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RESEARCH ARTICLE

CULTURE AND PRESERVATION OF GONOCOCCI: AN EXPERIENCE FROM TERTIARY CARE CENTRE

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ABSTRACT

Background: Gonorrhoea is one of the most prevalent sexually transmitted infection (STI) across the world. Isolation and preservation of gonococci is important to perform antimicrobial susceptibility testing and molecular characterization. **Objective:** To evaluate the preservation of gonococci by deep freezing, chocolate GC agar slope and lyophilization. **Methods:** The study was undertaken at State level STI/RTI Laboratoryat a tertiary care hospital. Isolation of gonococci was done from suspected cases of gonorrhoea. The culture isolates of gonococci were preserved by deep freezing, chocolate GC agar slope and lyophilization. **Results:** Out of 71 suspected cases of gonorrhoea, *N. gonorrhoeae* was isolated from 22 cases (30.9%). Preservation of all 22 isolates of *N. gonorrhoeae* were attempted by 3 methods.Out of 22 gonococcal isolates preserved by deep freezing (-70^0 C), 12 (54.5%), 06 (27.2%) and 03 (13.6%) isolates were revived at 3 months, 6 months and 8 months respectively by nutrient broth - glycerol method; 10 (45.5%), 04 (18.1%) and 01 (4.5%) were revived at 3 months, 6 months and 8 months respectively by preservation media (trypticase soy broth and yeast extract). Out of 22 gonococcal isolates, 18 (81.8%), 14 (63.63%) and 06 (27.27%) isolates were revived at end of 1 month, 3 months and 6 months by subculturing gonococcal isolates from chocolate GC agar slope. **Conclusion:** Chocolate GC agar slope is preferred method for preservation of gonococcal isolates over other methods in routine microbiology laboratories as its inexpensive, less laborious, doesn't require sophisticated equipment and recovery rate of gonococcal isolates is good upto 6 months.

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INTRODUCTION

STI/ RTI rank among the top five conditions for which sexually active adults seek health care in developing countries. Gonorrhoea is a STI, which if untreated can lead to complications and the probability of coinfection with other STIs may be high as HIV transmission is facilitated. CDC estimated that approximately 1.6 million new gonococcal infections occurred in the United States in 2018, and more than half occur among young people aged 15-24.[1] Gonorrhoea is the second most reported bacterial sexually transmitted infection in the United States. [2] However, many infections are asymptomatic, so reported cases only capture a fraction of the true burden. In India, the technical guidelines advocate syndromic management of STI/ RTI at all facilities providing STI/ RTI services, in addition to utilizing minimal laboratory tests at sites where there are laboratory facilities available without compromising the timely treatment of patients[3].

However, syndromic management needs to be validated periodically by etiological diagnosis and in addition, antimicrobial resistance for gonorrhoea, which is of global concern. Also, periodic prevalence of gonorrhoea among at-risk populations is essential to provide inputs for improved STI/ RTI services. Antimicrobial resistance in *N. gonorrhoeae* is the most significant challenge to controlling gonorrhoea. Following the spread of gonococcal fluoroquinolone resistance, the cephalosporins have been recommended for treatment of gonorrhoea.[4] The emergence of cephalosporin-resistant gonorrhoea would significantly complicate the ability of providers to treat gonorrhoea successfully, since we have few antibiotic options left.[5] It is of great importance to perform laboratory surveillance of antimicrobial resistance in *N. gonorrhoeae* in order to assess the effectiveness of locally recommended therapies. *N. gonorrhoeae* culture is the basic requisite to perform antimicrobial resistance and to evaluate suspected cases of gonorrhoea treatment failure.

[6]The GISP in the United States and GASP by WHO are sentinel surveillance systems to monitor trends in antimicrobial susceptibilities of *N.gonorrhoeae* strains.[7,8] Public health officials and healthcare providers use the data collected in these surveillance programmes to ensure that gonorrhoea is successfully treated with the right antibiotic. Only measurement of the in vitro susceptibilities of the infecting organism will provide objective information to help determine if a post treatment isolate is truly resistant to the antimicrobial agent being used to treat the infection. *N. gonorrhoeae* is a fastidious organism which grows only on few specific culture media. The growth requirements and behaviour of the gonococci are significantly different from many bacteria, necessitating modifications of common laboratory techniques. A fastidious organism, *N. gonorrhoeae* requires enriched media in a CO_2 atmosphere at 35^0C to 37^0C for growth. In addition, *N. gonorrhoeae* expresses potent autolysins whose activity increases following glucose depletion during stationary phase, leading to cell death.[9]Either isolation from clinical samples or maintenance of gonococcal cultures daily or long-term storage is a challenging task. We present here our experience of culture and preservation of gonococciat a tertiary care centre.

