Laboratory Diagnosis of Bacterial Keratitis in Alexandria Main University Hospital

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Abstract

INTRODUCTION: Blindness is and, apparently always has been, a problem in Egypt. Corneal blindness is a major public health problem worldwide according to the World Health Organization (WHO), which estimated that in every year, about 1.5–2.0 million new cases of monocular blindness in developing countries is secondary to corneal ulceration. Bacterial keratitis, because of its high incidence and potential complications, is one of the most threatening ocular infectious pathologies that can lead to severe visual disability or corneal perforations. To help avoiding the specific therapy risks of disease progression and the microbiological investigations being incomplete or misleading, other organisms as virus, fungi, and *Acanthamoeba* should be considered.

OBJECTIVES: To isolate and identify different bacterial agents causing keratitis and identify factors associated with bacterial keratitis.

SUBJECTS AND METHODS: This cross-sectional study was carried out to identify causative pathogens and to determine the demographic characteristics, predisposing factors of keratitis (corneal ulcer) and presenting at tertiary care hospitals in Alexandria, Egypt. A total of 100 cases were examined, samples (corneal swab and scrapings) were collected from clinically diagnosed corneal ulcer patients attending Ophthalmology outpatient clinic of Alexandria Main University Hospital from August, 2014 to May, 2015. Samples were processed by corneal smear microscopy (potassium hydroxide and Gram stains) and culture examination (5% sheep blood agar, sheep blood chocolate agar, Sabouraud dextrose agar and brain heart infusion).

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Guidelines

Media preparation and test performance [4]

Blood agar

It was prepared by dissolving the appropriate weight of the dehydrated powder in the appropriate volume of distilled water, as recommended by the manufacturer. It was heated to boiling, and then sterilized by autoclaving at 121°C for 15 minutes, left to cool to about 45°C before adding 5-7% of sterile blood. It was mixed thoroughly and poured aseptically into sterile petri-dishes then allowed to

solidify.

MacConkey’s agar

It was prepared by the same way as blood agar, except it was left to cool to 55°C after the sterilization process and then poured aseptically into sterile plates.

Xylose lysine deoxychocolate agar (XLD)

It was prepared by dissolving the appropriate weight of the dehydrated powder in the appropriate volume of distilled water and heated with frequent agitations until medium boiled but without overheating. The prepared medium was transferred immediately to a water bath at 50°C. As soon as the medium cooled, it was poured into plated. It was important not to prepare large volumes to avoid prolonged heating.

Acetamide Agar

It was prepared by adding the following media Per Liter Purified distilled water:

- Acetamide 0 gm
- Sodium Chloride 0 gm
- Dipotassium Phosphate (K_2HPO_4) 39 gm
- Monopotassium Phosphate ($KHPO_4$) 73 gm
- Magnesium Sulphate ($MgSO_4$ $7H_2O$) 5 gm
- Phenol red 012 gm
- Bacteriological Agar 15 gm

Brain heart infusion broth

It was prepared by dissolving the indicated weight of the dehydrated medium in the appropriate volume of distilled water by boiling. The prepared medium was mixed, distributed into final containers and Sterilized by autoclaving at 121°C for 15 minutes and then left to cool to approximately 45°C and aseptically poured into sterile tubes.

Nutrient agar

It was prepared by dissolving the indicated weight of the dehydrated medium in appropriate volume of distilled water by boiling. The prepared medium was autoclaved at 121°C for 15 minutes, then left to cool to 45°C, poured aseptically into sterile petri-dishes and allowed to solidify.

Sabouraud’s dextrose agar

It was prepared by dissolving the indicated weight of the dehydrated medium in appropriate volume of distilled water by boiling, with a final pH of 5.6 ± 0.2. Chloramphenicol is dissolved in 5 ml of 95% ethanol and then added to the heated agar before autoclaving. The prepared medium was sterilized by autoclaving at 121°C for 15 minutes, then left to cool to approximately 45°C and aseptically poured into sterile petri-dishes that were allowed to set and solidify.

Tryptone soya agar

It was prepared by dissolving the appropriate weight of the dehydrated powder of tryptone soya broth in the appropriate volume of distilled water and distributed into final containers where 0.5% of bacteriological agar was added. Sterilization carried out by autoclaving at 121°C for 15 minutes., left to cool to about 45°C, aseptically poured into sterile plates, and then allowed to solidify.

