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THE EVALUATION AND COMPARISON OF TWO TRANSPORT MEDIA FOR THE GROWTH, HOLDING AND TRANSPORT OF NEISSERIA GONORRHOEAE

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Abstract

Urethral discharge from men attending a dedicated STI clinic was collected and directly inoculated in a solid culture media and held in three types of semi-solid transport medium for varying periods and then cultured. The performance of the different media was compared for growth, holding and transport of *Neisseria gonorrhoae*. Amies'transport media performed much better than T-I with a viability of 62.4 % which was improved to 88.2% if used in combination with NYC at ambient temperature. However, the viability of subcultures decreased dramatically in all media by day 4 and 5/6 days with Amies at 25.8% and 14.0% respectively.

Keywords: Amies', NYC and T-I Culture media, *Neisseria gonorrhoeae.*

INTRODUCTION

The probability of success in the isolation of pathogenic Neisseria from clinical specimens is related to three factors, namely: the amount of care taken in obtaining good specimens and inoculating them correctly, the provision of a culture medium capable of growing demanding strains of Neisseria from small inocula and the inclusion of selective agents in the medium which are capable of preventing overgrowth of commensal organisms on the enriched medium but which will not inhibit the growth of the species required. Cultures from clinical materials often lose viability and become contaminated during shipment to the laboratory. Different culture transport systems such exist but are not frequently used. The Amies transport medium with or without charcoal have been used extensively to transport *Neisseria gonorrhoeae* and may preserve viability for up to 48 hrs. A Trans-isolate medium was used as a method of culturing which permit growth of the etiological agents of *Neisseria meningitidis* and allowed the cultures to survive adverse conditions in the field and during shipment for 2-4 weeks[1]. The medium protected *Neisseria meningitidis* at a temperature as high as 42^0C and as low as 4^0C.

This study will compare and evaluate Trans-isolate and Amies media for the growth, holding and transport of *Neisseria gonorrhoeae* from the clinic to the laboratory. This study is required to assist with the evaluation of both transport systems as a future means to send swabs to the STI Reference Centre (NICD/NHLS) from men with urethral discharge enrolled in surveillance studies in provinces outside of Gauteng. Depending on the results, such transport systems may be incorporated into future National Microbiological Surveillance Programme activities within South Africa. Additionally, in view of the substantial use of drugs for the treatment of gonococcal infections and increasing rates of resistance worldwide, it is important for each country to monitor antimicrobial resistance in *Neisseria gonorrhoea* as a core component of STI surveillance. The principal objective is to obtain data necessary for developing guidelines for treatment and to detect newly emerging resistance

The aim of this study was to evaluate several simple, inexpensive transport media for *Neisseria gonorrhoea* and to determine their ability to maintain viability of *N. gonorrhoeae* under various storage conditions in the laboratory. The most efficient transport media will then be used in a clinical peripheral setting from the reference laboratory in specimens obtained from patients with

clinically diagnosed *N. gonorrhoeae*.

MATERIALS AND METHODS

Urethral samples:

Three endourethral swabs were collected from 100 men with male urethral syndrome (MUS) attending public clinics in Alexandra Health Centre in Gauteng Province. One swab was directly inoculated into a plate of New York City (NYC) and two NYC slopes, the second and third swab were put directly into Amie's Transport and Trans-isolate transport medium and store at room temperature for 48 hours before being plated out on NYS media. The Trans-isolate, two NYC slopes and Amies' transport media were then evaluated and compared with the direct plating for growth, holding and transport of the organism. The order in which swabs will be taken will be the Trans-isolate first and the Amies' Transport swab second for even numbered enrolled patients and vice versa for odd numbered enrolled patients. The *Neisseria gonorrhoeae* isolates were confirmed by routine methods of Grams' stain, Oxidase and Phadebact agglutination tests.

Transport Media

The formulae of these media were as follows;

Modified New York City medium (NYC) comprised 72g Oxoid GC agar base/l (Oxoid Ltd). The medium was autoclaved at 121^0C for 30 minutes before cooling to 50^0C, at which supplements of Oxoid yeast autolysate, Oxoid L-Cat, dextrose, sodium bicarbonate, antibiotics (to inhibit practically all commensal organisms present in the specimen, including the saprophytic *Neisseria* species) and sterile laked blood were added. The NYC plates are used when there is immediate access to a laboratory and this medium is the selective medium of choice for the isolation of *N. gonorrhoeae* from specimens taken from sites such as urethra which contain mixed bacterial flora.

