

DEVELOPMENT OF MEDIUM FOR DIFFERENTIATION BETWEEN YERSINIA ENTEROCOLITICA AND OTHER BACTERIA

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ABSTRACT

A new dye containing medium, (Yersinia- 2, 3, 5-triphenyl tetrazolium chloride-rose bengal Agar, YTRA) was developed for differentiation between Yersinia enterocolitica and other bacteria. The YTRA medium was compared with cefsulodin irgasan novobiocin (CIN) medium, which is the most popular medium to differentiate and enumerate Yersinia enterocolitica. Several combinations of twenty three dyes were evaluated to develop the new medium. 2,3,5 Triphenyl tetrazolium chloride (0.05 g/liter) and rose bengal (0.05 g/liter) on Brain Heart Infusion Agar were found as the best combination for differentiation between Yersinia enterocolitica and other bacteria. On the slightly red YTRA medium, Yersinia enterocolitica produced metallic sheen colonies, whereas other growing bacteria produced red color colonies. The recovery rates of Yersinia enterocolitica from inoculated ground beef, and milk were significantly different between Cefsulodin-irgasan-novobiocin (CIN) and Yersinia- 2, 3, 5-triphenyl tetrazolium chloride-rose bengal Agar (YTRA) media. The recovery rates of heat injured Yersinia enterocloitica were also highly countable on YTRA medium.

INTRODUCTION

Yersinia enterocolitica is an important pathogen worldwide. In the past two decades, there have been many reports of outbreaks of yersiniosis with concomitant right lower quadrant abdominal pain (Attwood, 1987, Hoogkamp-Korstanje, and Stolk-Engelaar 1995). It has been reported that *Y. enterocolitica* invades the epithelium cells of the gastrointestinal tract to produce intestinal disease in animals and humans. It also produces a heat-stable enterotoxin that contributes to the symptoms of gastroenteritis (Coghlan, 1996). The association of human illness with consumption of *Y. enterocolitica*-contaminated food, animal wastes, and unchlorinated water is well documented (Aulisio *et.al*, 1982 and 1983). Some strains of *Y. enterocolitica* and related species produce an in vitro heat-stable enterotoxin (ST) that can be detected by intragastric injection of cultural filtrates in suckling mice and is very similar to Escherichia coli ST (Boyce *et. al*, 1979). Sefsulodin-irgasana by mannitol fermentation-novobiocin (CIN) agar medium was developed by Schiemann (1979) has usually been used for isolation and recovery of *Yersinia enterocolitica* bacteria from various

food products. It contains bile salts and antibiotics as selective agents and identifies the bacteri. Cefsulodin inhibits the growth of *Y. pseudotuberculosis* and other strains of Yersinia spp. (Fukushima and Gomyoda, 1986)

Furthermore, Fukushima (1987) developed new selective agar medium for accurate isolation of virulent *Yersinia enterocolitica* (VYE agar) from food samples highly contaminated with environmental *Yersinia* organisms, as well as for isolation from clinical specimens. VYE agar provided a quantitative recovery of 51 different strains of virulent *Y. enterocolitica* at 32 °C after incubation for 24 h. The cefsulodin, irgasan, josamycin and oleandomycin content of the medium resulted in a high selectivity, and the mannitol and esculin content provided some differentiation.

Agbonlahor *et. al,* (1982) has developed new differential and selective medium (DYS) agar for recovery of *Yersinia enterocolitica* from stools. The bile salts content of the medium resulted in high selectivity, and inclusion of arabinose, lysine, and arginine rendered *Y. enterocolitica* very distinct from Proteus spp., Pseudomonas spp., and other members of the family Enterobacteriaceae. This study aimed to evaluate new dye containing media for recovery of *Y. enterocolitica* from beef, milk and water.

MATERIALS AND METHODS

Culture and cell suspension

Microorganisms: The bacteria used in this study were obtained from culture collection, Botany and Microbiology Department, Faculty of Science, AlAzhar University, Assiut branch.

To prepare the cultures of the experiment, bacterial strains were transferred to 9.0 ml tryptic soy broth (TSB) and incubated at 37 ° C for 24 h. 1 ml of the incubated culture broth was transferred to 500ml polycarbonate centrifuge bottle containing 100 ml of sterilized TSB. After incubation, the cells were harvested by centrifugation at 5000 rpm for 10 minutes. The pellets were resuspended in 0.1% peptone water.

Preparation of dyes containing media

Twenty three dyes (Table 1) challenged twenty-one different bacterial genera, species and strains on Brain Heart Infusion Agar (BHIA), pH adjusted at 7.0 ±0.2 for developing a medium for differentiation between *Yersinia enterocolitica* and other bacteria. Cefsulodin irgasan novobiocin (CIN) medium was compared with Yersinia- 2,3,5-triphenyl Tetrazolium chloride-Rose Bengal Agar (YTRA), for recovery and determination of the growth rate of *Yersinia enterocolitica* from beef, milk and water.

