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# General Purpose Media (BNO) for Growing Fastidious Gram Negative (FGN) Bacteria

**Abdallah Bani Odeh[1], Issa Shtayeh[2], Mohammed Ayesh[3], Ismail Warad[4], and Sameer A. Barghouthi[1,3*]**

*[1]Medical Laboratory Sciences, Faculty of Graduate Studies, Al-Quds University, Jerusalem, Palestine.*
*[2]Central Public Health Laboratory, Ministry of Health, Ramallah, Palestine.*
*[3]Department of Medical Laboratory Sciences, Faculty of Health Professions, Al-Quds University, Jerusalem, Palestine.*
*[4]Department of Chemistry, An-Najah National University, P.O.Box 7, Nablus, Palestine.*

***Authors' contributions***

*This work was presented as a master's thesis and defended by author ABO under the supervision of author SAB at Al-Quds University in collaboration with author MA who provided several bacterial strains and helped in their morphological and cultural identification. Author IW suggested several ideas on media formulation. Author IS performed the real time PCR using his resources. All authors read and approved the final manuscript.*

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## ABSTRACT

General purpose media for growing fastidious pathogenic Gram negative (FGN) bacteria are limited to non-selective blood and chocolate agar. These suffer from excessive contamination with fungi, Gram positive, and Gram negative bacteria, especially *Pseudomonas* spp. MacConkey agar can be modified to grow *Haemophilus, Helicobacter, Campylobacter* and other FGN without the use of antibiotics. $BNO_{100}$ and $BNO_{70}$ media were based on MacConkey medium. BNO1-4 were Blood Agar Base containing crystal violet and no bile salts. All media were made as chocolate agar with 8% defibrinated sheep blood, yeast extract 0.5%, glycerol 0.5% (v/v), vitamins, and agar.

**Corresponding author: E-mail: bargsam@yahoo.com;*

*Campylobacter jejuni* ATCC29428 and *Haemophilus influenzae* were able to grow on all described media including BNO100. *Helicobacter pylori* ATCC43504 grew on all BNO media but not BNO100. Most BNO media were inhibitory for the growth of several Gram positive bacteria; *Streptococcus pneumoniae, Staphylococcus epidermidis, Bacillus atrophaeus* QUBC16, *Staphylococcus aureus* including three MRSA clinical isolates. *C. jejuni* and *H. pylori* were successfully grown on BNO media containing Dent's, Skirrow's, or Line's selective antibiotic-supplements. MacConkey based BNO Chocolate agar media may be used as general purpose selective media for Gram negative bacteria including FGNs. We recommend the utilization of $BNO_{70}$ and BNO2 for research and clinical sample processing involving FGN bacteria. The antibiotic-free BNO media reduced contamination with Gram positive and fungi. The incorporation of selective antibiotics and antifungal agents was compatible with the formulated BNO media; whereas *Pseudomonas* continued to pause a serious contamination problem.

*Keywords: Fastidious gram negative; MacConkey; new medium; crystal violet; bile salt; chocolate agar; sheep blood; Campylobacter jejuni; Helicobacter pylori; Haemophilus influenzae.*

## 1. INTRODUCTION

The slow growth of fastidious Gram negative bacteria (FGNs) (Table 1), their requirements for growth factors, and their relatively small colonies allow other organisms to outgrow FNGs. Hence, isolation and growing of FGNs is usually hindered by the outgrowth of other fast growing microbes including Gram positive, fungi, and Gram negative bacteria on non-selective media (Fig. 1). In addition, maintenance of pure FGN bacterial cultures on nonselective blood or chocolate agar is usually subject to being lost to other fast growing and swarming contaminating microbes as indicated by the incorporation of Skirrow's and Dent's antibiotics in selective media [1] and the incorporation of sodium chloride in MacConkey to inhibit gliding motility of bacteria such as *Proteus* therefore preventing its spreading over the entire agar plate [2].

MacConkey agar is a good selective medium against gram positive bacteria and most fungi and yeasts. The selectivity of the medium is due to its content of both crystal violet (1mg per litter) and bile salts [1-3].

The aim of this study was to formulate a general purpose medium or (media) that can support the growth of different FGN species while inhibiting Gram positive and fungi in the absence of antibiotics.

**Table 1. Fastidious gram negative***

| Genus | Species | Shape | Growth requirement | Reference |
|---|---|---|---|---|
| *Bordetella* | *pertusis, parapertusis, bronchiseptica* | Coccobacillus | Aerobic | [5] |
| *Brucella* | *abortus, canis, melitenesis, suis* | Coccobacillus | Aerobic | [6] |
| *Francisella* | *Tularensis* | Coccoid /rods | Aerobic | [7,8] |
| *Actinobacillus* | *actinomycetemco-mitans* | Spherical or rod-shaped | facultative anaerobic | [9] |
| *Haemophilus* | *aphrophilus, influenzae, segnis, paraphrophilus* | Coccobacillus | Factor V and or factor X Microaerophilic | [9] |
| *Legionella* | pneumophila. | Coccobacillus Coocus-Rods | Aerobic need cysteine and iron salt | [10, 11] |
| *Capnocytophaga* | *canimorsus* | Rods | Microaerophilic | [12] |
| *Helicobacter* | *pylori, spp.* | Spiral | Microaerophilic | [13] |
| *Campylobacter* | *jejuni , spp.* | Spiral | Microaerophilic | [1,5] |
| *Neiserria* | *meningitides, spp.* | Coccoid | Microaerophilic | [5] |