Amies'transport medium comprised 20g Oxoid Amies transport/l (Oxoid Ltd). The pH of the media was adjusted to 7.2 and autoclaved at 121^0C for 15 minutes before cooling at 50^0C and distributed into sterile test tubes. The agar was mixed at regular intervals while distributing to keep the charcoal evenly suspended.

Trans-isolate (*T-I) *medium is a modified Thayer-Martin medium with increased agar content and the addition of dextrose to ensure that toxic products produced by the Neisseria during growth are absorbed. It comprised 18g Oxoid GC agar base, 5g of Agar no.1 in 200ml of distilled water and supplements including phosphate buffers to prevent amine production causing changes in the pH that would affect the survival of the organism in the medium. It was then boiled to dissolve the agar and sterilized by autoclaving at 121^0C for 15 minutes. It is prepared in screw-capped glass bottles as agar slope which, after gassing with CO_2, is used as a selective transport medium. It is a biphasic medium that is useful for the primary culture of meningococci from CSF samples. It can be used as a growth or enrichment medium as well as a holding and transport medium for *Neisseria meningitidis.* The T-I medium need to be pre-warmed in an incubator (35^0C – 37^0C) or kept at room temperature (25^0C) before inoculation, Gloria et al. 1984.

These media as shown can be prepared from individual ingredients, however, it is very difficult to make a well-quality-controlled batch thus purchasing them from a manufacturer is recommended. It has been recognized that many modern commercial media perform with a high degree of reliability. Each batch of media should be checked for reactivity and for appropriate support of microbial growth, either by the manufacturer or in the local laboratory. These media are quite stable if stored in tightly sealed containers in a cool dark place so that the medium does not dry out. Each may be used for up to 1 year as long as no loss of volume, visible contamination or color change is observed. It is also recommended that prepared Amies medium that has been stored for longer than 9 months should be freshly steamed and the charcoal resuspended before use.

Primary Isolation Media

Recovery of *N. gonorrhoeae* after storage in various transport systems was studied using three selection transport/isolation media. These consisted of Trans-isolate medium, NYC, Amies transport medium kept at room temperature and another plate of NYC at 4^0C.

On-site inoculated plates for the primary isolation of *N. gonorrhoeae* were placed in a candle extinction jar for up to four hours and later incubated at 37^0C for 48 to 72 hrs. Oxidase-positive, gram-negative diplococci with typical colony morphology were presumptively identified as *N. gonorrhoeae* and subsequently confirmed with Phadebact monoclonal agglutination testing. This was used as a "gold standard" for comparison and evaluation of the transport, growth and holding of the other two media. Other inoculated plate on NYC was stored at room temperature as well as the Trans-isolate and Amies Transport media. The third inoculated plate on NYC was stored at 4^0C. They were subsequently inoculated on fresh NYC plate for 48, 72 and 96 hrs.

Laboratory Investigations

The sample was obtained from consecutive men with symptoms of urethral discharge attending a public health clinic in Alexandra township in region 8 of Gauteng, using a sterile calcium alginate swab by gently scraping

Table 1: Comparison of *Neisseria gonorrhoae* viability using different transport media over time to inoculation of NYC media in the laboratory (n=93).

Media	Time to inoculation	No +ve gonococcal samples	% Viability
	Day 2	58	62.4
	Day 4	24	25.8
AMIES	Day 6	13	14.0
	Day 2	34	36.6
	Day 4	6	6.5
NYC- RT	Day 6	6	6.5
	Day 2	31	33.3
	Day 4	3	3.2
NYC- Fridge	Day 6	2	2.1
	Day 2	46	49.5
	Day 4	9	9.7
T-I	Day 6	2	2.2

the mucosa or inserting the swab 1 to 2 cm into the anterior and directly inoculated on NYC medium by rolling the culture swab across a segment of the plate or preferably in a large "Z" pattern so that adequate area of the plate is inoculated. Streaking of the plate is carried out with a sterile wire loop to ensure adequate dispersion of the organisms. The plates were then incubated in a humid atmosphere containing 5-10% carbon dioxide at 37^0C and another NYC plate at room temperature. The first swab for even-numbered or odd-numbered enrolled patients were always inoculated on NYC plates while for Amies and Trans-isolate media, this swab was left inside the media and alternated between even-numbered and odd-numbered enrolled patients and these where stored at room temperature. The same laboratory methodology was employed for all media. Cultures were examined for growth after overnight incubation and then daily for 6 days or more.