Table 1. Dyes and their Sources

Dye	Source
Indigo Carmine- acid	Sigma lot 48C-0209
Orcien	Sigma lot 123H-757
Bismark brown Y- basic	Matheson Coleman & Bell
Erichrome black T-acid	Fisher lot 781614
Evans blue –acid	Sigma lot 44 H-3684
Crystal Violet-basic	Fisher
Rose bengal-acid	Sigma lot 102F-0671
Metanil yellow	Sigma lot 107 F-04231
Cresol red -acid	Fisher lot 765438
Aniline blue	Fisher
Victoria blue	Sigma
Nylomine red	ICI Americas inc.
Bromocersol purple	Sigma
Pararosaniline-basic	Sigma lot 72H-3663
Methyl green - basic	Sigma lot 14 H- 3626
Crystal violet	Fisher
Brilliant yellow-basic	Sigma lot 43 H-0739
Nile blue A	Sigma lot 12 F -0477
Auramine O	Sigma lot 82 F -0575
Methyl red	Sigma lot 12F-0426
Eosin Y	Sigma lot 45380
Cresol red	Aldrich Chem.co
2,3,5-tripheny tetrazolium chloride	Sigma lot 67H1640

Screening procedure

Multipoint miniaturized inoculation system was used to screen several organisms simultaneously on specified dye containing plates. Preparation of a master plate was done by transferring 250 µl of actively growing culture broth of the individual target microorganism into single "U" shaped wells of sterile 96-well microtiter plate. The microorganisms were then transferred from master plate onto surface of the dye containing BHI using the multipoint inoculation device.

Recovery of *Yersinia enteocolitica* from beef, milk and water.

One ml of *Yersinia enterocolitica* overnight culture (diluted to 7 $\log_{10}$ CFU ml^{-1}) was added to 99 gm of ground beef and/or 99 ml of milk and water, for artificial contamination. Samples were serially diluted and 0.1 ml of each dilution was spread on CIN, YTRA and tryptic soya plates by sterilized glass rod. The plates were incubated at 32°C for 24 hours.

Recovery of heat-injured *Yersinia enterocolitica.*

Broth cultures in tryptic soy broth (TSB) were separately incubated at 32°C for 24 h and diluted in peptone water to obtain viable cell counts 10^7 CFU/ml.

One hundred microliters of *Yersinia enterocolitica* culture suspension was added to, each of three test tubes containing 5 ml of peptone water . After inoculation, each tube was, immersed completely in a shaking water bath, and heated at 60°C for 1.5 min. The tubes were then cooled immediately in ice. Heat-injured cell suspensions were spread on YTRA and CIN and tryptic soy agar. The plates were incubated at 32°C for 24 h. and then the recovery was evaluated. (Kang.and Siragusa 1999 & Kang and Fung. 2000).

RESULTS AND DISCUSSION

On the slightly red YTRA medium, *Yersinia enterocolitica* produced yellow metallic sheen colonies is differentiable colonies whereas the other growing bacteria produced red color colonies on YTRA medium. Metallic sheen colonies of *Yersinia enterocolotica* may be related to unknown compound produced by *Y. enterocolitica* and reacted with 2,3,5- triphenyl Tetrazolium chloride while red color colonies of the other growing bacteria may be due to formation of formazan (Fig 2, Table 2)

The efficiency of YTRA medium may be related to the action of rose bengal as gram positive inhibitor (Fung and Miller, 1973) as shown in table 2, Tetrazolium structure is the five-member ring of one carbon and four nitrogen atoms. The dye may contain one or two of these structures as illustrated in Fig (1).

2-3-5-Triphenyl tetrazolium chloride (TTC) which turns red when reduced. This dye is also pH sensitive and the red coloration is observed only if the pH of the medium is above 6 or 7. Since the development of the red color is dependent on reduction of the dye as well as the pH of the medium, TTC can be used as either a redox indicator or a pH indicator. Medium in which TTC serves as a pH indicator was developed by Lederberg (1948) and has been modified by Winkleman and Clarke (1984). Medium in which TTC server as a redox indicator has been developed by Bachner and. Savageau, (1977).

Therefore all bacterial growth in the medium appears strictly pink or red and serves as a visual aide in the detection of bacterial motility.(Kelly and Fulton, 1953).