**Fastidious Gram Negative (FGN) bacteria were defined as Gram negative bacteria that did not grow on MacConkey agar but grew on blood (BA) or chocolate agar (ChA) [this study]*

It was reasoned that combining the selectivity of MacConkey medium with blood and other nutrients should allow the selective growth of Gram negative bacteria including FGNs while inhibiting Gram positive bacteria and saprophytic fungi. Such modifications should allow some, most, or all FGN bacteria to grow. Selectivity of such medium can be modified by incorporating other selective agents (e.g. Dent's, Skirrow's, or Line's antibiotics as selective antibiotics for *Campylobacter* and *Helicobacter* isolation) [1,4]. In this report we have tested several formulas; BNO100, BNO70, BNO1-4, and BNO 6-9 as potential selective media for FGN bacteria.

## 2. MATERIALS SAND METHODS

### 2.1 Bacterial Species

From the fastidious Gram negative bacteria compiled in Table 1, we selected three major FGN human pathogens as test-strains in the evaluation of newly formulated media in this study. FGN bacteria used in this study were: *Campylobacter jejuni* ATCC 29428 (Culti-loop, Thermo Scientific, Remel Europe, Ltd), *Helicobacter pylori* ATCC 43504 (Al-Quds University collection), and *Haemophilus influenzae* (clinical isolate, Palestinian Ministry of Health). Other used isolates: *Staphylococcus aureus*, three methicillin resistant *Staphylococcus aureus* (MRSA), and *Bacillus atrophaeus* (QUBC 16) [13].

### 2.2 Defibrinated Sheep Blood Collection, Treatment, and Antibiotic Stocks

Glass marbles (0.5 to 1 cm in diameter) were purchased from local toy stores, washed and placed in 500-ml large-mouth screw cap bottles (20 marbles/bottle) with 250 ml water and autoclaved. Water was drained off while hot (70° C) and allowing beads and bottle to dry before tightening the cap. Governmental slaughter house (Al-Biereh and Nablus, Palestine) have kindly collected sheep blood into the supplied bottles. Collected blood was shaken and defibrinated for 15 minutes, immediately 45 ml-aliquots were mixed with 225 µL of 200-fold concentrated Skirrow's antibiotic mix (2 mg /ml vancomycin, 1 mg/ml trimethoprim, 500 IU/ml polymyxin B, 20 mg /ml cycloheximide) [1] or 200-fold concentrates of Line's mix (2 mg /ml vancomycin, 1 mg/ml trimethoprim, 500 IU/ml polymyxin B, 20 mg /ml cycloheximide, 2 mg /ml rifampicin) [4]. The antibiotic stocks were made in 70% ethanol and sterilized using 0.45 µm syringe-filters (Schleicher & Schuell, Dassel, Germany) and stored at 4℃. Treated blood aliquots were kept for 24 h at 4℃ before use to allow antibiotics to kill possible contaminating organisms. Treated blood was then used within two weeks in media preparation. Chocolate agar plates (ChA) were used within 10-15 days [14].

### 2.3 Vitamin Mix Used as Media Enrichment (500X Concentrate; Table 2)

One tablet of multi vitamin weighing 0.626 g (Al-Quds Pharmaceutical Company, Palestine) and another tablet weighing 1.293 g (Spring Valley high potency B) were crushed into fine powder and dissolve in 50 ml of RO water, liquid phase was collected, the remaining solids were treated with 10 ml of absolute ethanol and pooled with the 50 ml extract, mixed and filtered through Whatman filter paper, then sterilized through 0.45 µm syringe fitted filter. Vitamin mix was aliquoted into 1 ml fractions into screw cap microfuge tubes and kept frozen at -20℃. Two ml were used per liter of medium at 48℃ just before pouring agar plates (Vitamin mix; Table 2).

**Table 2. Vitamins and minerals used as media enrichment (final concentrations)***

| Vitamins and minerals | Final concentration/L | Vitamins and minerals | Final concentration/L |
|---|---|---|---|
| A | 26.7 IU | E | 25 µg |
| B1/Thiamine | 1.8 mg | Folate | 5.0 µg |
| B12 | 0.5 µg | H/ biotin | 0.8 µg |
| B2 | 0.5 µg | Pantothonate | 1.9 mg |
| B3/niacin | 3.7 mg | Ca3(PO4)2 | 1.3 mg |
| B6 | 0.5 mg | Mg Phosphate | 0.7 mg |
| C | 0.8 mg | Mn.Glycerophosphate | 0.1 mg |
| D2 | 13.3 IU | Ferric Ammonium Citra | 0.1 mg |

**Filter sterile stock was 500× concentrated; 2 ml/L medium were used*

## 2.4 Preparation of Blood Agar and Chocolate Agar

Blood Agar Base (Difco-BBL, Swedesboro, NJ, USA) was used to prepare blood agar or chocolate agar (BA or ChA); 28 g of dehydrated medium, 5 g dehydrated yeast extract were suspend in 0.91 liter (L) of RO water then autoclave for 18 min. After cooling to 48℃, 8% (vol/vol) sterile defibrinated sheep blood was added slowly to agar, then vitamin mix (2 ml/L), and glycerol (10 ml/L). The 50% stock glycerol was made by mixing 1volume glycerol with 1 volume of deionised water mixed and autoclaved. Vitamins and glycerol were aseptically added to autoclaved media. Prepared agar plates were stored in an inverted position in plastic sleeves at 4℃. The plates were used within 2 weeks.

