Chapter Two

Materials and Methods

2.2. Samples:

2.2.1. Source of samples:

In this study which lasted for 2 years, 300 faecal samples from diarrhoeic calves were collected from different localities of Khartoum State. All samples were taken from the following sites:

1- Parlor 2 and 3, Shigla farms, Selate North and South farms, Ed babeiker farms, Mygoma Farms, Shambat farms, Helat kuku farms, University of Khartoum farm and University of Sudan for Science and Technology farm (in Bahry and East Nile localities).

2- El Ruduan Farms, Gabal Toureia Farms and El Fetaihab farms (in Omdurman Locality).

3- El Saig farms, Soba farms and El Azhari farms (in Khartoum and Gabal awleia localities).

Farms were visited during different seasons (dry, wet and cold seasons) of the years 2010, 2011and 2012.

2.2.2. Sampling procedure:

2.2.2.1. Questionnaire:

During surveillance of the above mentioned farms, the survey forms used contained information about farms and their owners, herd and calf diseases

including calf diarrhoea (morbidity, mortality and treatment).

2.2.2.2. Faecal sample:

Faecal samples were collected directly from the rectums of less than one month old, calves that showed clinical signs of diarrhoea, but had not been treated with antibiotics. Diarrhoeal samples of different colours were put in ice box containing ice and transported to the laboratory. In the laboratory samples were kept in a deep-freezer at -20^{O}C. On the next day samples were removed from the deep freezer and left on the bench to thaw. Samples were then subjected to bacteriological analysis (isolation, identification and characterization of the isolated *E. coli*).

2.2.3. Preparation of culture:

2.2.3.1. Collection of blood:

Blood for enrichment of media was collected by veni-puncture of the jugular vein of healthy donor sheep especially fetched for this purpose. Sodium citrate was added to the blood as anticoagulant.

2.1. Materials:

2.1.1.Media:

All media were prepared and sterilized according to the manufacturers.

2.1.1.1. Solid media:

2.1.1.1.1. Blood agar (Oxoid CM0271):

This consists of:

- Proteose peptone 15g
- Liver digest 2.5g
- Yeast extract 5g
- Sodium chloride 5g

- Agar No3 12g
- pH 7.4 (approx)

Forty grams of blood agar base were suspended in one liter of distilled water. This was brought to boiling temperature in a steamer to dissolve completely then mixed and sterilized by autoclaving at 121^{o}C under 15 lbs/in^{2} for 15 minutes, then cooled to 45–50^{o}C in a water bath before addition of 10% defibrinated ovine blood . This was mixed gently and dispensed as 15ml amount into sterile Petri dishes.

2.1.1.1.2. Blood agar slopes:

The slopes were prepared following the same procedure to prepare Blood Agar (Oxoid CM17). Then distributed in 10 ml amount into sterile MacCartney bottles and allowed to set in the slope position.

2.1.1.1.3. Eosin Methylene Blue agar (Oxoid CM0069):

This consists of:

- Peptone 10g
- Lactose 10g
- Dipotassium hydrogen phosphate 2g
- Eosin Y 0.4g
- Methylene blue 0.065g
- Agar 15g
- pH 6.8 (Approx)

The medium was prepared by dissolving 37.5g in one liter of distilled water. It was boiled to dissolve and then autoclaved at 121^{o}C for 15 minutes. After cooling, the mixture was poured aseptically in sterile Petri dishes.

2.1.1.1.4. MacConkey's agar (Oxoid CM0007):

This consists of:

- Peptone 20g
- Lactose 10g
- Bile salts 5g
- Sodium Chloride 5g
- Neutral red 0.075g
- Agar No.3 12g
- pH 7.4 (Approx)

The medium was prepared by dissolving 52 grams in one liter of distilled water. It was boiled to dissolve and then autoclaved at 121^{o}C for 15 minutes. After cooling, the mixture was poured aseptically in sterile Petri dishes.

2.1.1.1.5. Nutrient agar (Oxoid CM0003):

This consists of:

- Proteose Peptone 15g
- Liver digest 2.5g
- Yeast Extract 5g
- Sodium Chloride 5g
- Agar No3 12g
- pH 7.4 (approx)

Twenty eight grams of the powder of nutrient agar were suspended in one liter of distilled water, brought to the boiling temperature to dissolve completely in the steamer, mixed and sterilized by autoclaving at 121^{o}C under 15 lbs/in^{2} for 15 minutes, then cooled to 45–50^{o}C in water bath . This was dispensed into sterile Petri dishes in 15ml portion each.

2.1.1.1.3. Triple Sugar Iron agar (TSI) (Oxoid CM0277):

This consists of:

- 'Lab-Lemco' powder 3g
- Yeast extract 3g
- Peptone 20g
- Sodium chloride 5g
- Lactose 10g
- Sucrose 10g
- Glucose 1g
- Ferric citrate 0.3g
- Sodium thiosulphate 0.3g
- Phenol red 0.024g
- Agar 12g
- pH 7.4 (Approx)

The medium was prepared by dissolving 65 g in one liter of distilled water. It was boiled to dissolve and then autoclaved at 121^{o}C for 15 minutes. Medium was allowed to set in sloped form in sterile test tubes.

2.1.1.2. Semi-solid media:

2.1.2.1. Gelatin (LP0008):

A solution of 15% of gelatin was prepared, distributed into 5ml bijou bottles and sterilized by autoclaving at 121^{O}C for 15 minutes.

2.1.1.2.2. Hugh and Leifson's O.F medium (Hugh and Leifson, 1953):

This consists of:

- Peptone 2g
- Sodium chloride 5g
- K2HPO4 0.3g
- Agar 3g
- Bromthymol blue 0.2% aqueous solution 5g
- Glucose 10g

All the components above except, glucose were added to one liter distilled water then steamed to dissolve and then autoclaved at121^{O}C for 15 minutes. Glucose was sterilized by steaming for 10 minutes, and then it was added to the mixture. It was then distributed into sterile test tubes.

2.1.1.2.3. Simmons' citrate medium (Simmons, 1926):

This consists of:

- Ammonium dihydrogen phosphate 1g
- Sodium chloride 5g
- Dipotassium phosphate 0.3g
- Agar 15g
- Sodium citrate 2g
- Magnesium sulphate 0.20g
- Bromothymol blue 0.08g
- pH 6.9 (Approx)

All the components above were added to one liter distilled water, steamed to dissolve and then autoclaved at 121^{o}C under15 lbs/in^{2} for 15 minutes. After cooling the mixture distributed in 10 ml amount into sterile MacCartney bottles and allowed to set in the slope position.