Mueller-Hinton agar

As blood agar, it was prepared by dissolving the indicated weight of the dehydrated medium into the indicated volume of distilled water by boiling, left to cool to about 45°C, aseptically poured into sterile plates, and then allowed to solidify.

Mannitol salt agar

Principle

Mannitol salt agar is a selective and differential medium used for the identification and isolation of staphylococci. The high sodium concentration (7.5%) incorporated in this medium inhibits the growth of other bacterial organisms. *S. aureus is* able to ferment mannitol into acid, which is detected by the phenol red indicator.

Procedure

Mannitol salt agar plates were inoculated with the tested organism and incubated at 35-37°C for 48 hours (up to 72 hours).

Result

1. aureus grew on mannitol salt agar and produced colonies surrounded by yellow zones (mannitol fermentation alone is not used for presumptive identification of *S. aureus*).

Catalase test

Principle

This test demonstrates the ability of the organism to produce catalase enzyme, which breaks down hydrogen peroxide into water and oxygen bubbles.

Procedure

A portion of a bacterial colony from a fresh culture was placed on a clean glass slide using a wooden toothpick and a drop of hydrogen peroxide (3%) was added.

Result

A positive result was indicated by the formation of bubbles.

Coagulase test

Principle

This test demonstrates the ability of the organism to produce coagulase enzyme which reacts with fibrinogen in human plasma and forms clumps.

Procedure

- Slide coagulase test

A drop of rabbit plasma was placed onto one side of a dry, clean slide and a drop of distilled water was placed on the other side. A portion of an isolated colony was emulsified into each drop till forming a smooth suspension. The results were recorded after 5-10 seconds.

- Tube coagulase test

Few colonies were emulsified in 0.5 mL of rabbit plasma till forming a smooth suspension. The results were recorded after 4 hours incubation at 35^{o}C, and if negative the tube was further incubated at room temperature for 24 hours.

Result

- Slide coagulase: The test was considered positive when a coarse clumping became visible within 5-10 seconds in the plasma drop and no clumping in the water drop.
- Tube coagulase: Positive result was indicated by the formation of a clot of any size.

Oxidase test

Principle

This test determines the ability of an organism to produce oxidase enzyme that oxidizes the test substrate (tetramethyl-*p*-phenylenediamine dihydrochloride) into a colored end product (indophenol)

Procedure

A commercially available filter papers impregnated with the test substrate was inoculated with a portion of a colony (young culture) by using a wooden stick. The result was observed within only 10 seconds.

Result

A positive reaction was indicated by the production of dark blue to purple color within 5-10 seconds, while a negative reaction was recorded when there is no color.

Indole test

Principle

This test demonstrates the ability of the organism to split tryptophane amino acid and produce indole.

Procedure

An isolated colony of the suspected organism was used to inoculate a tube of tryptophane broth. The

broth was incubated at 37°C for 48 hours in ambient air. Then 1 mL of xylene was added, shaken well, and then left for a few minutes till the xylene raised to the surface. Then 0.5 mL of Ehrlich's reagent was gently added down the sides of the tube, so a ring was formed between the xylene layer and the aqueous layer.

Result

A positive result was recorded when a brilliant red ring was formed just below the xylene layer.

Methyl red-Voges Proskauer tests (MR-VP)

Principle

- Methyl red test: This test determines the ability of the organism to ferment glucose and to produce and maintain a stable acid product
- Voges-Proskauer test: This test determines the ability of the organism to ferment glucose and produce acetyl methyl carbinol (acetoin) (i.e.: neutral end product)

Procedure

An isolated colony (from a culture that was incubated for at least 48 hours at 37°C) was used to inoculate a tube of MR-VP medium. For MR test, 5 drops of MR-reagent was added, and the result was recorded right away. For VP test, 6 drops of solution A (α-naphthol) and 2 drops of solution B (potassium hydroxide) were added to 1 mL of MR-VP broth, and the result was observed within 5 minutes.

Result

- For MR test, a positive result was indicated by the production of a bright red color, while yellow color formation was recorded as a negative result.
- For VP test, a positive result was recorded when a bright orange-red color was produced at the surface within 5 minutes and intensified within 30 minutes, while a negative test appeared as yellow color.

Motility test

Principle

This test determines if an organism is motile or not.