For chocolate agar preparation, sheep blood (8%) was added and kept for 30 min at 70℃ with occasional shaking, then cooled to 48℃ before the addition of vitamins (2 ml/L) and 0.5% final concentration from an autoclaved stock prepared as 50% v:v in water. glycerol, then poured into Petri dishes.

## 2.5 Modifications of Blood Agar and Chocolate Agar

### 2.5.1 Selective chocolate with line's antibiotics (ChLines)

As with BA and ChA preparation, ChLine) was obtained when 5 ml of (200-folds) Line's antibiotic mixture were added to one liter of autoclaved medium at 48℃.

### 2.5.2 Chocolate MacConkey yeast glycerol vitamin medium (BNO100)

Fifty grams of dehydrated MacConkey agar and 5 g yeast extract were autoclaved in 910 ml RO water. Cooled to 70℃ in water bath, then defibrinated sheep blood was slowly added (8%, v/v) and mixed until chocolate color formed (~30 min). Cooled to 48℃ before the addition of glycerol (0.5% final concentration from 50% autoclaved stock) and 2 ml of vitamin mix. Plates were stored inverted in sealed plastic bags at 4-8℃.

### 2.5.3 Preparation of 70% chocolate MacConkey yeast glycerol vitamin medium ($BNO_{70}$)

As with $BNO_{100}$ except for reducing MacConkey dehydrates to 35 g, and addition of 10 g of Blood Agar Base dehydrates to form the 70% MacConkey ($BNO_{70}$).

### 2.5.4 Selective media with crystal violet (BNO1-5)

Crystal violet was added to a final concentration of 1 mg (BNO1), 0.75 mg (BNO2), 0.50 mg (BNO3), 0.25 (BNO4), or 0.0 mg (BNO5 was the control medium) to one liter of the prepared Blood Base Agar before autoclaving. Defibrinated sheep blood (8%; v/v) was added at 70℃ to make chocolate agar.

### 2.5.5 MacConkey 25% agar with different crystal violet concentrations (BNO6-9)

Thirty gram Blood Base Agar were mixed with 12.5 MacConkey dehydrated medium and 5g yeast extract. Then crystal violet stock was added to bring the final concentration to 1 mg (BNO6), 0.75 mg (BNO7), 0.50 mg (BNO8), 0.25 mg/L (BNO5 acted as control media; Table 4). Autoclaved medium was cooled to 70℃ to make chocolate medium as described above; vitamins and glycerol were added as well at 48℃.

## 2.6 Faecal Sample Processing

In order to remove growth inhibitors that may be found in faecal samples the following protocols were applied. One ml of freshly collected sewage sample (used within 1-3 h) was centrifuged at 12,000 rpm for 3 min in sterile microfuge tube, then the supernatant was discarded, and the pellets was washed three times each in 1 ml of 10 mM phosphate buffered saline (PBS; pH 7.2). Pellet was suspended in 1ml PBS, 10-µl were streaked on test agar media using sterile disposable loops.

A small portion of fresh stool (collected within the past 24 h, and kept at 4℃) was transferred into pre-weighed sterile snap top microfuge tube, weighed and washed in PBS buffer as with sewage samples. The washed pellet was reconstituted in PBS to produce a concentration of 0.1 g stool/ml PBS that was cleared by centrifuged at law speed (1000 rpm) for 30 s, 10-µl of the cleared supernatant were streaked on test medium plates using sterile disposable loops.

## 2.7 Helicobacter pylori Antigen Test

To confirm the identity of *H. pylori* grown on test-media, we used the HP-stool antigen test cassette (DiaLab, Neudorf, Austria).

**Table 3. BNO chocolate media and their composition**

| Per Liter* | MacConkey Agar | Blood Base Agar | Crystal violate | yeast extract | glycerol 0.5% (v/v) | Vitamin mix |
|---|---|---|---|---|---|---|
| $BNO_{100}$ | 50 g | 0.0 | ___ | 5 g | 5 ml | 2 ml |
| $BNO_{70}$ | 35 g | 10 g | ___ | 5 g | 5 ml | 2 ml |
| BNO1 | ___ | 40 g | 1.0 mg | 5 g | 5 ml | 2 ml |
| BNO2 | ___ | 40 g | 0.75 mg | 5 g | 5 ml | 2 ml |
| BNO3 | ___ | 40 g | 0.5 mg | 5 g | 5 ml | 2 ml |
| BNO4 | ___ | 40 g | 0.25 mg | 5 g | 5 ml | 2 ml |
| BNO5 | ___ | 40 g | ___ | 5 g | 5 ml | 2 ml |
| BNO6 | 12.5 g | 30 g | 1 mg | 5 g | 5 ml | 2 ml |
| BNO7 | 12.5 g | 30 g | 0.75 mg | 5 g | 5 ml | 2 ml |
| BNO8 | 12.5 g | 30 g | 0.50 mg | 5 g | 5 ml | 2 ml |
| BNO9 | 12.5 g | 30 g | 0.25 mg | 5 g | 5 ml | 2 ml |

***8%(v/v) of defibrinated and decontaminated sheep blood was added to make chocolate agar to all media in Table 3, ** Vitamin mix as in Table 2*