2.1.1.2.4. Urea agar base (Oxoid CM0053):

This consists of:

- Peptone 1g
- Glucose 1g
- Sodium chloride 5g
- Disodium phosphate 1.2g
- Phenol red 0.012g
- Potassium dihydrogen phosphate 0.8g
- Agar 15g
- pH 6.8 (Approx)

All the components above were added to one liter distilled water, steamed to dissolve and then autoclaved at 121^{o}C under15 lbs/in^{2} for 15 minutes. After cooling the mixture distributed in 10 ml amount into sterile MacCartney bottles and allowed to set in the slope position.

2.1.1.3. Liquid media:

2.1.1.3.1. Brain Heart Infusion broth (Oxoid CM0225):

This consists of:

- Calf brain infusion solids 2g
- Beef heart infusion solids 5g
- Protease peptone 10g

- Dextrose 2g
- Sodium chloride 5g
- Disodium phosphate 2.5g
- pH 7.4 (Approx)

An amount of thirty seven grams was added to one liter of distilled water, then mixed, distributed into 100ml conical flasks and sterilized by autoclaving at 121^{O}C for 15 minutes.

2.1.1.3.2. MacConkey's broth (CM0005a):

This consists of:

- Peptone 20g
- Lactose 10g
- Bile salts 5g
- Sodium chloride 5g
- Neutral red 0.75g

Forty grams were added to one liter of distilled water, distributed into 5ml bijou bottles fitted with Durham tubes and sterilized by autoclaving at 121^{O}C for 15 minutes.

2.1.1.3.3. Moeller arginine decaboxylase broth base (MacFaddin, 1985):

This consists of:

- Peptic digest of animal tissue 5g
- Beef extract 5g
- Bromcresol purple 0.01g
- Cresol red 0.005g
- Dextrose 0.5g
- Arginine 10g

- Pyridoxal 0.005g
- pH 6.0 (Approx)

Seventeen grams were added to one liter of distilled water, mixed well, distributed into 5ml bijou bottles and sterilized by autoclaving at 121^{O}C for 15 minutes.

2.1.1.3.4. Moeller KCN broth base (CAT No.1112) (Ewing, 1986):

This consists of:

- Sodium phosphate 5.64g
- Sodium chloride 5g
- Peptone 3g
- Potassium phosphate 0.225g
- pH 7.6 (Approx)

Fourteen grams were added to one liter of distilled water, and sterilized by autoclaving at 121^{O}C for 15 minutes. After cooling at 45-50^{O}C 15ml of a 0.5% Potassium cyanide solution was added and the mixture distributed into 5ml bijou bottles.

2.1.1.3.5. MRVP medium (Clark and Lubs medium) (CM0043):

This consists of:

- Peptone 7g
- Glucose 5g
- Phosphate buffer 5g
- pH 6.9 (Approx)

Seventeen grams were added to one liter of distilled water, mixed well,

distributed into 5ml bijou bottles and sterilized by autoclaving at 121^{o}C for 15 minutes.

2.1.1.3.6. Nitrate broth (MacFaddin, 1985).

This consists of:

- Beef extract 3g
- Peptone 5g
- Potassium nitrate (KNO3) 1g

The above components were added to one liter of distilled water, mixed well,

distributed into 5ml bijou bottles and sterilized by autoclaving at 121^{o}C for 15 minutes.

2.1.1.3.7. Nutrient broth (Oxoid CM0001):

This consists of:

- Lab. Lemco' powder 1g
- Yeast extract powder 2g
- Peptone 5g
- Sodium chloride 5g
- pH 7.4 (Approx)

Thirteen grams of the powder were dissolved in one liter of distilled water andsterilized by autoclaving at 121^{o}C under15 lbs/in^{2} for 15 minutes.

2.1.1.3.8. Peptone water (Oxoid CM0009):

This consists of:

- Peptone 10g

- Sodium chloride 5g
- pH 7.2 (Approx)

Fifty grams were added to one liter of distilled water, mixed well, distributed into 5ml bijou bottles and sterilized by autoclaving at 121^{O}C for 15 minutes.

2.1.1.3.9. Tryptophan broth (1% tryptophan) (Vracko and Sherris, 1963):

- Peptone or pancreatic digest of casein (trypticase) 10g
- Sodium chloride 0.5g

The above components were added to one liter of distilled water, mixed well, distributed into 5ml bijou bottles and sterilized by autoclaving at 121^{O}C for 15 minutes.

2.1.1.3.8. Api 20E suspension medium (#20110) (Crichton and Taylor, 1995):

Api 20E suspension medium consisted of distilled water.

2.1.1.4. Solutions and reagents:

2.1.1.4.1. Oxidase test reagent:

Tertramethyl-P-Phenylene diamine dihydrochloride reagent was prepared as 1% solution as described by Barrow and Feltham (2004).

2.1.1.4.2. Hydrogen peroxide solution:

It was prepared according to Barrow and Feltham (2004), as 3% solution and protected from light in a cool dry place. It was used for catalase test.

2.1.1.4.3. Reagents used in Voges and Proskauer test (Voges and Proskauer, 1998):

2.1.1.4.3.1. α-naphthol, 5% colour intensifier**.**

This consists of:

- α-Naphthol 5g
- Absolute ethyl alcohol 100ml

2.1.1.4.3.2. Potassium hydroxide, 40% oxidizing agent**:**

This consists of:

- Potassium hydroxide 5g
- Distilled water 100ml

2.1.1.4.4. Kovac reagent (Vracko and Sherris, 1963):

This consists of:

- Pure amyl or isoamyl alcohol 150ml
- P-Dimethylaminobenzaldehyde 10g
- Concentrated HCL 50ml

2.1.1.4.5. Methyl red pH indicator (MacFaddin, 1985):

This consists of:

- Methyl red 0.1g
- Ethyl alcohol 95% 300ml
- Distilled water 200ml

2.1.1.4.6. Reagent used in Nitrate reduction test (MacFaddin, 1985):

2.1.1.4.6.1. Reagent A:

This consists of:

- α-naphthylamine 5g
- Acetic acid (5N0, 30% 1L

2.1.1.4.6.2. Reagent B:

This consists of:

- Sulfanilic acid 8g
- Ethyl alcohol 95% 300ml

- Acetic acid (5N0, 30% 1L

2.1.1.4.7. Reagents used in Api 20E:

Reagents used in Api 20E were described by Crichton and Taylor (1995).