Procedure

An isolated colony of a young culture was inoculated into a semisolid agar tube by using a straight needle and stabbing the medium once in the center to only half of the medium depth. The tube was incubated at 37°C and was examined daily for up to 7 days.

Result

Motile organisms were able to produce a hazy diffuse growth that is spread through the medium from the site of inoculation, while non-motile organisms grew confined to the stab-line.

Triple sugar iron test (TSI)

Principle

TSI is used to determine whether a Gram-negative rod is able to ferment glucose and lactose or sucrose in order to produce acid and hydrogen sulfide (H_2S). The medium contains two indicators; phenol red for acid formation and ferrous sulfate for H_2S formation. When glucose is fermented the whole medium becomes acidic (yellow color) within 8-12 hours. After 18-24 hours, the butt remains acidic due to the anaerobic fermentation of glucose, while the slant reverts to the alkaline state (red color) due to the oxidation of the fermentation products (peptones into alkaline amines) and the formation of carbon dioxide and water under aerobic conditions of the slant.

Procedure

The medium (TSI) was inoculated by an isolated colony using a straight needle. The needle was stabbed once through the center till the end of the medium (without reaching the bottom) and then was used to streak the surface of the agar slant. It was then incubated at 35°C for 18-24 hours in ambient air. The results were not read beyond 24 hours of incubation.

Result

The results were recorded as the following:

- Alkaline slant/no change in the butt (K/K): (i.e. red slant/red butt) indicated that the organism is glucose, sucrose, and lactose non-fermenter.
- Alkaline slant/acidic butt (K/A): (i.e. red slant/yellow butt) indicated that the organism is only glucose-fermenter.
- Acidic slant/acidic butt (A/A): (i.e. yellow slant/yellow butt) indicated that the organism is glucose, sucrose and/or lactose fermenter.

The production of gas (carbon dioxide and hydrogen) was indicated by the presence of bubbles and/or cracks in the agar. The H_2S production was indicated by blackening of the medium butt.

Urease test

Principle

This test determines the ability of an organism to produce urease enzyme, which hydrolyzes urea into ammonia and carbon dioxide.

Procedure

The surface of urea agar slant was inoculated with an isolated colony of the suspected organism and then incubated at 35°C for 48 hours up to 7 days in ambient air.

Result

A positive result was recorded when the light orange color changed into magenta color, while a

negative result was indicated when there was no color change.

Citrate utilization test

Principle

This test is used to determine the organism's ability to utilize citrate as its sole source of carbon, so change the color of the test indicator from green to blue.

Procedure

The test was performed by streaking the slant of Simmon's citrate agar with an isolated colony of the suspected organism, and then incubating the tube at 37°C for 24 hours (up to 7 days).

Result

A positive result was indicated by the growth on the surface and/or change in the medium color from green to blue.

Before start

An information sheet for each patient was filled, data collected included personal data (name, age, sex, date of examination, occupation. etc.) and clinical data including (history of trauma, duration of symptoms, predisposing ocular conditions such as use of contact lens. etc.).

APPENDIX V

CORNEAL SCRAPING EXAMINATION, CULTURE & SENSITIVITY

Patient #:

If Gram positive organisms:

	TEST	PRINCIPLE / PROCEDURE	RESULT
	DAY ONE		
DAY 1	1. Direct Microscopic examination	· Swab of cornea on two slides.	_
	2. Culture ü On Blood agar (Solid enriched media)	· Culture on blood agar from corneal sample using Komura spatula. · Streak the agar in a C-shaped manner, streaking without burning. · Incubate at 37 ºC 24 hrs. · Incubate at 37 ºC in CO_2 24 hrs.	DAY 2 Colonies: Size: _ Shape:
	ü On Chocolate agar (non-selective, enriched growth medium) ü On Sabaraud dextrose agar (SDA)	· Method: Streak the agar in a C-shaped manner, streaking without burning. · Incubate at 20-25 ºC for 5 days.	_ _
	3. Culture on Brain Heart Infusion Broth (BHIB)	· It is a liquid medium rich in nutrients. · Incubate at 37 ºC 24 hrs.	
	DAY TWO		
DAY 2	1. Indirect Microscopic examination	· A loop full from BHIB or an isolated colony from the blood/chocolate agar.	_
	2. Culture - On Blood agar (Solid enriched media)	· Culture on blood agar from Brain heart infusion broth. · Method: primary inoculum, the next inoculum with burning loops, end with tail. · Incubate at 37 ºC 24 hrs.	DAY 3
	- On Chocolate agar (non-selective, enriched growth medium)	· Culture on chocolate agar from Brain heart infusion broth. · Incubate at 37 ºC 24 hrs.	_
	- On Sabaraud dextrose agar (SDA)	· Method: primary inoculum, the next inoculum with burning loops, end with tail · Incubate at 20-25 ºC for 5 days.	_
DAY THREE			