**Table 4. Growth profiles of all three FGN bacteria on BNO media***

| Medium | *Heamophilus influenzae* | *Helicobacter pylori* ATCC 43504 | *Campylobacter jejuni ATCC* 29428 |
|---|---|---|---|
| MacConkey | Negative | Negative | Negative |
| BNO100 | ++ | Negative | ++ |
| BNO70 | +++ | ++ | +++ |
| BNO1 | ++ | ++ | ++ |
| BNO2 | +++ | +++ | +++ |
| BNO3 | ++++ | ++++ | ++++ |
| BNO4 | ++++ | ++++ | ++++ |
| ChV [BNO5]** | ++++ | ++++ | ++++ |
| BNO6 | ++ | ++ | ++ |
| BNO7 | ++ | ++ | ++ |
| BNO8 | +++ | ++ | +++ |
| BNO9 | +++ | ++ | +++ |

**Increasing colony size (colony size; + to ++++), Negative indicates no growth.*
***BNO5 was the reference/control medium in this study; prepared from blood-base-agar (Table 3) with yeast, glycerol, and vitamins.*

### 2.8 Media Formulated and Tested in This Study

Composition of each formulated medium is illustrated in Table 3.

### 2.9 Storage and Recovery of FGN Bacteria

*Haemophilus influenzae, Helicobacter pylori,* and *Campylobacter jejuni* were stored in sterile screw-cap cryogenic tubes containing 1 ml of 25% glycerol made in autoclaved Luria broth (LB) at -70℃ was recovered on chocolate agar, colonies growing on the recovery plate were then streaked onto other plates and media (Table 4).

Other bacteria were obtained and used as fresh agar cultures from the Department's teaching laboratory; maintained and cared for by Department's staff and technicians in 25% glycerol at -70℃. *Bacillus atrophaeus* QUBC16 was kept as deep agar stab (0.75% soft LB-agar) in screw-cap microfuge tube and continuous subculturing onto nutrient agar plates.

## 3. RESULTS

### 3.1 Selectivity of BNO Media

As shown in Fig. 1, contamination of blood agar cultures usually lead to the loss of culture purity or complete loss of target colonies especially during clinical sample processing for pathogen isolation. Since blood or chocolate agar are the used general media for the isolation of the slaw growing FGN bacteria, loss of isolates to contamination represents a high probability.

**Fig. 1. Fungal overgrowth**

*Fungus can quickly over grow Campylobacter jejuni ATCC 29428 (arrow, small colonies) on blood agar (BA, left) with no selection. C. jejuni failed to grow on MacConkey (right) while fungal colonies grew poorly on MacConkey. This fungus was inhibited by cycloheximide (50 µg/ml)*

In order to develop a general purpose medium that is selective against Gram positive, fungi, and yeast, the first step was to show that modified MacConkey (BNO100, BNO70, and BNO1-9 media) were capable of inhibiting Gram positive bacteria.

*Staphylococcus aureas, S. epidermedis,* and *Streptococcous pyogen* failed to grow on BNO100 or BNO70, BNO1, BNO2, BNO6, or BNO7. However, BNO3, BNO4, BNO8, and BNO9 were not sufficiently selective against the tested Gram positive bacteria.

Inhibition was observed under aerobic and microaerophilic (candle-jar) conditions.

Control BA and CA plates showed good growth of tested Gram positive bacteria. Three additional clinical isolates of methicillin resistant *S. aureus* (MRSA) followed the same pattern of growth; they failed to grow on BNO100 or BNO70, BNO1, BNO2, BNO6, or BNO7.

One type of white fungus (Figs. 1 and 7) continued to grow on all blood containing media that was inhibited when cycloheximide (50 µg/ml was incorporated in OBN media).

### 3.2 FGNs Growth on $BNO_{100}$ and $BNO_{70}$

The ability of $BNO_{100}$ and $BNO_{70}$ to support the growth of FGNs was tested by streaking known FGN bacteria onto the test-media.

#### 3.2.1 Growth of *Helicobacter pylori* ATCC 43504

Pure culture of *H. pylori* was subcultured onto different media including $BNO_{100}$, $BNO_{70}$, BNO1-4. Bacterial growth was not observed on BNO100 but good growth was obtained on $BNO_{70}$ (Fig. 2). Bacterial growth was also obtained on BNO1-4 media with crystal violet (1 mg, 0.75 mg, 0.5 mg, and 025 mg/L respectively); BNO1-4 did not contain any bile salts. Table 4 shows the relative colony size of *H. pylori* to increase as crystal violet concentration decreased. BNO4 was considered non-selective since it allowed the growth of *Staphylococcus aureus,* which failed to grow on BNO1-3.

In addition to microscopic spiral appearance (Fig. 2B) of *H. pylori* grown on identity $BNO_{70}$ (Fig. 2A) and BNO1-4, cultures were further confirmed to be *H. pylori* using the antigen test kit (Fig. 3).