Envisaged outputs/outcome

Proportion of swabs in Trans-isolate and Amies' transport media yielding growth of *Neisseria gonorrhoeae* compared to the gold standard direct plating of swab one on NYC medium

Ethical clearance

Permission to conduct the evaluation and ethics approval was granted by the University of the Witwatersrand HREC (Protocol no. M10472 and M110402) and Johannesburg Metro Research Committee.

Funding

This work will be undertaken using existing NHLS funding for the STI National Microbiological Surveillance Programme

Statistical Analysis

A chi-square test was used to test for the difference between the overall performances of transport media in the laboratory–based study.

RESULTS

A total of 100 endourethral swabs were collected and 93 were Neisseria gonorrhoeae culture positive on direct plating and 7 were culture negative. The viability of *Neisseria gonorrhoeae* organisms is shown in Table 1: The Amies transport media was 62.4% sensitive in comparison to T-I media (49.5%) in maintaining the viability of the gonococci at day 2. Additionally, the sensitivity of NYC-RT (36.6%) and NYC-Fridge (33.3%) was also comparable. The combined use of Amies' with culture resulted in a 23.1% (62.4% to 88.2%) increase in viability compared to NYC culture alone. However, the viability of subcultures decreased dramatically in all the media by day 4 and 5/6 with Amies at 25.8% and 14.0% respectively (Figure 1).

The proportion of swabs in Trans-isolate and Amies' transport media yielding growth of *Neisseria gonorrhoeae* compared to the gold standard direct plating of swab one on NYC medium illustrate that Amies transport media is superior in term of holding and transporting the organism than Trans-isolate media. Thus direct plating together with Amies transport media should be used to transport *Neisseria gonorrhoeae* from remote areas to the referral laboratory.

DISCUSSION

Gonorrhoea is a sexually transmitted disease caused by *Neisseria gonorrhoeae*. It has been identified as a co-

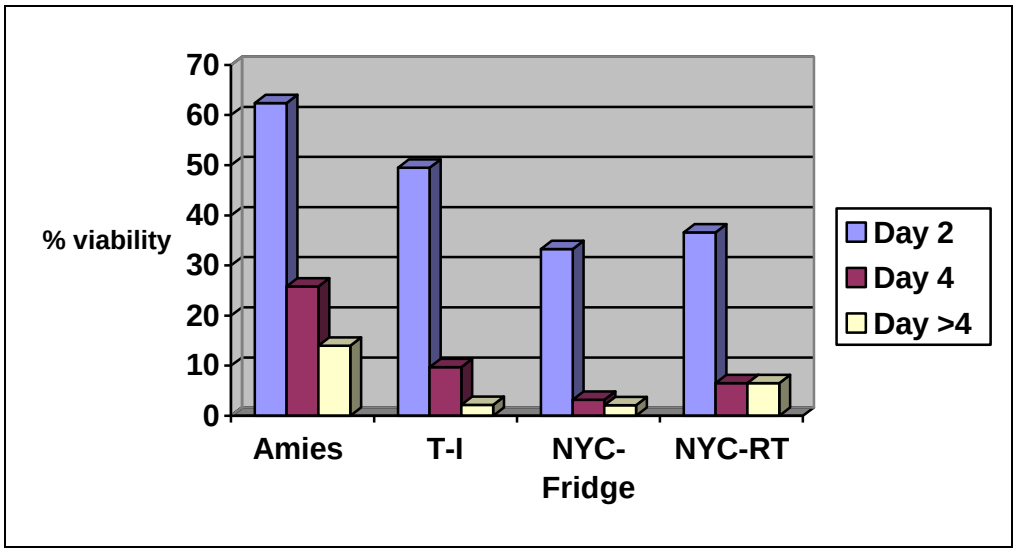

Figure 1: Comparison of *Neisseria gonorrhoae* growth in different media

factor in HIV transmission. The reasons for this are biological as well as behavioural Cohen et al. 1997; Wasserheit 1992. This new association provides an important reason for proper and timely treatment of gonorrhoea. Gonococcal infection usually produces purulent exudates, but signs and symptoms of the disease may either be absent or indistinguishable from those of chlamydial infection. Therefore, laboratory procedures are needed for diagnosis. It is a fastious bacterium that autolyses rapidly. In clinical specimens, the preservation and culturing of the organism is further complicated by the presence of numerous, faster-growing commensal organisms resulting in the loose of viability or become contaminated during shipment to a reference laboratory, Gloria et al. 1984. Ideally, clinical specimens should be directly inoculated onto suitable gonococcal culture selective medium and immediately incubated at 37^0C in 5 to 10% $C0_2$. However, practically the necessary facilities are not available in clinics or study sites. Protective transport systems have to be used to preserve the organism in clinical specimens during transfer to the laboratory for culturing. This may consist of non-nutrient, semisolid or buffered-agar medium Amies 1967; Stuart 1959. Some of this media were modified accordingly over years depending on the new growth requirements of the organism such as addition of certain antibiotics Faur et al., Previous studies by Symington et al showed that Gono-pak, containing CO_2 for support of gonococcal growth was comparable to Amies' medium for recovery of gonococci from clinical specimens up to 24 hrs but Gono-pak was superior for duration more than 24hrs, thus the effect of CO2 on *N gonorrhoea* growth Symington, 1975.

