LACTOSE FERMENTING *SALMONELLA* TYPHIMURIUM, *SALMONELLA* STRATFORD AND *SALMONELLA* BLEGDAM

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Abstract

The general concept that lactose fermenting *Salmonella* (lac+ *Salmonella*) are rare may be correct; however, these strains can become endemic in a given geographic area. Failure to detect these strains presents a public health problem.This study report cases of lactose fermenting *Salmonella* Typhimurium (4 isolates), *Salmonella* Stratford (2 isolates) isolated from diarrheic calves and *S.*Blegdam (one isolate) isolated from diseased chicken. The strains are strong lactose fermenter and resemble *Escherichia coli* on primary plating media and in triple sugar iron agar.The aim of this study was to compare the capacity of 4 different selective media:- Xylose Lysine Desoxycholate agar (XLD), novobiocin – brilliant green – glucose agar (NBG), modified semisolid Rappaport –Vassiliadis agar (MSRV) and Bismuth sulphite agar (BS) to detect the growth of lactose fermenting *S.* Typhimurium, *S.* Stratford and *S.*Blegdam inoculated in milk replacer. They were cultured, in parallel, on these media, both by direct cultivation and after selective enrichment in selenite broth. The performance of BS was the excellent medium of all media used followed by XLD in combination with lysine iron agar (LIA). On MSRV only two strains grew and only one produced swarming. On NBG after 48h in the cases where there was growth, the number of colonies was much lower and the rest were inhibited. Antibiotic sensitivity pattern differed between lactose -positive *Salmonella* isolates and lactose-negative *Salmonella* Typhimurium standard strain ATCC 14028. Lactose-positive strains showed higher degree of resistance than lactose-negative strain. The differences in resistance were seen in the case of erythromycin, chloramphenicol, streptomycin and tetracycline.

INTRODUCTION

Salmonellosis continues to be a major health problem, and its diagnosis most often involves direct detection of bacteria by culture or, by PCR after enrichment. Isolation of *Salmonella enterica* on selective culture media is different from PCR in that it allows further identification of the bacteria and antibiotic susceptibility testing, which are important for disease control (*Gaillot et al.,1999)***.** Thus, wide variety of selective differential agar media have been developed for this purpose.

Characteristically salmonellae are non-fermenters of lactose and this inability to ferment lactose is one of the important laboratory diagnostic criterions to distinguish them from other members of the family *Enterobacteriaceae.* However some

salmonellae are known to ferment lactose, in this aspect Chugh., (1978) suggested that this characteristic found in these *Salmonella* is attributed to Lac + plasmid either as a separate entity or in conjunction with drug resistance. Chander *et al*. (1980) suggested that certain *Salmonella* serotypes viz. *S*. Anatum, *S*. Tennessee, *S*. Newington, *S*. Schwarzendgrund, *S*. Typhimurium and *S*. Oranienburg ferment lactose, they have attributed this atypical behavior in Salmonella to plasmids .

Farmer *et al*. (1984) found that about 1% of *Salmonella* enteric and 15-85 % *Salmonella* arizonae do ferment lactose; also, Mackie and McCartney., (2011) recorded that lactose positive *Salmonella* comprise less than 1% of the genus *Salmonella* . The recent revision of ISO 6579 for *Salmonella* testing is a result of the growing incidence of lactose positive *Salmonella* spp isolated from cases of food poisoning.

Most laboratories use only enteric plating media which contain lactose, so these types of colonies were omitted in diagnostic examination for *Salmonella*, thus their identification would pose a problem for most clinical laboratories.

The purpose of the present study was to compare the capability of four plating media that does not key on lactose fermentation for isolation of lactose-fermenting strains of *Salmonella.* The second aim, was to compare the antibiotic sensitivity between lactose-fermenting strains of *Salmonella* and non-lactose fermenting strain (*Salmonella* Typhimurium ATCC 14028).