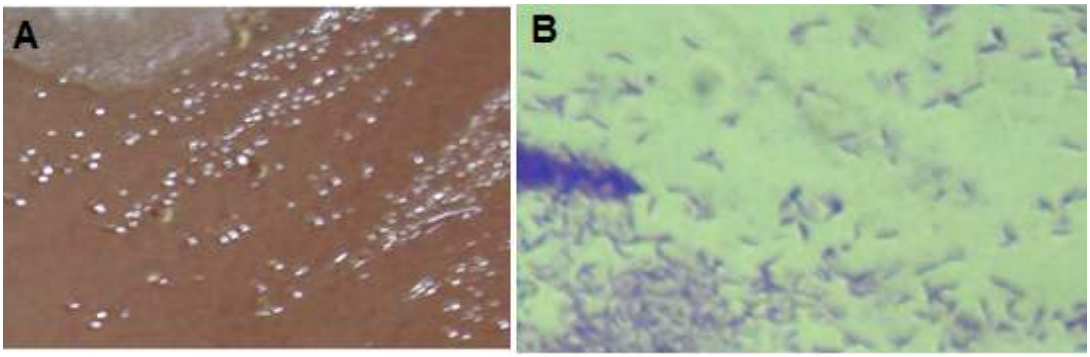


**Fig. 2. Spiral forms of *Helicobacter pylori***

*H. pylori ATCC 43504 (A) Grown on $BNO_{70}$ (section 2.5.3). B: Simple crystal violet smear showing S-shaped and curved bacterial cells but not coccoid forms; oil 1000x*

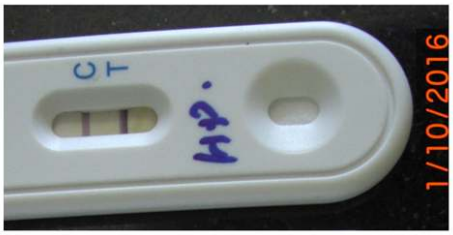


**Fig. 3. *H. Pylori* antigen test**

*Positive antigen test of colonies grown on BNO 70 and BNO1-4. Lower band represents the test result relative to internal control (upper band). BNO100 did not support the growth of this ATCC 43504 strain*

In addition, coccoid formation on $BNO_{70}$ as well as OBN5 was evident (Fig. 4). Coccoid formation was used to monitor purity of *H. pylori* and *Campylobacter jejuni* since contaminants usually do not show this type of morphogical transformation from Spiral form (S-shape) to coccoid form (also known as viable but non-culturable forms (VBNC) [1].

### 3.2.2 Growth of *Campylobacter jejuni* ATCC 29428 on $BNO_{100}$ and $BNO_{70}$

*Campylobacter jejuni* ATCC 29428 was cultured on different types of media prepared in this study (Table 4), it was able to grow on all chocolate media with or without vitamins. It grew well on $BNO_{100}$ and $BNO_{70}$ but not on MacConkey (Fig. 1; Table 5).

It also grew on BNO100 supplemented with Dent's selective antibiotics or Line's selective antibiotics. Growth was in candle jars kept at 37℃ for 3-5 days or longer for coccoid formation test.

S-shaped bacterial cells were observed after 13 days when *Campylobacter jejuni* ATCC 29428 was grown on BNO100 medium as judged by microscopic examination (Fig. 5). On Chocolate agar coccoid forms were abundant after 5 days of subculture.

The bacterium grown on BNO100, BNO70, and BNO1-4 was confirmed to be *Campylobacter jejuni* using real time PCR (Palestinian Ministry of Health) for *C. jejuni* and *Haemophilus influenza* (Fig. 6).

### 3.2.3 Growth of *Haemophilus influenzae* on $BNO_{100}$ and $BNO_{70}$

*H. influenzae* stored in 25% glycerol at -70℃ was recovered on chocolate agar. Colonies were then subcultured onto the different types of media (Table 4). The cultured plates were incubated at 35℃ in a candle jar for three days. After the incubation period, good growth was observed on BNO media including BNO100; but no growth on MacConkey.

When *H. influenzae* growth on chocolate agar and BNO100 agar were observed; small translucent colonies of *H. influenza*e with few fungal contamination on the surface of BNO100 medium. On BNO5, fungal contamination quickly spread to cover the plate on the next day (Figs. 7). Fungal contamination was a significant problem in this work, as fungal growth spreading over chocolate and blood agar which usually resulted in loss of bacterial cultures such as *H. influenzae*. The identity of suspected *H. influenzae* which was grown on BNO100 (Fig. 8) was confirmed by RT-PCR (Fig. 9).

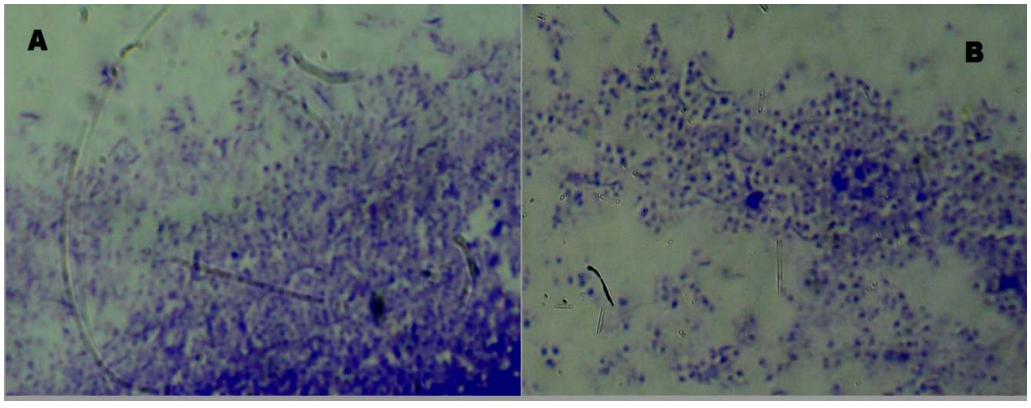


**Fig. 4. Spiral and coccoid forms**

*4-day old culture of H. pylori grown on $BNO_{70}$ (A), notice the presence of spiral and coccoid forms, and BNO5 (B), notice the near absence of spiral form*