Microscopy and culture are the two methods used for diagnosis. Recently developed tests using molecular or probe technology, such as examination of urine by PCR, allow diagnosis to be made without the need for culture. However, culture recovery of the *Neisseria gonorrhoeae* remain important for the diagnosis of gonorrhoea, and is essential for surveillance of antimicrobial susceptibility as well as for epidemiological studies Wade and Graver 2005.

Transport of specimens:

Although inoculation of bacteriological media in clinic setting is optimal, it can prove impractical or impossible in some healthcare settings. Thus a healthcare strategy that distances patient testing from diagnostic laboratories reinforces the need for transport media. If adequate laboratory facilities are not available for inoculation, the specimen should be inoculated in a suitable protective medium for transport to the laboratory to preserve Neisseria gonorrhoeae Symington, 1975.

If standard culture media are available at the collection site, the specimen can be inoculated at the site and placed in an atmosphere containing 5% CO_2 at 37ºC. The inoculated plates can then be transported at a convenient time.

For transport over longer periods only growth/transport medium can be used. However, results are not satisfactory if transit takes more than two days. Transport media are used for the transport of swab specimens to prolong the survival of microorganisms, especially *N. gonorrhoeae,* between collection and culture. Gonococci do not remain viable for long periods on swabs in transport medium, therefore transit time should be kept to a minimum, preferably less than six hours.

Survival of gonococci is influenced by the composition of the inoculum, such as the presence of pus and other organisms which may overgrow the gonococci; and the type of swab. Charcoal, present in certain transport

media to neutralize toxicity may interfere with Gram staining of smears, and hence these swabs are not recommended. Therefore, in the absence of charcoal, suitable swabs (serum or bovine albumin-coated cotton swabs) should be used to minimize inhibitors.

The swab should be inoculated into a non-nutrient transport medium such as Stuart or Amies. These can be left at room temperature. The isolation rate after transport of specimens in a non-nutrient transport medium at room temperature (25ºC) is approximately 100% within 12 hours, and more than 90% within 24 hours, although the number of colonies decreases markedly. Combination swab/transport packs are available commercially. A selective growth and transport medium such as Transgrow can also be used if available. These are, however, expensive.

