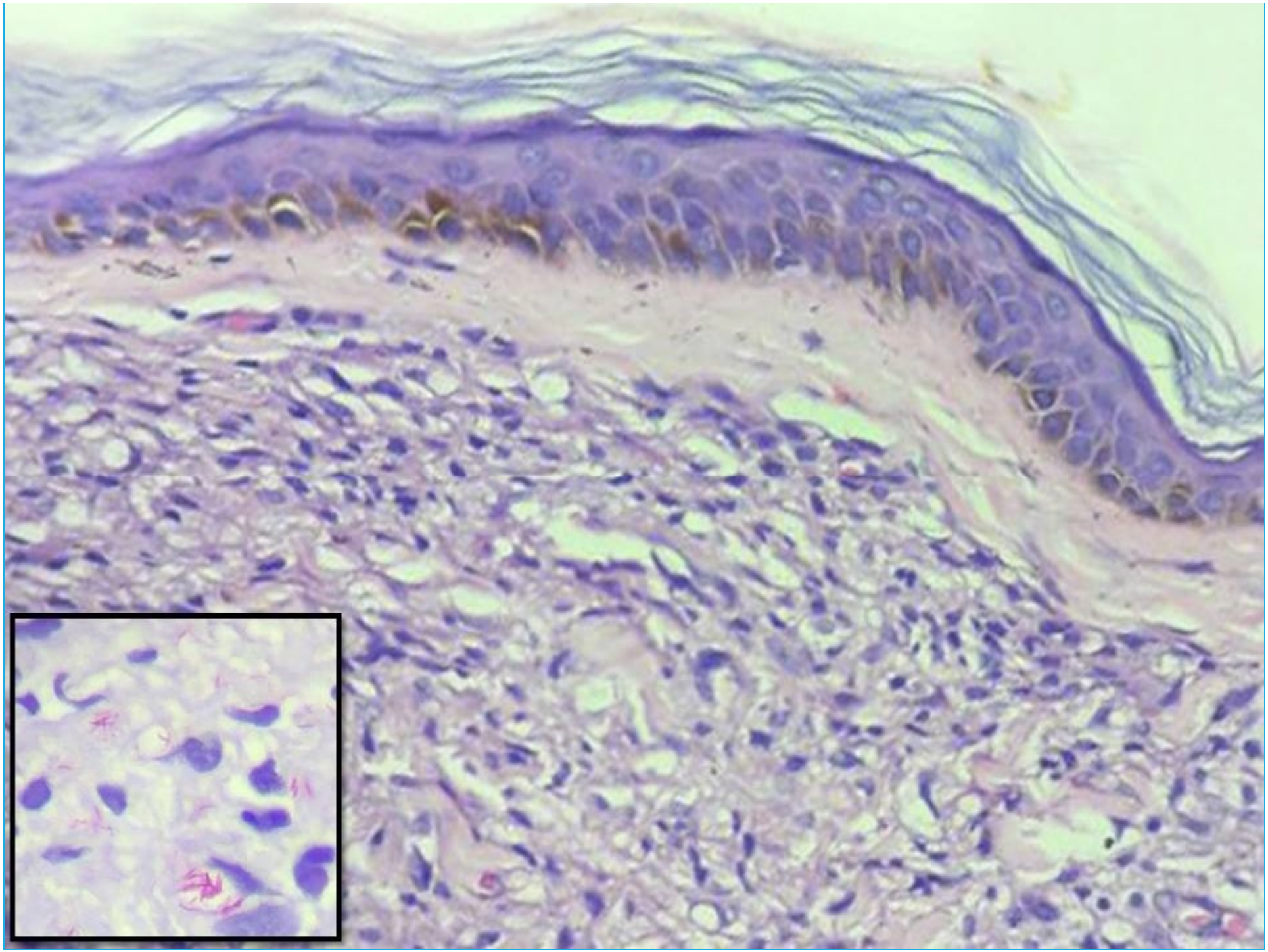


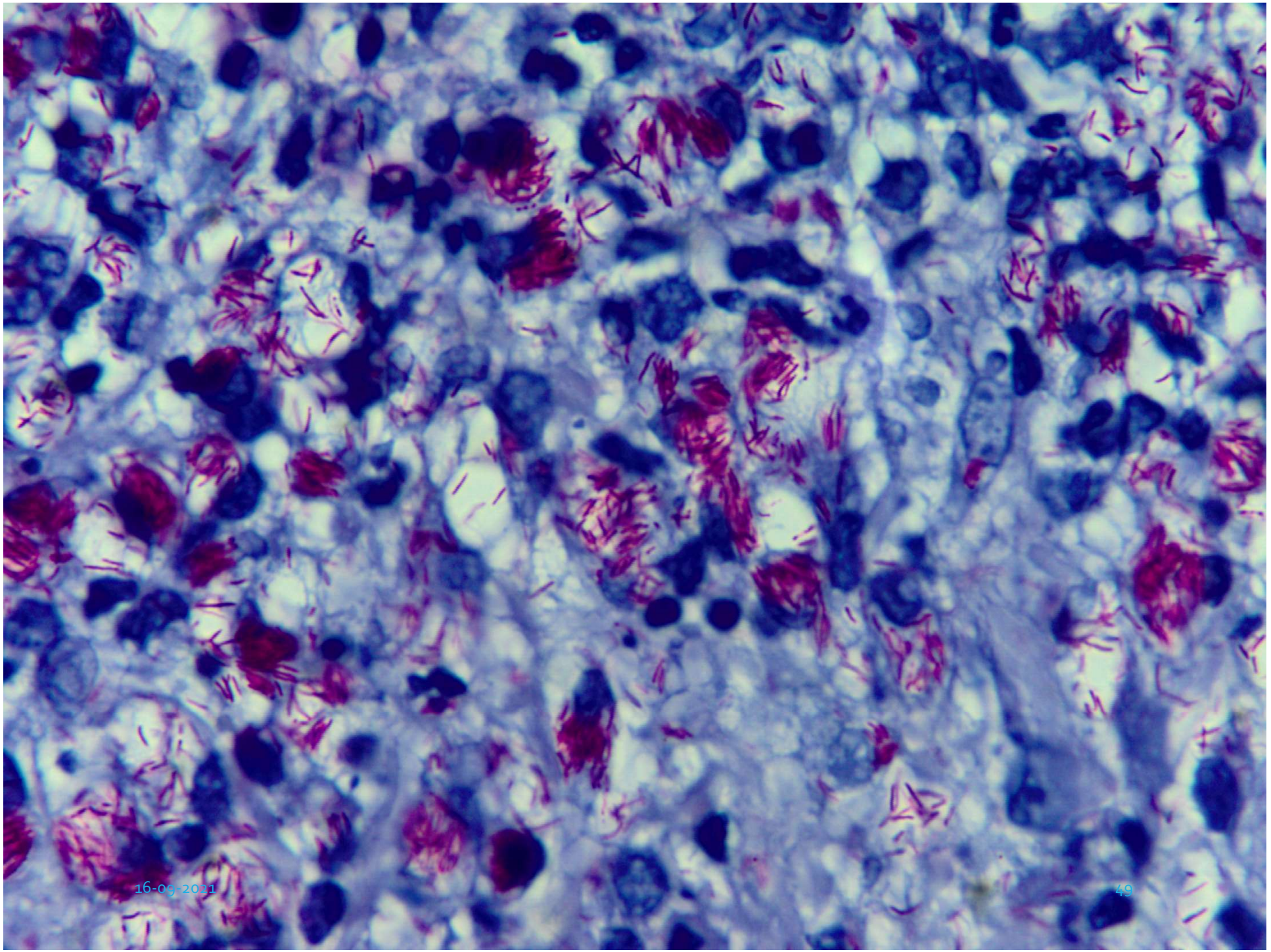
Lepromatous leprosy

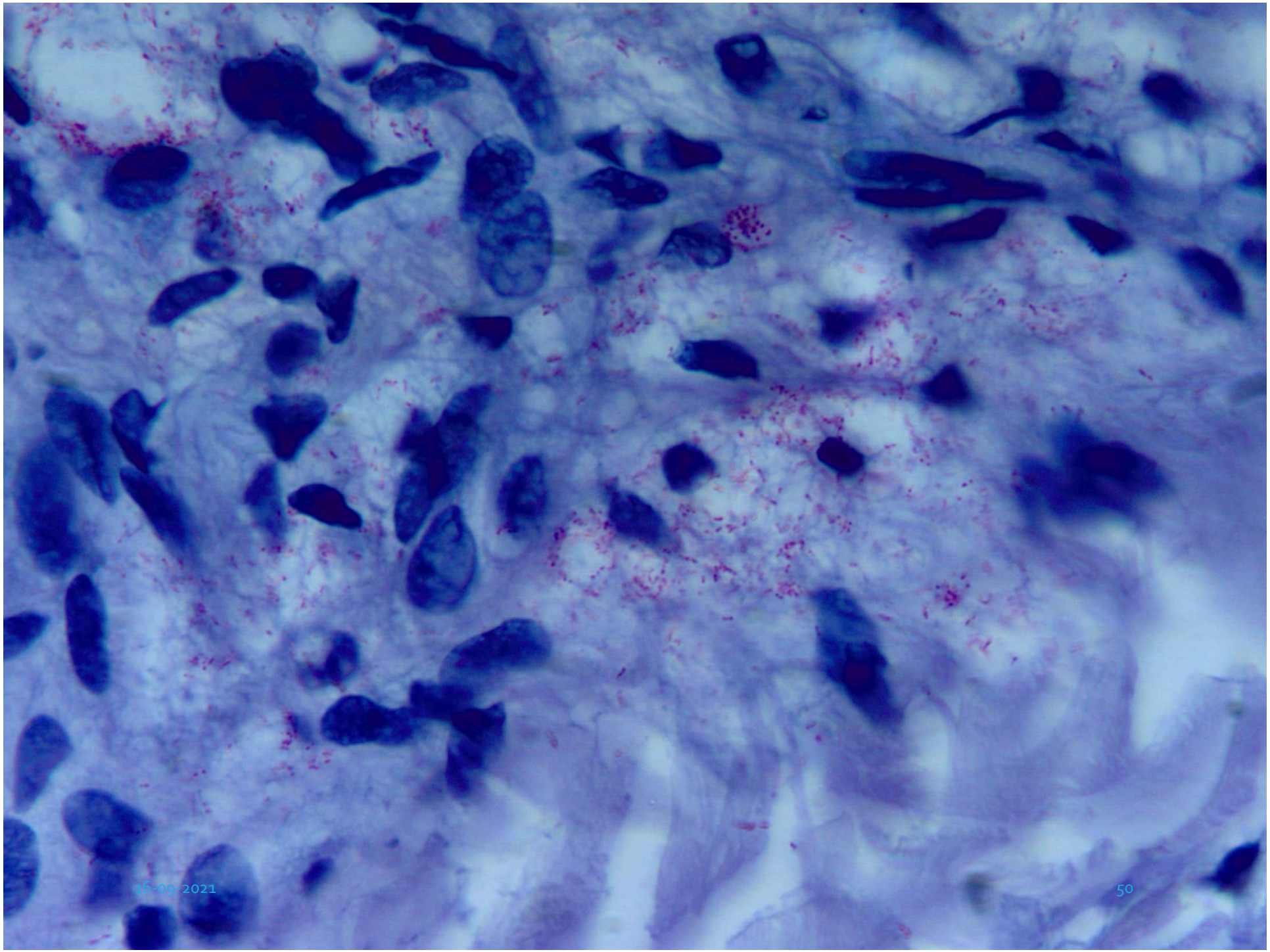
- Foamy macrophages around blood vessels and along neuro-vascular bundles are seen
- Thin atrophic epidermis with grenz zone
- Few lymphocytes, numerous solid stained bacilli
- After treatment, all solid bacilli are fragmented into granular forms.