**Table 5. Modified MacConkey agar***

| FGN Isolates | BNO100 | BNO70 | Blood A | Chocolate A | McCon | McB | BNO5 Line** | BNO5 |
|---|---|---|---|---|---|---|---|---|
| SW1 | + | + | + | + | Neg§ | + | Neg | + |
| SW; Ode1¶ | + | + | + | + | Neg | + | + | + |
| Chicken stool | + | + | + | + | Neg | + | Neg | + |
| Cat stool | + | + | + | + | Neg | + | Neg | + |
| Soil sample | Neg§ | Neg | + | + | Neg | Neg | Neg | Neg |
| *H. pylori* Ag-positive stool | + | + | + | + | Neg | + | + | + |
| *H. pylori* ATCC 43504 | Neg | + | + | + | Neg | + | + | + |
| *C. jejuni* ATCC 29428 | + | + | + | + | Neg | + | + | + |

**Samples grown on BNO5 then isolates subcultured onto BNO100 or BNO5 and other media. No growth (neg); positive growth (+); SW: Sewage sample isolates.*

***BNO5 with Line's antibiotics, reference positive and negative control media (Blood agar, chocolate agar and MacConkey), §Neg indicates negative bacterial growth on the indicated medium*

*¶16S amplicon obtained by the universal method from isolate Ode1 was sequenced but BLAST alignment did not produce any close match [13].*

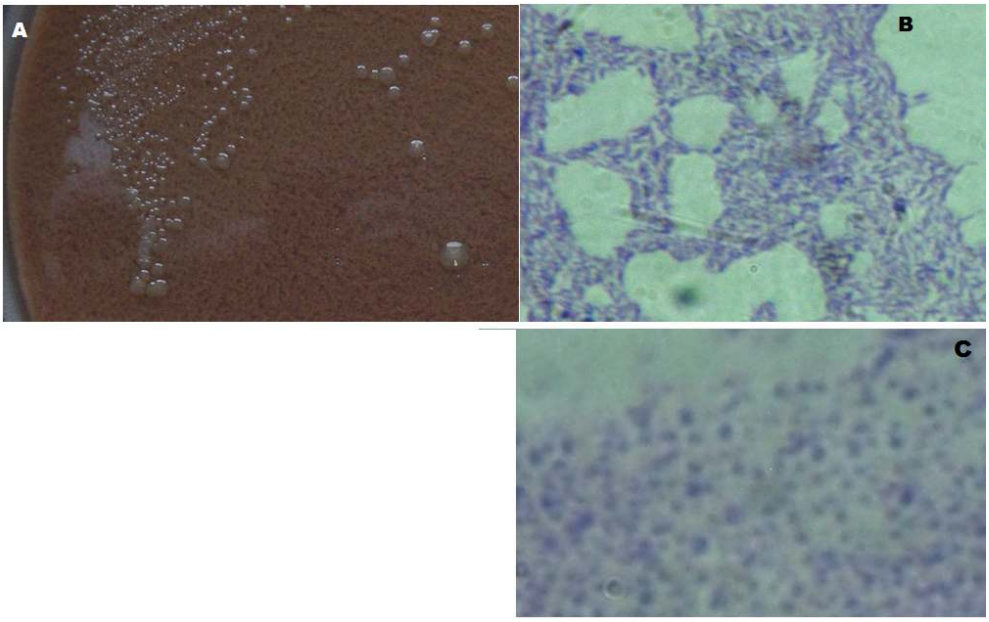


**Fig. 5. *Campylobacter jejuni ATCC* 29428**

*Campylobacter jejuni grown on the BNO100 in a candle jar. A: 3-days old colonies, B: Simple crystal violet stain of 4-days old colonies taken from (A). Notice the absence of coccoid forms. C: 4-day old culture from control plate BNO5 mostly showing coccoid forms and few spiral cells*

## 4. DISCUSSION

The fact that fastidious Gram negative pathogenic bacteria belong to different genera and species paused several obstacles in formulating a selective medium that may satisfy the growth requirements for some, most, or all fastidious Gram negative bacteria (FGN; Table 1).