	1. Indirect Microscopic examination	· Isolated Colony from cultured plate.	-
	2. Catalase test	· Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H_2O_2.	-
DAY 3	3. Coagulase test	v ***Slide Coagulase Test Procedure(done to detect bound coagulase or clumping factor)*** v ***Tube Coagulase Test Procedure (****done to detect free coagulase)* • Incubate tube at 35°C in ambient air for 4 hours.	-
	4. Culture on Mannitol salt agar (If suspect staphylococcus spp.)	· Incubate at 37 ºC 24 hrs.	DAY 4
	5. Novobiocin Susceptibility	· Novobiocin is an amino-coumarin antibiotic which can be used to differentiate *S. aureus* from some CoNS.	DAY 4

If Gram negative organisms:

DAY TWO

Day 2	1. Microscopic examination	· Isolated Colony from cultured plate/ BHIB, Heat fix. · Gram stain; Then examine by oil immersion lens (100x).	
	2. Culture ü On Blood agar (Solid enriched media) ü On Chocolate agar (non-selective, enriched growth medium) ü On MacConkey's agar (Selective & Differential media) ü On Nutrient agar ü On Cetrimide agar(Selective & Differential media)[If suspect pseudomonas spp.] ü On Sabaraud dextrose agar (SDA)	· Culture on blood agar from Brain heart infusion broth. · Streak the agar with burning. · Incubate at 37 ºC 24 hrs. · Method: primary inoculum, the next inoculum with burning loops, end with tail · Incubate at 37 ºC in CO_2 24 hrs. · Contain inhibitor substances (bile salts, crystal violet) · PH indicator Neutral red (red in acid) · Fermentable sugar is Lactose. · Method: primary inoculum, the next inoculum with burning loops, end with tail · Incubate at 37 ºC 24 hrs. · Nutrient agar is a general purpose medium supporting growth of a wide range of non-fastidious organisms. · Plates are usually inoculated by streak or spread method from non-selective medium or directly from the specimen. Incubate the plates at 35- · 37°C for up to 48 hours. · Method: primary inoculum, the next inoculum with burning loops, end with tail. · Incubate at 20-25 ºC for 5 days.	DAY 3 DAY 4 -5 DAY 5-7
	3. Triple Sugar Agar test (TSA) DONE ON GRAM NEGATIVE RODS ONLY (enteric pathogens) ü Inoculate by: Stab + Streak the slant) ü Incubate at 37 ºC 24 hrs.	Test the ability of the organism to : 1. Ferment glucose (0.1 %-constitutive enzyme); 2. Utilize lactose – sucrose (1% each - inducible enzymes). 3. Anaerobic respiratory process that use Sulfur as final electron acceptor to produce hydrogen sulfide (Black precipitation). 4. Protein [Aerobic process –upper slant if deaminate become red color]. 5. Indicator: phenol red (yellow in acid). 6. Sulfur source: a) Organic amino acid. b) Inorganic Ferrous sulfate.	DAY 3
	4. Oxidase test	· This test depends on the presence of cytochrome oxidase in bacteria.	
	5. Motility test	· Non biochemical test	
	6. IMViC a) Indole	· Ability of the organism to spit Indole form tryptophan amino acid by tryptophanase enzyme in tryptophane broth. ü Incubate at 37 ºC 24 hrs. ü Add 0.1 xylol (shake), add Kovac's reagent.	DAY 3
	b) Methyl Red	· Test mixed acid producers. · The bacteria maintain stable acid end products from glucose fermentation (large amount of acid from glucose fermentation that overcomes the buffering action. ü Inoculate buffered glucose broth. ü Incubate at 37 ºC 2-5 days. ü Add Methyl red reagent & shake.	DAY 4
	c) VP	Test butylene glycol producers. Test the ability of bacteria to produce NEUTRAL end products from fermentation of glucose. ü Inoculate buffered glucose broth. ü Incubate at 37 ºC 2-5 days. ü Add VP reagent – wait 15 min with open cap [Don't shake]	DAY 4
	d) Citrate	· Test the ability of the organism to utilize Citrate as sole source of carbon & energy by citritase enzyme. · Streak the slant of simmon citrate agar by inoculated loop. · Incubate at 37 ºC 24 hrs.	DAY 3
	7. Urease hydrolysis test	· Test the ability of the organism to hydrolyze urea by urease enzyme producing alkaline product. · Incubate at 37 ºC 24 hrs.	Day 3