MATERIALS AND METHODS

Isolates:

Lactose-fermenting strains of *Salmonella* were isolated from diarrheic calves and chicken fecal samples. Primary isolation was made directly on MacConkey agar and Salmonella Shigella (S.S) agar medium, incubated aerobically at 37 °C. After overnight incubation, the isolated lactose fermenting colonies were cultivated on triple sugar iron agar (T.S.I). After overnight incubation, the isolated separate colonies were identified by colonial morphology, Gram staining and traditional biochemical tests, (Oxidase test, indole, urea and citrate tests) as well as commercial standard biochemical methods (API 20 E strip bioMerieux) for *Enterobacteriaceae* was used. Isolates identified biochemically as suspected salmonellae were confirmed serologically by agglutination. *Salmonella* isolates were serotyped after overnight culture on nutrient agar slopes (Oxoid) by agglutination with anti-O and anti-H antisera (SIFIN, Berlin, Germany) by using the Kauffmann-White scheme (Popoff**.,**

2001). Spontaneous agglutination was ruled out by testing the isolates with saline without the antisera.

Culture media:

Four selective media that does not key on lactose fermentation, Xylose Lysine Desoxycholate agar (XLD), novobiocin – brilliant green–glucose agar (NBG), modified semisolid Rappaport – Vassiliadis agar (MSRV) and Bismuth sulphite agar (BS), were obtained from Oxoid, Ltd and Conda. They were supplied in powder form and prepared according to the manufacturer's instructions. All media were tested for sterility and culture response using the following reference strains: *Salmonella* serovar Typhimurium ATCC 14028 and *E. coli* ATCC87739. Prepared media were stored in a refrigerator and used within 7 days of preparation. Selenite broth (bioMerieux) was used for sample enrichment. Mueller-Hinton plates were used as growth controls

Inoculation:

Lactose - fermenting strains of *Salmonella* were inoculated separately one cfu /25 g milk replacer. They were streaked onto the four media, both directly and after selective enrichment in selenite broth, as follows. Briefly: 25 g of inoculated milk replacer samples was suspended in 225 ml of 0.1% peptone water and incubated for 24 h at 37°C; then subculture onto four selective culture media plates and spread with sterile loops for single colonies growth. In MSRV, the inoculum was plated in the center of the plate, and a reading taken after 24 h of incubation at 43°C to detect swarming. Concurrently, 1 ml of each peptone suspension was inoculated into 10 ml selenite broth to be incubated overnight at 37°C and then sub cultured onto four media agar plates (50 µl per plate). All plates were incubated for 18 to 24 h at 37°C aerobically. Suspected colonies (lac+ colonies , with and without black center) were subculture on lysine iron agar (LIG) and Triple sugar iron agar (T.S.I) for screening of lactose -positive colonies for H2S production.

Antimicrobial susceptibility testing

The disc diffusion method (NCCLS., 2002) was used for antimicrobial susceptibility testing. Fourteen different antibiotics were used: streptomycin (10ug), chloramphenicol (30ug), tetracycline (30ug), norfloxacin (10ug), levofloxacin (5ug), tobramycin (10ug), amikacin (30ug), ampicillin (10ug) gentamicin (10ug), sulphamethoxozole (25ug), amoxycillin (25ug), nalidixic acid (30ug), cefoperazone (75ug) and erythromycin (15ug).

RESULTS

Isolation of lactose positive *Salmonella* serovars from diarrheic calves and chickens .