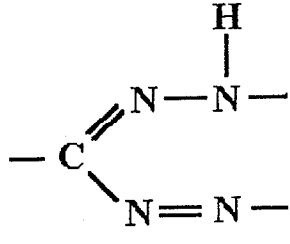
After 24 h of incubation, the different tested Y. enterocolitica strains grew well on CIN and YTRA media. YTRA medium was effective for the inhibition of all gram-positive bacteria but not for some gram-negative bacteria. Furthermore, the growth rate of tested Yersinia enterocolitica on YTRA medium was higher than that on CIN medium.

Microbial counts of Yersinia enterocolitica from inoculated ground beef, milk and water, showed significance difference between YTRA and CIN media.

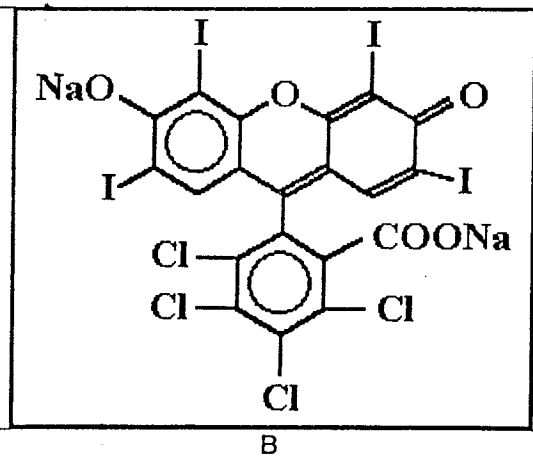
Table 2. Cultural Characteristics of *Yersinia enterocolitica* and other bacteria on YTRA medium after 24 h at 32 °C.

Organism	Cultural characteristics of the colonies		
	Growth	Colony color	average size (mm)
Yersinia enterocolitica			
Serotype O: 3	+	Metallic sheen	3.1
Serotype: O: 5	+	Metallic sheen	3.1
Serotype O : 8	+	Metallic sheen	3.1
Serotype O: 9	+	Metallic sheen	3.1
Yersinia pseudotuberclosis	+	Red	2.2
Yersinia intermedia	-		
Yersinia frederiksenii	+	Red	1.3
Yersinia kristensenii	+	Red	1.8
Escherichiacoli O157:H7	+	Red	2.1
Enterobacter aerogenes	+	Red	1.9
Enterobacter cloacae	+	Red	2.1
Klebsiella oxytoca	+	Red	2.3
Klebsiella pneumonia	+	Red	1.8
Proteus mirabilis	+	Red	2.0
Proteus vulgaris	+	Red	2.0
Pseudomonas aeruginosa	+	Red	0.6
Pseudomonas flurescencs	+	Red	0.6
Serratia marcesens	+	Red	2.4
Gram Positive			
Bacillus subtilis	-	-	-
Bacillus cerus	-	-	-
Staphylococcus aureus	-	-	-
Streptoccus pyogenes	-	-	-

+= growth , - = no growth



A



B

Fig 1: A, Tetrazolium compounds (general formula). B, Rose Bengal (Lillie, 1980.)

Approximately 90% *Yersinia enterocolitica* recovered from milk on YTRA agar while 79 % on CIN agar, 90.5 % *Yersinia enterocolitica* recovered from ground beef on YTRA agar while 74.5 % on CIN agar and 85.5% *Yersinia enterocolitica* recovered from water on YTRA agar while 72 % on CIN agar (table 3).
The recovery rate % was calculated by the following:

(*Y.enterocolitica* /Total Viable count X100%)

Table 4 showed the recovery rate of heat injured *Yersinia enterocolitica* strains from buffered peptone on YTRA and CIN Agar. On YTRA agar, the recovery rate of *Y. enterocolitica* were 90.25 % before heat treatment and 70.25% after heat treatment whereas on CIN were 78.25% before heat treatment and 46.25% after heat treatment.

Table 3. Recovery rate (%) of *Yersinia enterocolitica* on YTRA and CIN media

Yersinia enterocolitica strains	Media					
	YTRA			CIN		
	Sources			Sources		
	Milk	Beef	water	Milk	Beef	Water
Serotype O: 3	91	93	82	83	80	77
Serotype: O: 5	89	86	87	79	72	72
Serotype O : 8	86	91	83	81	76	73
Serotype O: 9	94	92	90	73	70	66
Mean %	90	90.5	85.5	79	74.5	72

Table 4. Recovery rate of heat injured *Yersinia enterocolitica* strains on YTRA and CIN **media**

Yersinia enterocolitica strains	YTRA		CIN	
	B. T[a]	A .T[b]	B. T[a]	A .T[b]
Serotype O: 3	93	73	84	52
Serotype: O: 5	89	70	80	50
Serotype O : 8	91	73	72	43
Serotype O: 9	88	65	77	40
Mean %	90.25	70.25	78.25	46.25

hree measurements before or after treatment (60°C for 1.5 min.).