2.1.1.4.7.1. TDA (#70400) for the detection of tryptophane deaminase**:**

This consists of:

- Ferric chloride 43g
- Distilled water 100ml

2.1.1.4.7.2. James (#70540) for the detection of indole**:**

This consists of:

- Compound J 2183 0.5g
- Hcl N qsp 100ml

OR

2.1.1.4.7.3. IND (#70410) for the detection of tryptophane deaminase**:**

This consists of:

- Paradimethlaminobenzaldehyde 5g
- Isoamyl alcohol 75ml
- Hcl 37% 25ml

2.1.1.4.7.4. NIT1 (#70440)**:**

This consists of:

- Sulfanilic acid 0.8g
- Acetic acid 5N 100ml

2.1.1.4.7.5. NIT2 (#70450)**:**

This consists of:

- N; N-dimethyl-1-naphthylamine 0.6g
- Acetic acid 5N 100ml

2.1.1.4.7.6. VP1 (#70420**):**

This consists of:

- Potassium hydroxide 40g
- Distilled water 100ml

2.1.1.4.7.7. VP2 (70430**):**

This consists of:

- α - Naphthol 6g
- Ethanol 100ml

2.1.1.4.8. Reagents used in Fimbrex kits (Thorns *et al*., 1989):

- Test latex: Reagent 1 (R1) 7ml
- Control latex: Reagent 2 (R2) 7ml
- Positive control antigen 7ml
- Suspension buffer 10ml

2.1.1.4.9. Reagents used in *Vibrio cholera* and *E. coli* Heat-labile enterotoxin test kid (Ristaino *et al*., 1983):

- **TD921** sensitized latex (Latex suspension sensitized with specific antibodies (rabbit IgG) against *V. Cholerae* enterotoxin).
- **TD922** latex control (Latex suspension sensitized with non-immune rabbit gloulins.
- **TD923** Enterotoxin control (Dried *V. Cholerae* enterotoxin).
- **TD924** Diluent (Phosphate buffered saline contain bovine serum albumin).

2.2.4. Sterilization:

2.2.4.1 Hot air oven:

Glassware like Petri dishes, pipettes, tubes, flasks and glass rods were sterilized in hot air oven at 160^{O}C for one hour.

2.2.4.2. Autoclaving (121^{O}C 15lbs/in2):

Screw capped bottles, rubber caps, tips of micropipette, media solution etc were sterilized in autoclave at 121^{o}C for 15 minutes. Sugar media were sterilized at 110^{o}C for 10 minutes.

2.2.4.3. Disinfection of bench:

Solution of 70% alcohol was used for bench sterilization.

2.2.5. Cultural methods:

2.2.5.1. Primary isolation:

A loopfull of faecal sample was streaked onto blood agar, McConkey's agar, and Nutrient agar and then the streaking over the plate was completed using the wire loop.

2.2.5.2. Incubation of culture:

All inoculated solid and liquid media were incubated aerobically at 37^{o}C for 18-24 hours.

2.2.5.3. Examination of cultures:

Cultures on semi-solid media were examined grossly for colonial morphology and haemolysis on blood agar. Whereas, broth media were checked for turbidity, change in colour, accumulation of gases in CHO media and for sediment formation.

2.2.5.4. Gram's stain:

From one colony on each plate one half was taken with a sterile loop, emulsified in a drop of normal saline on a clean microscopic slide. The smear was allowed to dry and then fixed by passing the slide over a flame. The slides

were placed on the rack and flooded with crystal violet stain for one minute and rinsed with water. They were then

covered by iodine for one minute and rinsed with water. Acetone was poured and immediately the slides were rinsed with water. The slides were counter stained with diluted carbolfuchsin for one minute and rinsed with water again and allowed to dry by blotting with filter paper. A drop of immersion oil was added to each slide and examined under microscope. Colonies which showed Gram–positive cocci, Gram positive bacilli and Gram-negative bacilli were subcultured on nutrient agar.

2.2.5.5. Subculturing and purification:

Purification was based on the characteristics of colonial morphology and smear. This was obtained by subculturing of a typical discrete colony on blood agar plate. Pure cultures were preserved on slants of blood agar and dorset egg media at 4^{o}C.

2.2.6. Biological and biochemical identification:

The purified isolates were identified according to criteria described by Smith (1967) Barrow and Feltham (2004) which include: Gram's reaction, presence or absence of spores, shape of organism, Motility, colonial characteristics on different media, aerobic and anaerobic growth, sugars fermentation ability and biochemical tests.

2.2.6.1. Staining of smear:

This was done according to Barrow and Feltham (2004) to observe the shape, colour, size and arrangement of stained organism.

2.2.6.2. Catalase test:

A drop of 3% aqueous solution of hydrogen peroxide was placed on a clean glass slide. A drop of the culture under test grown on nutrient agar was added and mixed with the hydrogen peroxide. Production of gas bubbles indicates positive results for the catalase test.

2.2.6.3. Oxidase test:

The tested bacteria were grown on nutrient agar. A disk of tetramethyl -p–phenylene diamine dihydrochloride was put on a clean slide. The tested organism was picked with sterile bent glass rod and rubbed on the disk. A violet color that developed within 5-10 seconds indicates a positive result.

2.2.6.4. Oxidation Fermentation test (O.F):

Two tubes containing (O.F) media were inoculated vertically by the tested culture. One tube was covered by sterile paraffin oil and the other left. The uncovered tubes were incubated at 37^{o}C up to seven days and examined daily. The development of a yellow color was an indication for positive results. Development of the yellowish color in the tube covered with paraffin oil was an indication of fermentation reaction and in opened tube an indication of oxidation reaction.

2.2.6.5. Motility test:

Motility media was inoculated by stabbing the isolated bacteria with a sterile wire loop. The media was then incubated for up to 3 days together with uninoculated media as control. The growth of non-motile organism was confined to the stab, while the motile one was distributed out the stab.

2.2.6.6. Glucose breakdown:

Each Bijou bottle containing 1% glucose in peptone water and indicator was inoculated with the tested organism. Change in colour indicated positive reaction. If gas is produced, it will accumulate on the top of Durham's tube.