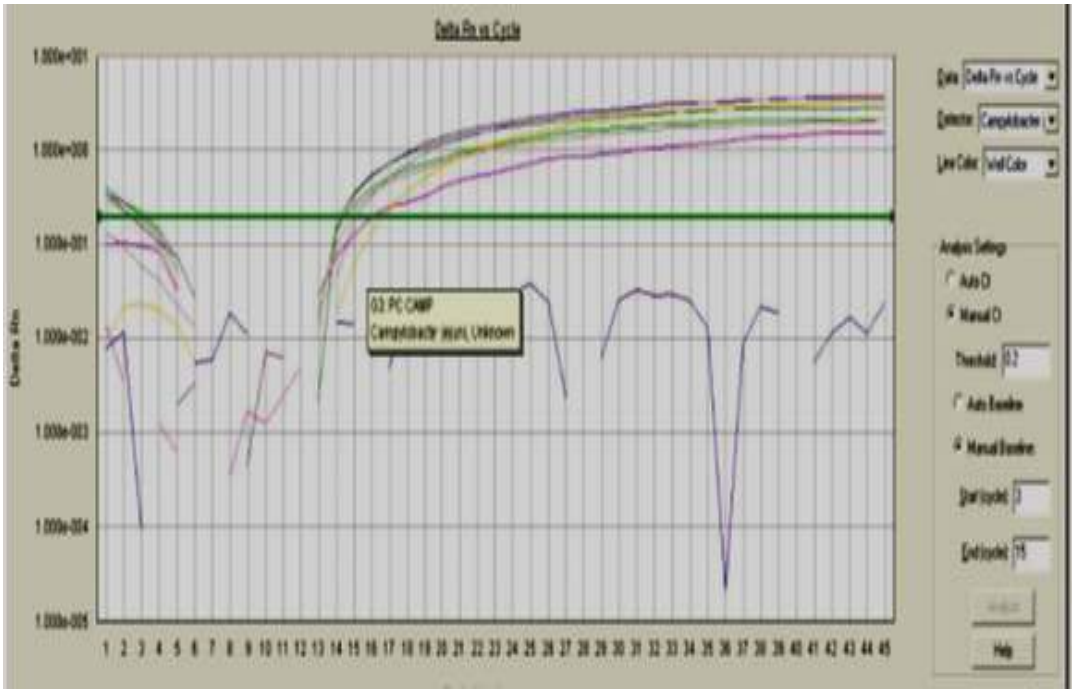

**Fig. 6. Real Time PCR for *Campylobacter jejuni*. Cultured on $BNO_{100}$, $BNO_{70}$, and BNO1-5**

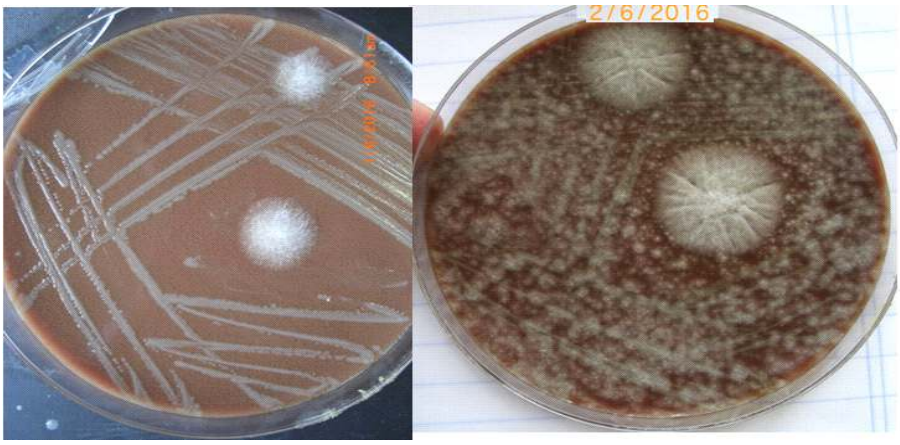


**Fig. 7. Contamination of chocolate agar**

*Fungal contaminated H. influenzae grown on regular chocolate agar, and the loss of the isolate due to fungal over growth on the following day*

## 4.1 Incorporation of MacConkey Agar

Since MacConkey medium has proven its efficiency in growing most non-fastidious Gram negative bacteria, while selecting against and inhibiting the growth of most Gram positive bacteria and saprophytic fungi, it was a first choice as a base medium to be modified in the quest of formulating a general purpose medium capable of accommodating the growth of fastidious Gram negative bacteria (FGN). Currently, selective media used to isolate specific FGN incorporate a number of antibacterial and antifungal antibiotics which renders them suitable only for the specified organism. In addition, the use of antibiotics may increase the risk of selecting antibiotic resistant pathogens. Replacement of such selective media with MacConkey (i.e. bile salts and crystal violet, or similar agents) is suggested. To enrich MacConkey medium, blood was incorporated inBNOs to satisfy most FGN pathogenic bacteria;

known to grow on blood (BA) or chocolate agar (ChA). In addition, MacConkey agar inhibits swarming motility of *Proteus* and possibly gliding as well as swarming of other Gram negative bacteria. It is known that the selectivity of MacConkey II, also known as cysteine-lactose-electrolyte deficient medium (CLED) which is an improved form of MacConkey is due to its content of bile salts and crystal violet [2,15].

#### 4.1.1 Carbon source in MacConkey

Since the carbon source utilized by this diverse group of known and unknown FGN bacteria cannot be predicted, a common intermediate of the glycolytic pathway was used to fulfil and support the requirement for carbon and energy sources. Glycerol was added to the medium at a moderate single concentration throughout this study (a final concentration of 0.5%; 5 ml/L Ca~1.23 mM) [16]. However, there is no strong evidence that *H. pylori* can use glucose as a carbon and energy source and the amino acids alone cannot supply sufficient energy to *H. pylori.* In the absence of glucose, addition of high concentrations of any single amino acid does not enhance growth [17]. The rational for using glycerol instead of D-glucose was two folds; first glucose may facilitate and accelerate the growth of fast growing contaminants therefore reducing the chances for the growth of FGNs. Second, chances for the accumulation of fermentation products (due to the microaerophilic anvirionment) by contaminants may adversely affect FGN growth.