[a]B. T, before treatment, [b]A .T, after treatment

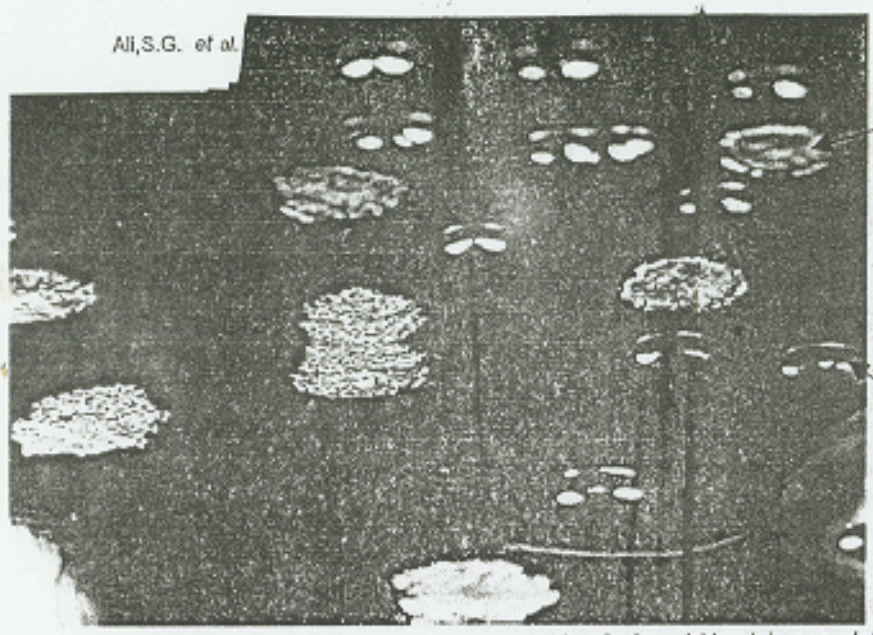


Fig 2: Mixed culture of *Yersinia enterocolitica* O: 3 and *Yersinia pseudotuberclosis*

In conclusion, these data indicate that, the YTRA agar is an efficient medium for recovery of *Yersinia enterocolitica* from beef, milk , water and sublethally heat-injured *Yersinia enterocolitica*.This new dye containing media can be applied for detection of pathogenic organisms in food ,water and dairy products.In regard to its efficiency for differentiation between the virulent *Yersinia enterocolitica* and other bacteria, it can be applied in food products safty tests.

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بيئه غذائيه ذات صبغة جديدة للتمييز بين بكتريا اليرسينيا لنتيروكوليتيكا وغيرها من البكتيريا الاخرى

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اشتملت هذه الدراسة على الحصول على بيئه غذائيه تحتوى على أصباغ جديدة للتمييز بين بكتريا اليرسينيا انتيروكوليتيكا والأنواع البكتيرية الأخرى وتضمنت الدراسة استخدام ثلاثة وعشرون صبغة على اثنين وعشرين سلالة بكتيريه تمثل اليرسينيا انتيروكوليتيكا والأنواع البكتيرية الأخرى. وقد تم تطوير بيئه غذائيه ا حتوت على نوعين من الاصباغ وعرفت ب ٥،٣،٢ تراى فينيل تيترا زوليم كلوريد البنجال البنفسجى (YTRA) و قد تم مقارنتها مع وسط سيفسيولدين ايرجاسان نوفوبايوسين (CIN) والذى يعتبر اكثر شيوعا من ناحية استخدامه لاختبار وعد اليرسينيا انتيروكوليتيكا .

وقد كان افضل تركيز لكل من ٥،٣،٢ تراى فينيل تيترا زوليم كلوريد والبنجال البنفسجى هو ٠،٠٥جم/لتر على الوسط Brain Heart Infusion Agar لغرض التمييز بين اليرسينيا انتيروكوليتيكا والأنواع البكتيرية الأخرى حيث اظهرت مستعمرات بريق معدنى لليرسينيا انتيروكوليتيك بينما الأنواع البكتيريات الاخرى المختبره السالبه لصبغة جرام قد تلونت باللون الاحمر بينما البكتريات موجبة لصبغة جرام لم تنمو على الاطلاق.

كان معدل الانتعاش فى اللحم المفروم واللبن والماء ذو نسبه عالية بين YTRA و CIN. أيضا كان معدل انتعاش الخلايا المتضررة من المعاملة الحرارية فى عينات اللحم المفروم واللبن والماء عالى جدا على YTRA وواضح بين الوسطان المستخدمان .