2.2.6.7. Fermentation of carbohydrates:

Isolates were inoculated into peptone water with sugars, incubated at 37^{O}C and examined daily for up to 7 days. Acid production was detected by the change of bromothymol indicator colour from blue to yellow.

2.2.6.8. Methyl red reduction (MR):

MR-VP media were inoculated and incubated at 37^{O}C for two days. Two drops of methyl red solution were added and the tube was shaken and examined. A positive MR reaction was indicated by the appearance of a red colour at the surface.

2.2.6.9. Voges-Proskauer reaction:

MR-VP media were inoculated and incubated at 37^{O}C for two days. 0.6ml of 5% alph-naphthol solution and 0.2ml of 40% KOH aqueous solution were added and the tube was shaken and sloped, then examined after 15 minutes and one hour. A positive VP reaction was indicated by the appearance of a strong red colour at the surface of the slope.

2.2.6.10. Indole production:

Tryptophan broths were inoculated and incubated at 37^{O}C for two days. 0.5ml of Kovacs' reagent was added, shaken and examined after one minute. A red colour in the reagent layer indicated indole production.

2.2.6.11. Urease activity:

Urease slopes were heavily inoculated and incubated at 37^{O}C for up to 5 days. A red colour indicated urease activity.

2.2.6.12. Citrate utilization:

Citrate slopes were heavily inoculated and incubated at 37^{O}C for up to 7 days. A blue colour indicated citrate activity.

2.2.6.13. Gelatin hydrolysis:

Gelatin media was inoculated with a needle all the way to the bottom, incubate at 25^{O}C for one week then placed in the fridge for about 30 minutes to determine liquification.

2.2.6.14. Eijkman's test (Oxoid):

Each Bijou bottle containing MacConkey's broth was inoculated with the tested organism and incubated at 44^{O}C in water bath for 18-24 hours. Change in colour indicated positive reaction. If gas is produced, it will accumulate on the top of Durham's tube.

2.2.6.15. Arginine decaboxylase test (MacFaddin, 1985):

Each Bijou bottle containing decarboxylase broth was inoculated with the tested organism and incubated at 37^{O}C in water bath for 18-24 hours. Purple or violet colour indicated positive reaction.

2.2.6.16. Potassium cyanide test (Ewing, 1986):

Each Bijou bottle containing KCN broth was inoculated with the tested organism and incubated at 37^{O}C in water bath for 18-24 hours. Turbidity indicated positive reaction.

2.2.6.17. Nitrate reduction test (MacFaddin, 1985).

Each Bijou bottle containing nitrate broth was inoculated with the tested organism and incubated at 37^{O}C in water bath for 18-24 hours. 0.2ml of reagent A and 0.6ml of reagent B were added and after 5 minutes the test was read. The appearance of a red or pink colour indicated a positive test. Negative results were confirmed by adding a small amount of zinc dust.

2.2.7. Confirmation of *E. coli* identification:

This was performed by using of Api 20E and Vitek 2 identification systems.

2.2.7.1. API 20E (BIOMERIEUX, France):

According to Crichton and Taylor (1995), API 20E is a standardized identification system for Enterobacteriaceae and other Gram-negative rods which uses 23 miniaturized biochemical tests and a data base.

1- Principle:

The API 20E strip consists of 20 microtubes containing dehydrated substrates. These microtubes are inoculated with a bacterial suspension, prepared in API 20E medium that reconstitutes the tests. During incubation metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The reactions were read according to the reading table

and the identification is obtained by referring to the analytical profile index or using the identification soft-ware.

Tests included in API 20E strips were:

Ortho-nitro-phenyle-galactoside (ONPG), Arginine (ADH), Lysine (LDC), Ornithine (ODC), Sodium citrate (CIT), Sodium thiosulphate (H_2S), Urea (URE), Tryptophane (TDA), Indole (IND), Sodium pyruvate (VP), Kohn's gelatin (GEL), Glucose (GLU), Mannitol (MAN), Inositol (INO), Sorbitol (SOR), Rhamnose (RHA), Sucrose (SAC), D-melibiose (MEL), Amygdalin (AMY), Arabinose (ARA), Oxidase (OX), Nitrate (NO3-NO2), Motility (MOB), MacConkey growth (MAC) and Oxidation-fermentation test (OF).

2- Content of the kit (kit for 25 tests):

- 25 API 20E strips.
- 25 incubation boxes.
- 25 ampules of API 20E medium.
- API 20E reagents.
- 25 report sheets.
- 1 API 20E instruction manual.

3- Preparation of the strips:

The incubation boxes (trays and lids) were prepared and 5ml of distilled water was distributed into the honey–combed wells of the trays to create a humid atmosphere. The strains references were recorded on the elongated flab

of the trays. The strips were removed from their packaging and placed in the incubation boxes.

4- Preparation of the inoculum:

The organisms were sub cultured onto blood agar and incubated at 36^{O}C ± 2 for 18–24 hours. They were checked to be belonging to *Enterobacteriaceae* Family (morphology, Gram stain, catalase, oxidase, etc.) and they were pure cultures. Ampoles of API 20E medium were opened and homogeneous bacterial suspension prepared. These suspensions were used immediately after preparation.

5- Inoculation of the Strips:

Both tubes and cubules were filled with the inoculated API 20E media by using a pipette. Anaerobiosis was ensured in the ADH, LDC, ODC, URE and H_2Stests by filling the cupules with mineral oil to form a convex meniscus. The incubation boxes were closed and incubated at 36^{O}C ± 2 for 18–24 hours.

6- Reading the strips:

After the incubation period, the reaction was developed by adding one drop of each of the following reagents and then results were read by referring to the reading tables.

- **V.P test:** one drop of each of **VP1** and **VP2** reagents was added. After 10 minutes, a bright pink or red color indicated a positive reaction.

- **NO2 test:** one drop of each of **NIT1** and **NIT2** reagents was added to the GLU. After 2 minutes, red color indicated positive reaction.

- **TDA test:** Add one drop of **TDA** reagent. A dark brown color indicated positive reaction.

- **IND test:** one drop of **James** reagents was added. A pink colour indicated a positive reaction.

7- Interpretation of results:

Identification was obtained with the numerical profile. On the results sheets, the results were separated into groups of three and a value of 1, 2 or 4 was indicated for each. By adding together the values corresponding to positive reaction within each group, a 7–digit profile number was obtained. Identification was performed by using Api 20E manual. Positive and negative tests were interpreted according to table (1).