Lactose fermenting *colonies* were isolated by primary plating medium onto MacConkey agar and S.S agar after 24 hr of aerobic incubation at 37°C. Isolates are strong lactose fermenter and resemble *Escherichia coli* on MacConkey agar , S.S and in triple sugar iron agar. It was identified using colony morphology, Gram staining, Oxidase test, indole, urea, citrate, and API 20E. It was identified biochemically as suspect *Salmonella* serovars *by* API 20E. It was confirmed *as Salmonella* Typhimurium after positive agglutination reaction with *Salmonella* antisera polyvalent A-S and A-I and antiserum somatic O 1,4,(5),12 flageller antigen phase 1 :I and phase 2 :1 and 2. *S.* Stratford somatic O1,3,19 flageller antigen phase1:I phase 2: 1 and 2. and *S.* Blegdam, somatic O9,12 flageller antigen phase 1:g,m,q phase 2:

Comparison *of* four plating media for the isolation of lactose-fermenting strains of *Salmonella:*

Selenite broth enrichment giving maximum suppression of contaminating organisms. Selenite broth gave a lower occurrence of false positive results than 0.1% peptone water, A false-positive lactose ferment colony was a colony that looked like lac + *Salmonella* on the XLD, NBG, MSRV and bismuth sulfate agar plates but that was not *Salmonella* upon further biochemical characterization and serological typing.

On XLD , lactose-fermenting strains of *Salmonella* grow on this medium after 24 hr of incubation in the form of colorless colonies with or without black center. On the triple sugar iron agar the reaction was acid slant / acid but /gas.

On LIA they gave a positive reaction to the test for lysine decarboxylase purple color and H2S production in the form of a black precipitate from all colonies that subsequently were identified as *S.* Typhimurium , *S.* Stratford and *S.* Blegdam.

In NBG *,* none of the strains of Lac + *Salmonella* were capable of growing within 24 h of incubation. After 48 h in the cases where there was growth, the number of colonies was much lower and the rest were inhibited.

On Modified Semisolid Rappaport Vassiliadis agar (MSRV), only two *S.* Typhimurium strains grew , one with swarming and the other without .

On bismuth sulfate agar, all serovars grow on this medium after 24 hr of incubation in a form of black, medium sized colonies, with some metallic brilliance and characteristic blackening of the medium under colonies.

Table 1. Growth of 4 isolates of *S.* Typhimurium, 2 isolates *S.* Stratford and one isolate *S.* Blegdam on four culture media.

Serovars	XLD	NBG	MSRV	B.S
S. Typhimurium				
1	Colorless colonies with black center	Growth at 48h	Growth without swarming	Black colonies with metallic sheen
2	Colorless to opaque colonies with black center	Growth with reduction at 48 h	Growth with swarming	Black colonies with metallic sheen
3	Colorless colonies without black center	Growth at 48h	No growth	Black colonies with metallic sheen
4	Colorless colonies with black center	Growth with reduction at 48 h	No growth	Black colonies with metallic sheen
S. Stratford				
1	Colorless colonies without black center	Growth with reduction at 48 h	No growth	Black colonies with metallic sheen
2	Colorless to opaque colonies without black center	Growth with reduction at 48 h	No growth	Black colonies with metallic sheen
*S.*Blegdam	Colorless to opaque colonies without black center	Growth at 48h	No growth	Black colonies with metallic sheen

XLD: Xylose Lysine Desoxycholate,– **NBG**: novobiocin brilliant green–glucose agar **MSRV**: modified semisolid Rappaport –Vassiliadis agar- **BS**: Bismuth sulphite agar

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Antimicrobial susceptibility testing

Salmonella Typhimurium, *S.* Stratford and *S.* Blegdam showed higher degree of resistance than *Salmonella* Typhimurium standard strain. The differences in resistance were seen in the case of erythromycin, chloramphenicol, streptomycin, and tetracycline. Both lactose-positive and standard strain were sensitive to norfloxacin, nevofloxacin, tobramycin, amikacin, gentamicin, cefoperazone, and nalidixic acid. They were resistant to amoxycillin, sulphamethoxazole and ampicillin.