MATERIALS AND METHODS

Prospective cross-sectional study was conducted at State level STI/ RTI laboratory at a tertiary care hospital in Bengaluru, Karnataka from 2017-2019. Institutional ethical clearance and written informed consent from the patients were obtained. 71 patients suspected of gonococcal infection were included in the study. Specimens (urethral swab from men and endocervical swab from women) were obtained from 71 patients with symptoms of urethritis /cervical discharge attending STI clinic of our tertiary care hospital and were processed at State level STI/ RTI laboratory. Samples were subjected to Gram's stain, inoculated onto Chocolate GC agar and NYC Medium with NYC supplement, and incubated in a candle jar at 36^0C with 5% CO_2 for 24–72 h. The colonies suggestive of *N. gonorrhoeae* were presumptively identified as per standard guidelines. Preservation of all grown isolates of *N. gonorrhoeae* were attempted by 3 methods as per standard guidelines.[10]

Preservation of bacteria by deep freezing (-70^0 C) by

- **Nutrient broth plus 20% glycerol-** both the ingredients were sterilized by autoclaving at 15 psi (121^0 C) for 15 minutes and distributed in1.0 ml aliquots in small test tubes and stored at 2-8^0 C prior to use. Bacterial suspension was prepared using a heavy inoculum of bacteria and inoculated in aliquots using pipette, the suspension was mixed well and then transferred to a labelled cryo vial before placing in a freezing box at –70^0 C.
- **Trypticase Soy broth plus yeast extract-**Ingredients (Trypticase Soy Broth, Becton Dickinson 30 g, Yeast Extract 3.0 g, Agar No 2, Lab M 0.5 g, Distilled water 700 ml) were mixed and sterilized at 121^0 C for 15 mins, cooled to <50^0 C, horse serum was added and mixed. pH was adjusted to 7.5 + 0.1 and 1 ml was dispensed in sterile tubes for freezing and stored at 2- 8^0 C. Bacterial suspension was prepared using a heavy inoculum of bacteria and inoculated.

Preservation on chocolate GC agar slopes: Strains of *N.gonorrhoeae* were stored on chocolate agar slopes overlaid with paraffin oil for up to 3 months or more until a more permanent means of storage was undertaken. A fresh, pure culture of the gonococci stored was heavily inoculated onto a 3ml volume chocolate GC agar slope in a polycarbonate (plastic) screw-top Bijou (5 ml volume) bottle and incubated with the screw cap loosened for a minimum of 24 hours in a CO_2 enriched atmosphere or until visible growth was present on the agar surface. Sterile liquid paraffin was then used to completely fill the agar lope, the screw cap lid was fully tightened and the Bijou bottles slope was then stored at 35-36^0 C until the *N.gonorrhoeae* were tested or forwarded elsewhere (Figure 1)

Figure 1. Chocolate GC Agar containing Gonococcal isolate

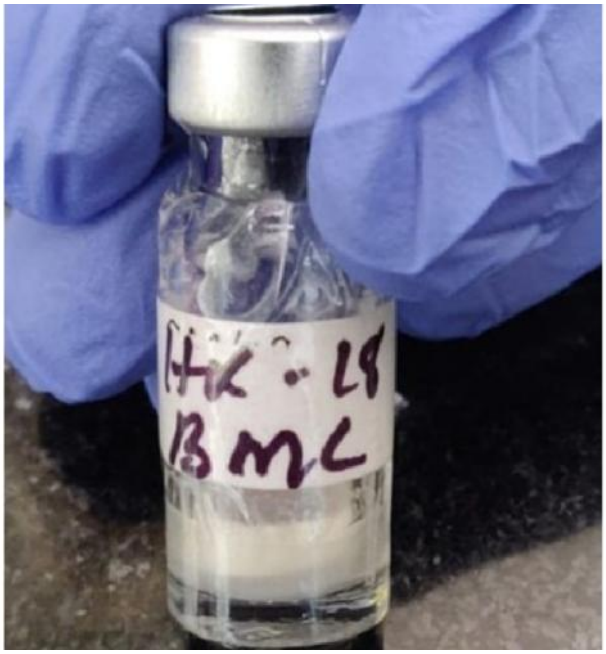

Figure 2. Lyophilized Gonococcal isolate

- **Lyophilization:** The gonococcal isolates were sent to WHO Apex Regional Reference Laboratory for GASP at Vardhman Mahavir Medical College and Safdarjung Hospital.
- **For lyophilisation:** 7.5% glucose horse serum (7.5 gm of glucose to 100 ml horse serum) was sterilized and 1ml was put in each test tube. Each tube was inoculated with a very heavy growth from half a plate, vortexed and distributed into 3 ampoules, freeze dried and lyophilized. (Figure 2). Recovery of cultures were done on chocolate GC agar and NYC agar as per standard guidelines.