Table (1)

(API 20E Reading table)

Test	positive	negative	Test	Positive	Negative
ONPG	yellow	Colourless	**INO**	yellow	Blue/blue-green
ADH	Red/orange	yellow	**SOR**	yellow	Blue/blue-green
LDC	orange	yellow	**RHA**	yellow	Blue/blue-green
ODC	Red-orange	yellow	**SAC**	yellow	Blue/blue-green

CIT	Blue-green/green	Pale green/yellow	**MEL**	yellow	Blue/blue-green
H_2S	Black deposit	Colourless/greyish	**AMY**	yellow	Blue/blue-green
URE	Red/orange	yellow	**ARA**	yellow	Blue/blue-green
TDA	Dark brown	yellow	**OX**	violet	colourless
IND	Pink/red	Pale green/yellow	**NO_3**	red	yellow
VP	Pink/red	colourless	**NO_3/Zn**	yellow	red
GEL	Diffused black	Restricted black	**MOB**	motile	Non motile
GLU	yellow	Blue/blue-green	**MAC**	presence	absence
MAN	yellow	Blue/blue-green	**OF**	yellow	green

2.2.7.2. VITEK 2 (BIOMERIEUX, France) (Funke *et al.*, 1998):

1- Principle:

The BioMerieux® introduced Vitek 2, the new generation of Vitek® microbiology analyzers and it associated colorimetric ID-GN card in 1997. The Vitek 2 system is developed on fluorescence-based technology and designed for the totally automated identification of wide range of micro organisms including gram-negative & gram positive bacteria,Neisseriacea and yeasts in clinical or industrial samples. There are different marked cards containing 64 chambers for identification tests or antibiotic susceptibility testing (AST). In clinical microbiology Vitek®2 used as an auto analyzer system for the identification (ID) and antibiotic susceptibility testing (AST) of the bacteria in clinical samples (Figure 1).

There are 64 micro spaces in this series of the reagent cards that each microchamber contains a special substrate for individual tests. The chambers are limited between two clear walls on both sides of the card. This structure provides possibility of optical observation of contents inside chamber. Observation takes place once every 15 minutes for each chamber, by an electronic sensor. The walls are permeable to oxygen and needed oxygen level inside of micro chambers, could be provided throw walls where the sealing is protected also.

Different tests exists in different cards as expected; for instance acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances (antibiotics) known as antibiotic susceptibility test (AST).

For GN cards, 47 biochemical tests, some assimilation tests and one negative control well (well No 1) are located in these micro spaces. The GN card is based on established biochemical methods and developed substrates measuring carbon source utilization, enzymatic activities, and resistance. Each card has a transfer tube used for inoculation and has bar code that contains information on product type, lot number and a unique identifier that can be matched to the sample during loading the card onto the system. The GN card is used for the automated identification of 135 taxa of the most significant fermenting and non-fermenting Gram-negative bacilli.

A transmittance spectrophotometer is used to read of test reactions using different wavelengths. During incubation, each test reaction is read every 15 minutes to measure either turbidity of bacterial mass growth or colour products of substrate metabolism.

2- Examination requirements:

1- Vitek 2 system (BioMerieux®) whitch is consisted of 3 chambers:Filling and sealing chamber, the viteck 2 reader-incubater chamber and Computerized ID-GNB database for interpretation of results.

2- Purified Gram-negative young culture.

3- 0.45% saline solution (BioMerieux®) for bacteria suspending.

4- Magnetic steerer.

5- ATB 1550 densiometer (biomerieux).

VI-GN identification cards:

The identification card for Gr –ve bacilli (ID-GNB card) for the vit 2 system is a 64-well plastic card containing 41 fluorescent biochemical tests, including 18 enzymes tests for aminopeptidases and aminosidases. Substrates used for detection ofaminopeptidases are usually coupled with 7-amino-methylcoumarin (7AMC); substrates for detection of aminosidases are usually coupled with 4-methylumbelliferone (4MU). The 18 tests substrates are as follows: 4MU-α-arabino-pyranoside, 4-MU-α-D-galactoside, α-L-glutamic acid-7AMC, 4MU-β-D-cellobiopyranoside,4MU-β-D-galactoside, 4MU-β-D-glucoside, 4MU-β-D-glucuronide, 4MU-β-D-Mannopyranoside, 4MU-N-acetyl-β-D-glucosaminide, 4MU-N-acetyl-β-D- galactosaminide, 4MU-β-D-
xyloside, glutaryl- glycyl-arginine-7AMC, γ-L-glutamic acid-7AMC, 4MU-phosphate, L-proline-7AMC, L-pyroglutamic acid-7AMC, L-lysine-7AMC and Z-arginine-7AMC.Furthermore, the ID-GNB card includes 18 fermentation tests (adonitol, L-arabinose, D-cellobiose, D-galacturonate, D-glucose, glucose-1-phosphate, D-glucuronate, inositol, 5-kito-gluconate, D-maltose, D-mannitol, D-melibiose, palatinose, D-raffinose, L-rhamanose, sucrose, D-sorbitol and D-trehalose, 2 decaboxylase tests (Ornithine and lysine), and miscellaneous tests (urease and utilization of malonate and tryptophane deaminase) (Figure 2).

3- Identification Process with Vitek 2:

A sterile plastic stick applicator was used to take pure colonies from culture media and transfer a sufficient number of them to plastic test tubes. Test tubes contained about 3.0 ml of sterile saline to suspend the microorganism in. After mixing by shaker in order to produce a homogenous suspension of bacteria, the turbidity of suspension was adjusted to (0.50-0.63 MacFarland) by addind proper amounts of saline or bacteria. The density (turbidity) of the suspension was checked by using ATB 1550 densiometer (biomerieux). The time between preparation of the suspension and card filling was less than 30 minutes.

GN cards were loaded (inoculated) with bacterial suspensions using a vacuum chamber in machine. Test tubes containing the samples were placed into a cassette (a special test tube rack) and the identification card was placed in the neighboring place while inserting the transfer tube into the corresponding suspension tube. The cassette could accommodate up to 10 test tubes. The filled cassette was placed into a vacuum chamber station inside the vitek 2 analyzer machine. The vacuum was applied then the air was recharged into the station, the bacterial suspension was forced through the transfer tube into micro-channels that filled all the test wells. Inoculated cards were passed by a mechanism, which cut off the transfer tube and sealed the card prior to loading into the circular incubator. The incubator could accommodate up to 60 cards. The card was automatically filled by a vaccum device and automatically sealed. It was manually inserted in the viteck 2 reader-incubater module (incubator T^{o} , 35.5^{o}C). and every card automatically subjected to a kinetic fluorescence measurement every 15 minutes. All used cards were automatically discarded in waste container.