#### 4.1.2 Addition of sheep blood

Two forms of the medium were used at the beginning; a blood agar and chocolate agar. However since it was observed that chocolate agar has outperformed blood agar;

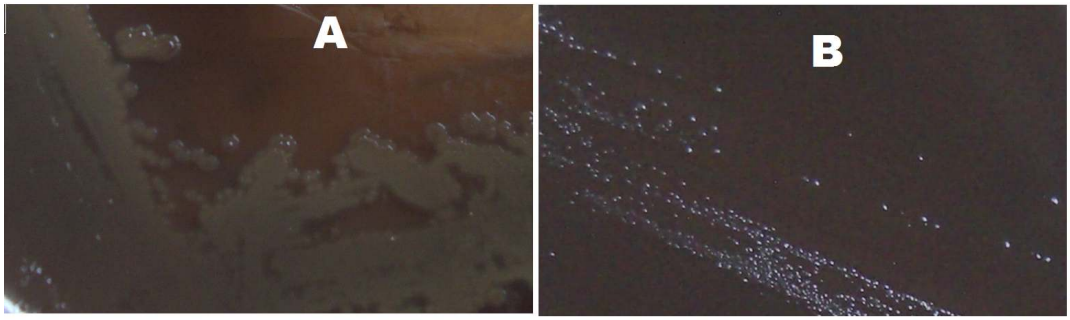


**Fig. 8. Growth of *Haemophilus influenza*e on BNO100. (A) BNO5 and (B) BNO100**

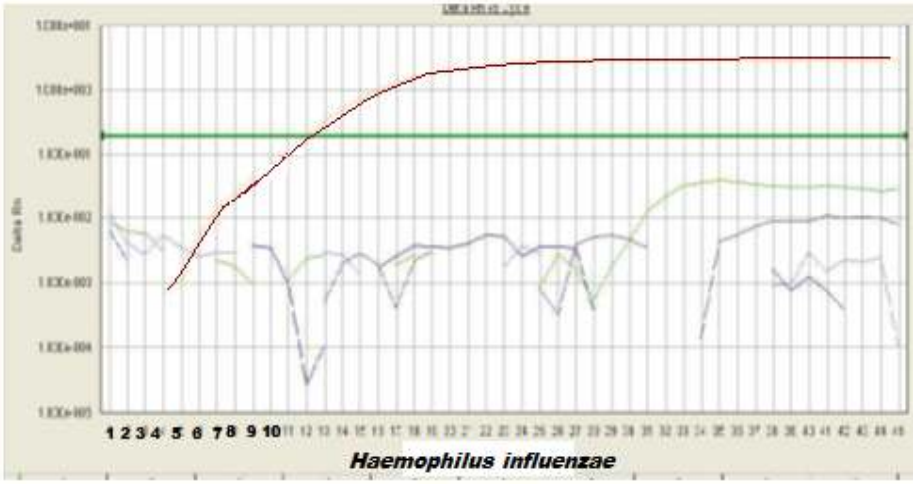


**Fig. 9. *Haemophilus influenzae* Real time PCR (RT-PCR)**
*Confirmatory test for colonies grown on $BNO_{100}$.(the PCR curve was accentuated manually for clarity)*

less contamination which may have been due to heating at 70℃ for 30 min. Two-week old blood agar plates often failed to support the growth of *Helicobacter* unlike chocolate agar counterpart which supported its growth; the shelf-life of chocolate agar appeared to be longer than that of blood agar. It is also known that *Haemophilus influenzae* is routinely cultured on chocolate agar supplemented with vitamins and iron in the form of a vitamin mix (Bio-X, IsovitaleX). Shelf life for Chocolate Agar with IsoVitaleX and Bacitracin is one week when stored at 2-8℃ [18,19]. Accordingly, BA utilization was discontinued with BNO media were all made as chocolate agar.

#### 4.1.3 Manipulation of Crystal Violet and Bile Salt Concentration

The formulation of the $BNO_{70}$ medium (Table 4) was the result of failing growth of *H. pylori* on full strength MacConkey; $BNO_{100}$. Manipulation of bile salt concentration and crystal violet in the media labelled BNO 1-9 (Table 4) showed that bile salt had negatively impacted the growth of all three bacteria, crystal violet also affected bacterial growth when supplied at high concentration (1mg/ L) as in medium BNO1 (Table 4). Improved growth for all three species was observed in BNO2-BNO4 which had no bile salts and had reduced crystal violet. 50% bile esculin chocolate agar showed slow growth of the three FGNs (*Campylobacter jejuni*, *Haemophilus influenzae*, and *Helicobacter pylori*); no growth took place on 100% bile esculin made as chocolate agar (not shown).

## 5. CONCLUSION

This work has unravelled the relationship between three important pathogenic bacteria and their sensitivity to bile salts and crystal violet. This work has achieved its goals by formulating selective media that preferentially supports the growth of fastidious Gram negative bacteria over Gram positive bacteria. It is recommended that $BNO_{70}$ be used for the initial isolation of these bacteria which should be maintained on medium BNO2 or 3 later on to avoid devastating frequent contamination such as that shown in (Figs. 1 and 8) which was observed several times when plain BA or BNO5 were used. This phenomenon was avoided when MacConkey base was used in BNO media; although it did not prevent the contamination, it did control spreading of the fungi across the plate surface. BNO media were antibiotic-free, they controlled the growth of Gram positive and saprophytic fungi therefore reducing dependency on antibiotic-use in controlling contaminants. However BNO media were compatible with the incorporation of selective antibiotics such Line's, Skirrow's, Dent's selective mixes, and cycloheximide [1].

An important outcome that may prove to be of value in differentiating *Campylobacter* from *Helicobacter* by simply growing *Campylobacter jejuni* on both $BNO_{100}$ and $BNO_{70}$ whereas *Helicobacter pylori* may grow on $BNO_{70}$ but not on $BNO_{100.}$ (Table 4).

Future work should explore the application of more non-specific selective agents and focus on controlling *Pseudomonas* contamination which presented a real challenge during this work.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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