Table 2. Antimicrobial susceptibility testing

Antibiotic	*Salmonella* Typhimurium isolates				*S.* Stratford isolates		*S.*Blegdam isolate	*Salmonella* Typhimurium standard strain
	1	2	3	4	1	2		
1-Amoxycillin	R	R	R	R	R	R	R	R
2-Amikacin	S	S	S	S	M	M	S	S
3-Ampicillin	R	M	R	R	R	R	R	R
4-chloramphenicol	R	M	R	R	R	R	R	S
5-cefoperazone	S	S	S	S	S	S	S	S
6-Erythromycin	R	R	R	R	R	R	R	S
7-Gentamicin,	S	S	S	S	M	M	S	S
8-Levofloxacin	S	S	S	S	S	S	S	S
9-Nalidixic acid .	S	S	S	S	S	S	S	S
10-Norfloxacin	S	S	S	S	S	S	S	S
11-Streptomycin	R	R	R	R	R	R	R	S
12-Sulphamethoxazole	R	R	R	R	R	R	R	R
13-Tetracycline	R	R	R	R	R	R	R	S
14-Tobramycin	S	S	S	S	S	S	S	S

S: sensitive M: moderate R: resistance

DISCUSSION

Biochemical tests form an important backbone for the identification of *Salmonella* even though, organisms of the *Salmonella* group have most of the tests similar to family *Enterobacteriace* biochemically and antigenically, they differ in one of the important property i.e. inability of lactose fermentation. However. There are certain *Salmonella* serotypes, which can ferment lactose and therefore, raise a possibility of wrong reporting of strains during routine laboratory analysis, which is based on lactose fermentation.

In this study, lactose fermenting colonies were isolated on MacConkey agar and S.S agar media after 24 hr of incubation at 37°C. It was identified biochemically as suspected *Salmonella* serovars and confirmed serologically as *Salmonella* Tyhimurium, *Salmonella* Stratford and *Salmonella* Blegdam. It is important to point out that all isolates were positive for ONPG test by using API 20, they gave yellow color. ONPG is used for detection of B-galactosidase activity . This compound is normally colorless. However, if B-galactosidase is present, it hydrolyzes the .ONPG molecule into galactose and orthonitrophenol. The latter compound has a yellow color. Galactosidase is required for lactose utilization. In this aspect, (Timoney *et al.,* 1980) isolated lactose-positive and chloramphenicol-resistant strain of *Salmonella*

Typhimurium of high virulence from an outbreak of enteric and septicemic salmonellosis in veal calves. Also, Tyc *et al*. (1989) suggested that lactose –fermenting stains of *Salmonella* belonged most frequently to the following genera : S. Agona, S. Enteritidis, S. oranienburg, S. Typhimurium, and S. Goldcoast.

In this study, it was found that lac+ strains exist although this study did not record any result on the percent of occurrence of lac+ *Salmonella*. Information on the true incidence of lac+ *Salmonella* is needed. Ewing (1972 and 1986) reported that lac+ Salmonella comprised less than 1% of all *Salmonella* examined at the center for disease control, However, the actual incidence may be higher because most laboratories use only enteric plating media which contain lactose and lac+ *Salmonella* are indistinguishable from *E. coli* on these media.

Generally speaking, true incidence of lac+ Salmonella should come from many sources widely distributed geographically and from the points of view of human clinical and veterinary microbiologists, it is important to recognize that lac+ strains exist and then to used particularly useful method for detecting lac+ *Salmonella*.

The accurate differentiation between lactose and non-lactose fermented *Salmonella* must be made on the basis of medium does not key on lactose fermentation and serological agglutinations. Therefore, the aim of this work was to study the behavior of lactose-positive *S.* Typhimurium, *S.* Stratford and *S*. Blegdam in 4 differential Plate media that does not key on lactose fermentation. It is important to point out that isolated lac+ Salmonella were inoculated in milk replacer samples not in animal feed samples. In this aspect (Donough *et al*., 2000) suggested that the newly acquired ability of *Salmonella* to use lactose may confer on it a selective advantage in an environment where lactose is plentiful, i.e., milk replacer in the environment and intestine of the calf.