4- Interpretation of results:

The results were interpreted by the ID-GNB database after the incubation period.The list of tests in GN cards used in this study is shown in table (2).

Fig. (1) Vitek2 Compact autoanalizer machine

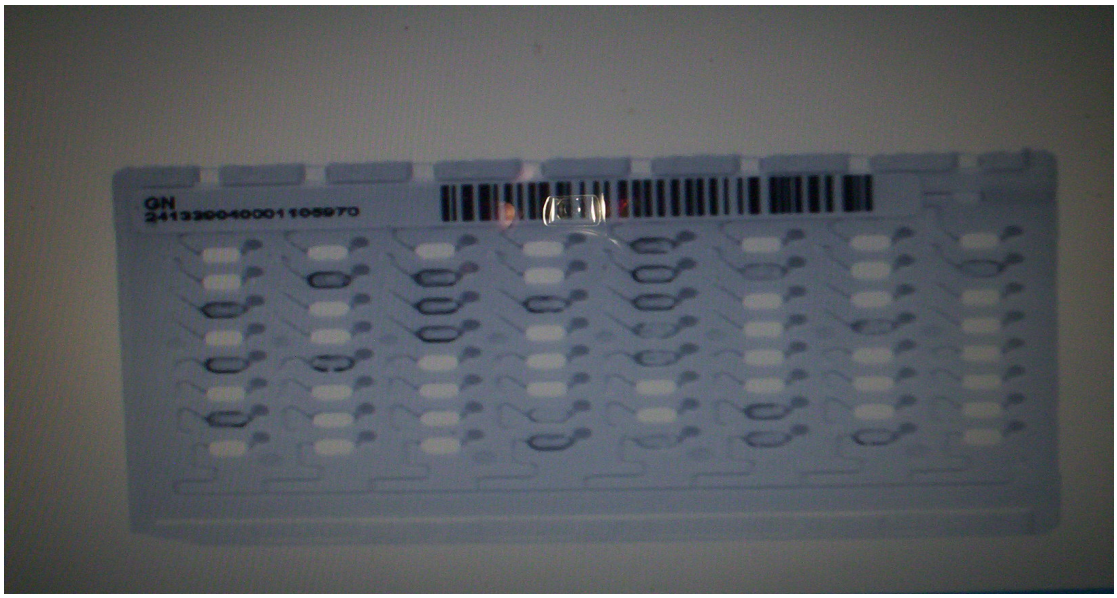

Fig. (2) A sample of GN cards used for Vitek 2 autoanalizer machines

Table (2)

(Test substrates on GN card)

Well	Test	Amount	Well	Test	Amount
2	Ala-phe-pro-Arylamidase (APPA)	0.0384mg	21	β-xylosidase (BXYL)	0.0324mg
3	Adonitol (ADO)	0.1875mg	22	β-alanine arylamidase pNA (BALap)	0.0174mg
4	L-pyrrolydonyl-arylamidase (PyrA)	0.018mg	23	L-proline Arylamidase (ProA)	0.0234mg
5	L-arabitol (lARL)	0.3mg	26	Lipase (LIP)	0.0192mg

7	D-cellobiose (dCEL)	0.3mg	27	Palatinose 0.0192mg (PLE)	0.3mg
9	β-galactosidase (BGAL)	0.036mg	29	Tyrosine arylamidase (TyrA)	0.0276mg
10	H2S production (H2S)	0.0024mg	31	Urease (URE)	0.15mg
11	B-N-actyl-glucoseaminidase (BNAG)	0.0408mg	32	D-sorbitol (dSOR)	0.1875mg
12	Glutamyl Arylamidase pNA (AGLTp)	0.0324mg	33	Sucrose (SAC)	0.3mg
13	D-glucose (dGLU)	0.3mg	34	D-tagatose (dTAG)	0.3mg
14	γ-glutamyl-transferase (GGT)	0.0228mg	35	D-trehalose (dTRE)	0.3mg
15	Fermentation/glucose (OFF)	0.45mg	36	Sodium citrate (CIT)	0.054mg
17	β-Glucosidase (BGLU)	0.036mg	37	Malonate (MNT)	0.15mg
18	D-maltose (dMAL)	0.3mg	39	5-keto-D-Gluconate (5KG)	0.3mg
19	D-mannitol (dMAN)	0.1875mg	40	L-lactate alkalinisation (lLATK)	0.15mg
20	D-mannose (dMNE)	0.3mg	41	α-glucosidase (AGLU)	0.036mg

Table 2 (Continued)

Well	**Test**	**Amount**	**Well**	**Test**	**Amount**
42	Succinate alkalinisation (SUCT)	0.15mg	53	L-histidine assimilation (lHISa)	0.087mg
43	β-N-acetyl-galactosaminidase NAGA)	0.0306mg	56	Coumarate (CMT)	0.126mg
44	α-galactosidase (AGAL)	0.036mg	57	β-glucoronidase (BGUR)	0.0378m

					g
45	Phosphatase (PHOS)	0.0504mg	58	O/129 resistance (comp. vibrio) (O129R)	0.0105mg
46	Glycine arylamidase (GlyA)	0.012mg	59	GLU-Gly-Arg-Arylamidase (GGAA)	0.0576mg
47	Ornithine decarboxylase (ODC)	0.3mg	61	L-malate assimilation (lMLTa)	0.042mg
48	Lysine decarboxylase (LDC)	0.15mg	62	Ellman (ELLM)	0.03mg
52	Decarboxylase base (ODEC)	N/A	64	L-lactate assimilation (lLATa)	0.186mg

2.2.8. Detection of pathogenic mechanisms of *E. coli*:

2.2.8.1. FIMBREX kits (Thorn *et al.*, 1989).

These are commercially available kits for in vitro detection of the fimbrial adhesions of ETEC. It is monoclonal antibodies (Mab) based Immuno Assay. Monoclonal antibodies were coated onto either K88, 987p, K99, and F41 latex particles at concentration of 10% (W/V) by standard method.