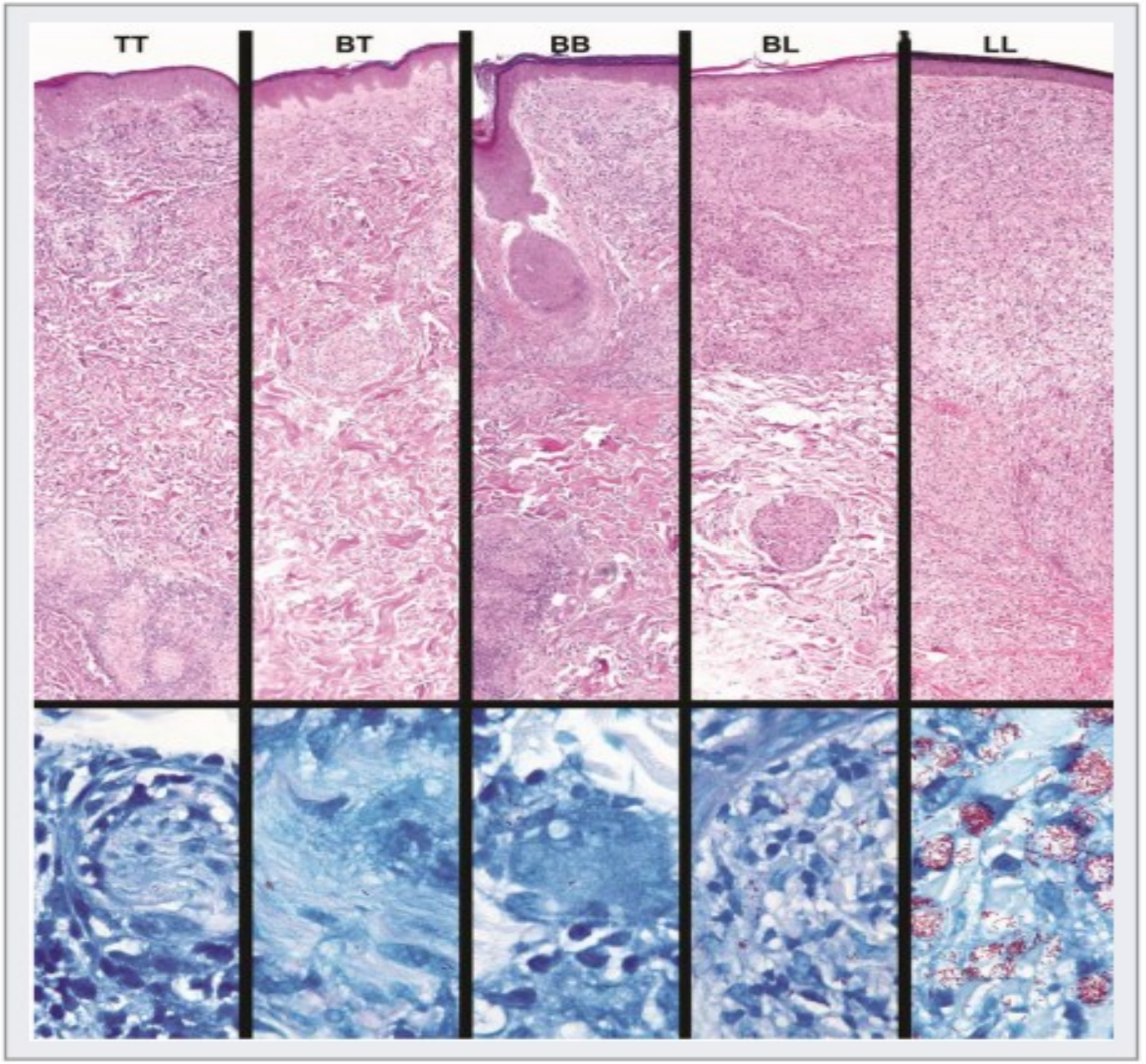











TT
BT
BB
BL
LL

Take home

Molecular biology

- Diagnosis and antimicrobial resistance detection
- Quantification
- Viability assay
- High resolution melt analysis

Histopathology

- Lymphocytes
- Epitheloid cells
- Langhans giant cells
- Foamy macrophages
- M. leprae bacilli

Thank you