RESULTS

Out of 71 suspected cases of gonorrhoea, *N. gonorrhoea* was isolated from 22 cases (30.9%). The colonies were tiny measuring around 0.5-1mm, translucent. The growth was better appreciated on NYC media than on Chocolate GC agar. Preservation of all 22 isolates of *N. gonorrhoeae* were attempted by 3 methods as described earlier. Out of 22 gonococcal isolates preserved by deep freezing (-70^0 C): 12 (54.5%), 06 (27.2%) and 3 (13.6%) isolates were revived at 3 months, 6 months and 8 months respectively by nutrient broth plus glycerol method; 10 (45.5%), 04 (18.1%) and 01 (4.5%) were revived at 3 months, 6 months and 8 months respectively by preservation media (trypticase soy broth and yeast extract). Out of 22 gonococcal isolates, 18 (81.8%), 14 (63.63%) and 6 (27.27%) isolates were revived at end of 1 month, 3 months and 6 months by subculturing gonococcal isolates from chocolate GC agar slope. Out of 22 gonococcal isolates, 12 (54.5%) isolates could be transported to Apex laboratory for lyophilization. Out of 12, only 8 gonococcal isolates were revived at Apex laboratory and lyophilized; rest 4 isolates were overgrown by bacterial contamination during transportation. All 8 lyophilized isolates were revived on Chocolate GC agar and NYC agar. The *N. gonorrhoeae* isolates were subcultured once every 48 hours on GC agar and NYC media. The isolates could be maintained for an average of about one month before the isolate was lost during subculture.

DISCUSSION

N. gonorrhoeae is a fastidious organism which grows only on enriched media such as GC agar, Columbia agar and selective media such as NYC medium with NYC supplement and Columbia agar with supplement. Both enriched and selective media to be used to isolate gonococci from clinical specimens such as urethral swab, endocervical swab, oral swab and anorectal swab. The clinical specimens may contain normal bacterial flora which may grow along with gonococcal isolates or sometime overgrow gonococcal isolates.[10]It is important to identify gonococcal colonies on culture media among other normal bacterial flora and perform preliminary test such as Gram stain, super-oxal and oxidase test for preliminary identification and then perform subculture or plate out of presumed gonococcal colonies on selective media.[10] Once gonococcal isolates are obtained in pure form on culture media, they are confirmed as *N.gonorrhoeae* by standard biochemical tests.[9]It is important to preserve *N. gonorrhoea*e as long as possible so as to perform antimicrobial susceptibility testing and quality control. It also helps to perform molecular analysis such as sequencing and to infer about pathogenic markers, virulence markers, drug resistance markers as well as to obtain data on epidemiological strains isolated from different geographic area and different population groups. This data helps in policy making about testing and treatment guidelines at national and international level. As *N. gonorrhoeae* is a fastidious organism, it is an uphill task to preserve gonococcal isolates for longer duration and retrieve viable gonococci whenever required.[11] Various methods have been mentioned as preservation techniques if liquid nitrogen facility is unavailable.[10]The revival of gonococcal isolates was comparable at end of one month by sub culturing from chocolate agar slope as well as by subculturing every 48 hours on enriched and selective media compatible for gonococci.

It is easier to subculture on chocolate agar slope one time and incubate at 35^0 C in bacteriology incubator as compared to laborious technique of subculturing every 48 hours which consumes time, resources and manpower. In this study, retrieval of viable gonococci was shown to better be with chocolate GC agar slope at 3 months followed by deep freezer preservation by nutrient broth plus glycerol media and then by trypticase soy and yeast extract media. At 6 months, revival of gonococcal isolates was almost equal from chocolate GC agar slope and deep freezer preservation by nutrient broth and glycerol media followed by trypticase soy broth and yeast extract media. Deep freezer preservation by nutrient broth plus glycerol proved to be better over trypticase soy and yeast extract media for revival of gonococcal isolates after 6 months of preservation.

This shows that chocolate GC agar slope can be used as a preferred method for preservation of gonococci upto 6 months. This method is advantageous as less resources are used, require one time inoculation and incubation in bacteriology incubator which is commonly available in the microbiology laboratory. Among the methods of preservation by deep freezing, revival from nutrient broth plus glycerol was better compared to trypticase soy broth and yeast extract. Trypticase soy broth and yeast extract methods require horse serum which is costly and not available routinely in microbiology laboratory. Both these techniques require availability of deep freezer, a high-end equipment not commonly found in most of the microbiology laboratories.

We conclude that preservation of gonococcal isolates on chocolate GC agar slope is preferred method as it has good revival rates up to 6 months, less laborious, less resource intensive and does not require sophisticated equipment. This study shows that preservation of gonococcal isolates need not be confined only to reference laboratories but can be done at microbiology laboratories where there is lack of liquid nitrogen, lyophilizer and deep freezer.

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Conflict of Interest: None

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