Fimbrix is a rapid agglutination test and it should be used for identification of the fimbrial adhhesins of *E. coli* grow on an appropriate medium (Figures 3, 4, 5).

1- Kit contents:

- Test latex: Reagent 1 (R1) 7ml
- Control latex: Reagent 2 (R2) 7ml

- Positive control antigen 7ml
- Suspension buffer 10ml

2- Test requirements:

1- Bacteriological wire loops or sticks.

2- Rocking device or orbital shaker.

3- *E. coli* young culture.

3-Reagents preparation:

Reagents were stored at 2^{o}C to 8^{o}C. They were first allowed to reach room temperature (approximately 30 minutes). Then bottles were shacked to resuspend R1, R2 and the positive control antigen before use.

Reagents used in this study were reagent kits for the identification of K88 (F4), K99 (F5) and 987p (F6) fimbrial adhesions. They were obtained from Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, KT 15 3NB, UK.

4- Method:

1- Fimbrex test reaction card was marked with the test identification.

2- One drop of suspension buffer was added to reagent circle 1 and reagent circle 2, in the test column.

3- An even suspension of the test organism was made in each of the drops of buffer on the card.

4- One drop of the positive control antigen was added to reagent 1 circle and one drop to reagent 2 circles in the control column.

5- One drop of test latex (R_1) was added to each circle in the reagent 1 column.

6- One drop of control latex (R_2) was added to each circle in the reagent 2 coulmn.

7- Each circle was mixed thoroughly using a clean mixing stick (e.g. handle of a bacteriological loop) and the mixture was spread to fill each circle.

8- Cards were rocked gently for up to 3 minutes.

9- Macroscopic agglutination was examined.

5- Analysis of results:

The reaction was checked with the positive control antigen. Valid tests were those only showed agglutination with R_1 and not with R_2.

Positive results showed agglutination with R_1 within 3 minutes and lacked agglutination with R_2.

Negative results showed no agglutination with R_I or R_2 after three minutes.

When agglutination occurs with both R_1 and R_2 within three minutes, the test considered invalid and that an indication of auto-agglutination. Auto-agglutination indicates that the test organism is not suitable for testing by this method. Usually this indicates that the test organism will be found to be in the rough phase and therefore untypeable.

Fig. (3) Fimbrex K99 test kit

Fig. (4) Fimbrex 987p test kit

Fig. (5) Fimbrex K88 test kit

2.2.8.3. Detection of heat-labile (LT) enterotoxin (Carroll *et al*., 1990):

Certain strains of *E. coli* are known to produce enterotoxins. These ETEC strains are common cause of of diarrhoea in developing countries and of travelers' diarrhoea. ETEC strains produce one or both of two different enterotoxins, a heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). The LT eterotoxin has antigenic structures similar to those found on *Vebrio cholerae* enterotoxin (CT). Antiserum taken from rabbits, immunized with CT and LT, will therefore react with both CT and LT. The VET-RPLA test was

designed for the detection LT or CT in culture fluid. A positive result given in the test indicates that the organism produces the relevant enterotoxin. The technique of reversed passive latex agglutination (RPLA) enables soluble antigen such as bacterial toxins to be detected in agglutination assay (Figure 6).

In a standard agglutination assay, soluble antibody reacts with particular antigen such as bacterial cells. However, in a reverse agglutination assay the antibody, which is attached to particles, reacts with the soluble antigen. The particles (in this case, latex) do not, themselves, play a part in the reaction and they are therefore passive. The cross-linking of the latex particles by the specific antigen/antibody reaction results in the visible latex agglutination reaction.

2.2.8.3.1. Principle of assay:

Polystyrene latex particles are sensitized with purified antiserum taken from rabbits immunized with purified *Vebrio cholerae* enterotoxin. These latex particles will agglutinate in the presence of *Vebrio cholerae* enterotoxin (CT) or *E. coli* heat-labile enterotoxin (LT). A control reagent is provided which consists of latex particles sensitised with non-immune rabbit globulins. The test is performed in V-well microtitre plates. Dilutions of the culture filtrate are made in two rows of wells, a volum of the latex suspension is added to each well and the contents mixed. If either toxin is present, agglutination occurrs which resulted in the formation of a lattice structure. Upon setting, this forms a diffuse layer on the base of the well. If the enterotoxins are absent or at concentration bellow the assay detection level, no such lattice structure can be formed and, therefore, a tight button will be observed.

3- Storage:

The VET-RPLA kit (Oxoid) was strored at 2^{o}C to 8^{o}C.

4- Preparation of enterotoxin:

Enterotoxin was prepared according to Giannella (1976).

5- Materials required:

1- Microtitre plate (V-well) and lid.

2- Fixed or variable pipette and tips (25µl).

3- Orbital shaker.

4- Moisture box.

6- Components of the kit:

- **TD921** sensitized latex (Latex suspension sensitized with specific antibodies (rabbit IgG) against *V. Cholerae* enterotoxin).

- **TD922** latex control (Latex suspension sensitized with non-immune rabbit

gloulins.

- **TD923** Enterotoxin control (Dried *V. Cholerae* enterotoxin

- **TD924** Diluent (Phosphate buffered saline contain bovine serum albumin).
- **Instruction leaflet.**

7- Assay method:

1- The latex reagents and diluents were ready issued for use. The latex reagents were thoroughly shaken before use to ensure a homogeneous

suspension. To reconstitute the enterotoxin control, 0.5ml of diluents (TD24) was added to each vial. Vials were shaken gently until the contents were dissolved.

2- The plate was arranged so that each row consisted of 8 wells. Each sample needed the use of 2 such rows.

3- A pipette was used to dispense 25µl of diluent in each well of the two rows except for the first well in each row.

4- 25µl of test sample was added to the first and second well on each row, 25µl was picked up and doubling dilutions was performed along each of the 2 rows. The 7^{th} well was left to contain diluents only.

5- 25µl of sensitized latex was added to each well of the first row.

6- 25µl of latex control was added to each well of the second row.

7- To mix the contents of each well, the plate was rotated gently by hand and no spillage occurred from the wells.

8- To avoid evaporation, plates were covered and put in moisture place. The plates were left at room temperature for 24hours. The plates were placed on black background for the duration of incubation to help subsequent reading.

9- Each well in each row was examined for agglutination against the dark background.

8- Interpretation of test results:

The agglutination pattern was judged by comparison with the illustrated results classified as (+), (++) and (+++) and considered positive.