STUDY ANALYSIS

The results of this study demonstrated that Amies transport medium was the most efficient of the two transport systems evaluated for preservation of gonococci in clinical specimens. This may allow the cultures to survive adverse conditions in the field and during shipment. In a separate study by Dangor et al, Amies' transport maintained the viability of *Haemophilus ducreyi* for up to three days at 4^0C which decreased with time thus showing its ability in preserving other STIs than gonococci[11]. When testing for ability of a medium to support growth, a small inoculum will give greater assurance that the medium is adequate for recovery of a small number of organisms from a clinical specimen. There is a conflicting recommendations for commercial transport systems in terms of storage temperature for the recovery of *Neisseria gonorrhoeae*. Other shows that overgrowth and killing of the organism in transport media by contaminating bacteria may be inhibited by refrigeration while it is unclear whether refrigeration is detrimental to its recovery. This cold enrichment technique that results in selective slowing of the metabolism of the competing microflora was also demonstrated in other studies Dangor et al., 1993; Smith and Moore 1988. Some studies have compared the survival of clinical strains of *Neisseria gonorrhoeae* in charcoal transport swabs held at ambient temperature (20-22^{0}C) and at 4^0C, Wade and Graver 2005. Sng et al found better survival at lower temperatures in Amies medium Sng et al. 1982 and Arbique et al found that refrigeration improved recovery and survival and optimum temperature varied with the system employed, Judy et al., 2000. Taylor and Phillips notes a marked variation in results held at room temperature before inoculation and failure rates increased with time Taylor and Phillips 1980. Generally, most studies came to the same conclusion that compared to ambient temperature, refrigeration does not compromise the recovery and that storage at 4^0C offers the potential benefit of reducing overgrowth and elimination of the organism by contaminating normal flora, Wade and Graver 2005. In a study by Wade et al to investigate whether auxotypes of *Neisseria gonorrhoeae* differ in their abilities to survive in plain and charcoal containing transport media, significant variability and differences in survival in plain transport medium and the value of charcoal both for reducing this variability and for improving recovery was determined. Wade and Graver 2003; Olse et al. 1999; Thompson and French 1999. The CO_2 environment still plays a significant role in the survival of *N. gonorrhoae*. In a study by Symington, Jembec chambers with tablet-generated CO_2 containing inoculated NYC provided a selective environment that protected and maintained the viability for extended periods, allowing a reasonable time for postal transit of clinical specimens to the laboratory Symington 1975; Brown 1974. Even though some of the media such as T-I may have some value in the bacteriological diagnosis of gonorrhoea, CO_2 content may be variable and different batches of the medium may vary in heir ability to support the growth. Condensation that accumulates inside the bottle may contribute to the spreading of contaminants and inoculation and sub-culturing of colonies through the narrow bottle neck may be difficult, Symington 1975; Chapel et al. 1974. However, each laboratory must ensure adequate control of the media and reagents it uses. Quality control (QC) includes the selection of satisfactory reagents, the preparation of media according to approved formulations or specific manufacturer's instructions, and the use of well-characterized reference strains to check prepared media, Developing World 2003; NCCLS 1982. In addition, the availability of these tests does not assure the quality, thus quality assurance procedures for specimen collection and aseptic techniques must be in place for these tests to provide consistent and reliable results.

CONCLUSION AND RECOMMENDATIONS

The proportion of swabs in Trans-isolate and Amies' transport media yielding growth of *Neisseria gonorrhoeae* compared to the gold standard direct plating of swab one on NYC medium illustrate that Amies transport media is superior in term of holding and transporting the organism than Trans-isolate media. Thus direct plating together with Amies transport media should be used to transport *Neisseria gonorrhoeae* from remote areas to the referral laboratory with the aim of increasing yield of positive gonococcal cultures. However, when evaluating media combinations as shown in Figure 2, Amies' + NYC stored at 4^0C and Amies' + T-I yielded promising results especially if temperature requirements can be maintained.

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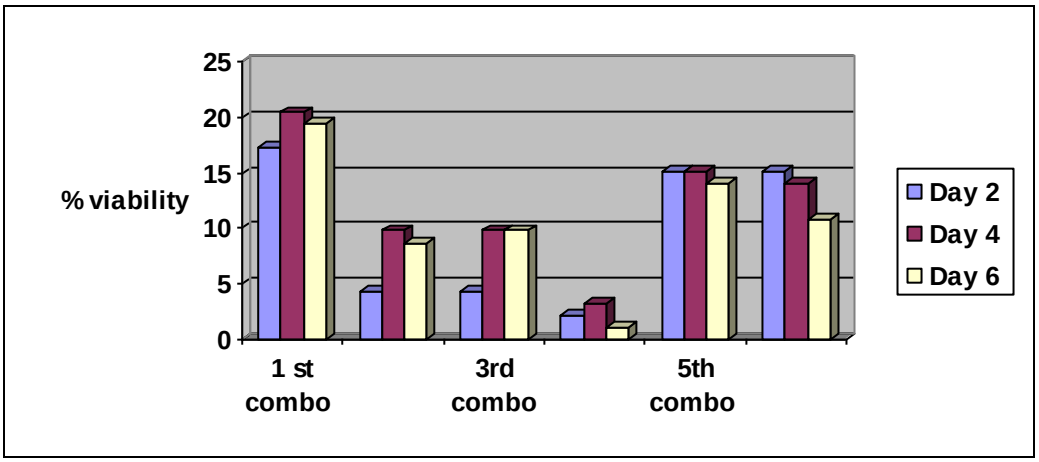
Figure 2: Media combination for the growth and maintenance of *Neisseria gonorrhoeae* (6 combinations)



Keys: 1st combo = NYC RT +Amies, 2nd combo = NYC RT+T-I
3rd combo = NYC RT + NYC Fridge, 4th combo = NYC Fridge + T-I
5th combo = NYC Fridge + Amies, 6th combo = T-I + Amies

Diagnostic Media Production for the preparation of the media and recipes manuscript for the media. We are grateful to the management of the Alexandra Health Centre for the facilities and participants for their willingness to participate in this study.

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