DAY THREE

	Antibiogram		**DAY 4**
	Generic name	**Trade name**	
	Penicillin		
	1. Oxacillin (OX)		
	Cephalosporin 2nd		
	2. Cefoxitin (FOX) 2nd		
	3. Ceftriaxone (CRO) 3rd IM	Rociphin, Cefotrix, Cefaxon	
	4. Cefotaxime (CTX) 3rd	Claforan	
	5. Ceftazidime (CAZ) 3rd	Fortum	
	Glycopeptide		
	6. Vancomycin (VA)		
	Aminoglycoside		
	7. Gentamycin (CN)	Apigent , genoptic , Cidomycin	
	8. Tobramycin (TOB)	Tobralex, Tobral, Tobrin	
	9. Amikacin (AK)		
	10. Neomycin		
Day 3	**Fluoroquinolones**		
	11. Ciprofloxacin (CIP) 2nd	Ciloxan , Ciprofar , Ciprocin , Cipro	
	12. Ofloxacin (OFX) 2nd	Optifox,Oculofox,Oflox,Oflicin,Ofloxin	
	13. Norfoxacin (NOR) 2nd	OptoQ3	
	14. Lomefoxacin (LOM) 2nd	Okacon, Orchacin	
	15. Levofloxacin (LEV) 3rd	Levaquin	
	16. Moxifloxacin (MOX) 4th		
	Macrolide		
	17. Erythromycin (E)	Erythromycin	
	18. Azithromycin (AZM)	Zithromax	
	19. Clarithromycin (CLR)		
	Tetracyclin		
	20. Doxycyclin (DO)	Vibramycin	
	21. Tetracyclin (TE)		
	Bacteriostatic		
	22. Fusidic acid (FD)	Fucithalmic	
	23. Chloramphenicol (C)	Isoptofenicol	
	24. Polymyxin B (Pb)	Polyfax, Polytrin	
	25. Clindamycin (DA)		
	DAY 4 (In case of MRSE/MRSA)		**DAY 5**
	OXACILLIN RESISTANCE SCREENING AGAR BASE (ORSAB)	· Oxacillin Resistance Screening Agar Base is a nutritious and selective medium containing peptones for growth, a high salt concentration and lithium chloride to suppress non-*staphylococcal* growth with mannitol and aniline blue for the detection of mannitol fermentation.	

Materials

- brain Heart Infusion Broth Oxoid CM1135-UK by Contributed by users
- blood agar base Oxoid CM0271- UK by Contributed by users

Protocol

Step 1.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2823109/

Warnings

It was recommended to cease antibiotic prior to corneal scraping but it was hard to fulfill in this study.

Complete initial ocular examination was carried out under slit lamp biomicroscope with special attention paid to the characteristics of ulcer in terms of:

- Site, size, shape, edges, margins, floor and depth of ulcer.
- Presence or absence of hypopyon.
- Associated local risk factors such as blepharitis, dacryocystitis, dry eyes etc.

After ocular examination, topical anaesthesia “benoxinate hydrochloride” 0.4% was applied and wait 3-5 minutes for draining of anaesthetic to ensure that adequate material was obtained and to avoid perforation of the eye. Corneal scrapings were performed under strict aseptic techniques by an ophthalmologist using a flame sterilized Kimura spatula. Four different calcium-alginate sterile swabs were used to transfer the scrapped materials from the spatula blade to the prepared media. Ulcers were scraped from the leading edge and base. After taking corneal scrapings patient was advised to put one drop of the broad spectrum antibiotic and antifungal medications that included 4th generation fluroquinolones and Natamycin 5% respectively.[7, 99]