Results in the row of wells containing latex control were negative. In case of non-specific agglutination, results were interpreted as positive, provided that their action with sensitized latex was positive to a higher dilution of test sample than that seen with the control latex. The last well in all rows was negative.

9- Limitations of the test:

The sensitivity of the test kid in detecting LT was 1-2ng/ml. Enterotoxin present at concentrations lower than this would, therefore, gave negative results.

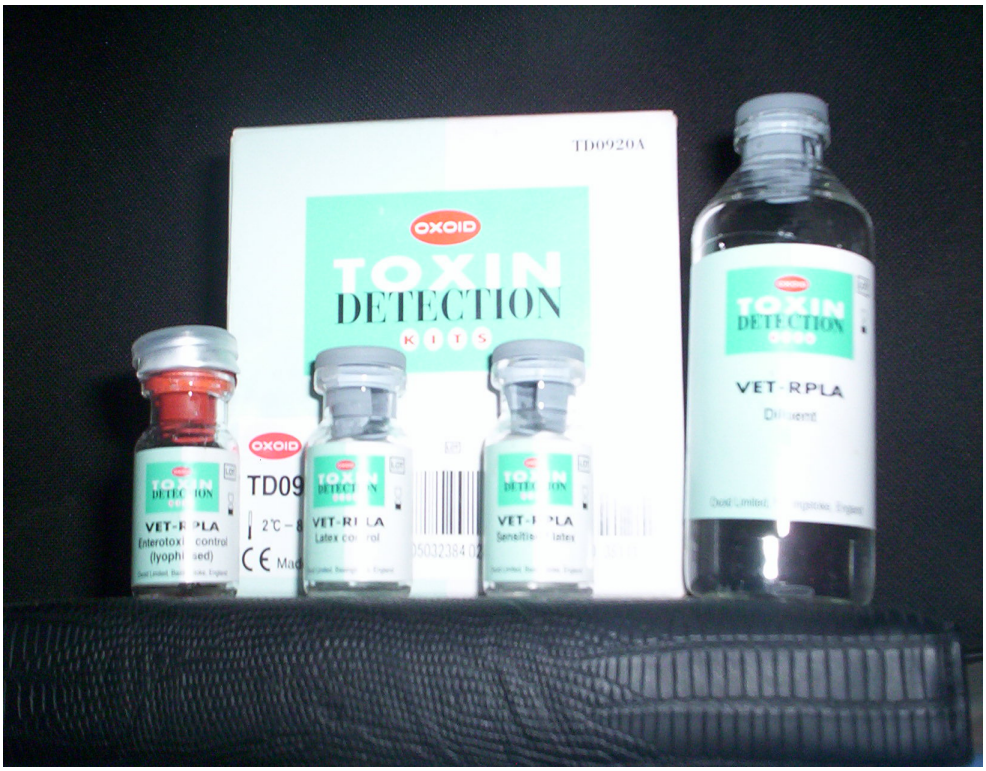

Fig. (6) VET RPLA toxin detection kit for detection of heat-labile enterotoxin

2.2.8.2. Detection of STa enterotoxin:

2.2.8.2.1. Suckling mouse test (SMT):

This biological method described by Dean *et al* (1972) and standardized by Giannella (1976). The principal of the test is that, the injection of STa preparation into the stomach of 2-4 days old suckling mouse causes fluid accumulation in the intestine.

2.2.8.2.1.1. Toxin preparation:

Volumes of 100ml of Brain Heart Infusion Broth in 100ml conical flasks were inoculated with a loop swept lightly a cross several colonies of *E. coli* grown on blood agar plates. The flasks were incubated in a water path shaker (100rpm) at 37^{o}C for 24 hours. The broth cultures were coldly (4^{o}C) centrifuged at 4000rpm for 15 minutes and the supernatant was collected. This supernatant constituted the STa which was aseptically stored at 4^{o}C before use.

2.2.8.2.1.2. Sterility of toxin:

This was done by streaking two loopful of the prepared toxin onto blood agar plate and incubated at 37C^{o} overnight, any growth seen, the toxin was discarded.

2.2.8.2.1.3. Mice inoculation:

Two days after birth infant mice were inoculated with 0.1ml STa into the stomach using a special canula. After inoculation the mice were kept at room temperature for 4 hours and then decapitated or killed. The abdomen was

opened (after killing of mice); the small intestines were examined for distension and then removed by forceps. The intestines were then weighted using a sensitive balance and the ratio of gut weight to the body weight was calculated. Ratios of less than 0.070 were considered negative. Those in range of 0.070-0.090.were considered doubtful positive and those over 0.090 were positive.

2.2.8.6 Antibiotic sensitivity test:

Oxoid antimicrobial susceptibility test discs:

This was performed by the standard Disc Diffusion method (CruickShank *et al*, 2007). The organisms were subcultured onto Blood agar and incubated at 37^{O}C for 18-24 hrs. They were diluted in sterile normal saline tubes and homogenous bacterial suspensions (0.5 MacFarland) were prepared. After drying Nutrient agar plates, 2 ml of diluted culture were spread evenly over the surface of the media. Excess fluid was aspirated and the plates were allowed to dry. Oxoid discs (Basingtoke, Hampshire, England) of antimicrobial drugs were applied to the surface of the medium and pressed gently using sterile forceps. They were incubated at 37^{O}C for 24-48 hours. Zones of inhibition were measured in (mm) to determine whether the organism was sensitive or resistant (Jacoby and Archer, 1991) (Table 3).

Table (3)

Zone size interpretation chart: (Quinn *et al*, 1999)

Antimicrobial agents	Resistant ≤	Intermediate	Moderately Susceptible	Susceptible ≥
Ampicillin 10µg	28 mm	-	-	29 mm
Cephalothin 30µg	14 mm	-	15-17 mm	18 mm
Chloramphenicol 30µg	12 mm	13-17 mm	-	18 mm

Ciprofluxacin 15µg	15 mm	-	16-20 mm	21 mm
Erythromycin 15µg	13 mm	14-22 mm	-	23 mm
Gentamycin 10µg	12 mm	13-14 mm	-	15 mm
Kanamycin 30µg	13 mm	14-17 mm	-	18 mm
Penicillin 10µg	28 mm	-	-	29 mm
Tetracyclin 30µg	14 mm	15-18 mm	-	19 mm
Sulphamethoxazole-trimethoprim 75µg	10 mm	-	11-15 mm	16 mm