Xylose Lysine Desoxycholate medium relies on Xylose fermentation, lysine decarboxylation and production of hydrogen supplied .*Salmonella* exhaust the xylose and decarboxylate the lysine, thus altering the pH to alkaline. In case of Lac+ *Salmonella,* high acid level produced by fermentation of lactose and sucrose, prevents Lysine –positive *Salmonella* from reverting the pH to an alkaline value and blackening of the medium until after 24 to 48 h of incubation. Lac+ Salmonella isolated on XLD medium as colorless colonies with or without black center (Suspect colonies) were then

inoculated into lysine iron agar and triple sugar iron agar for screening of lactose -positive colonies for H2S production. In this aspect Ewing*.,* (1986) suggested that the only recognized groups of *Enterobacteriaceae* which regularly decarboxylate lysine rapidly and which produced large amount of hydrogen supplied are members of the *Salmonella* and Arizone groups.

It is important to point out that screening of lactose -positive colonies for H2S production was not possible with triple sugar iron agar, the reaction was acid slant / acid but /gas. However, in lysine iron agar, H2S production in the form of a black precipitate was visible from all colonies that subsequently were identified as lac + *Salmonella*. This result could be explained by Donough *et al*. (2000) who suggested that the black iron sulfide precipitate that would normally be seen from non – lactose – fermenting salmonellae in T.S.I medium was dissolved due to acid production by lactose fermenting *Salmonella* in this medium.

From this result it was concluded that using the combination of XLD and LIA medium a greater discrimination can be made between lactose fermenting *Enterobacteriaceae (Escherichia coli*, Proteus, Kebsiella and Citrobacter) and lactose fermenting *Salmonella.*

Novobiocin –brilliant green -glucose agar is based on H2S production and glucose fermentation. The result was not good with NBG because there is the drawback that most strains were not capable of growing within 24h and after 48 h, in the cases where there was growth, the number of colonies was much lower

Modified semisolid Rappaport Vassiliadis agar is a selective enrichment medium for the isolation of *Salmonella.* MSRV is based on the phenomenon of Swarming, exhibited by motile bacteria in low concentrations of agar (Aspinall *et al* ., 1992).

In bismuth sulfite agar medium, bismuth sulfite acts together with brilliant green as a selective agent by suppressing the growth of coliforms, whilst permitting the growth of salmonellae and the Interpretation of the colors is easy. The few false –positive cultures obtained on bismuth sulfite agar medium were due to *Pseudomonas* species, which could be easily distinguished from salmonella species by the oxidase test. Thus, there is a clear –cut differentiation of lac+ *Salmonella* species from accompanying organisms. On the basis of these results, bismuth sulfite agar medium can be recommended for the isolation of lac+ strains *Salmonella* species.

In conclusion, bismuth sulfite agar medium is excellent for the detection and presumptive identification of lac+ Salmonella in the routine analysis of laboratories for diagnostic examination of salmonella. Moreover, bismuth sulfite agar medium is more specific than XLD agar and therefore, reduces considerably the work load in diagnostic laboratory.

In this study the antibiotic sensitivity result indicated that Lactose-positive strains showed higher degree of resistance than lactose-negative *Salmonella* Typhimurium standard strain. The differences in resistance were seen in the case of erythromycin, chloramphenicol, streptomycin, and tetracycline. In this aspect, Zimmer (1989) found that the antibiotic sensitivity pattern differed between lactose-positive and lactose-negative strains. Lactose-positive strains showed higher degree of resistance than lactose-negative strains. The differences in resistance were seen in the case of chloramphenicol, doxycycline, gentamicin and tetracycline. Both lactose-positive and lactose-negative strains were sensitive to colistin, neomycin, nitrofurantoin and nalidixic acid. They were resistant to ampicillin, cloxacillin, rifampicin, streptomycin, sulfathiazole and biseptol.

Generally speaking, in this study lac+ strains of *Salmonella* were isolated from diarrheic calves and diseased poultry. These lactose-positive strains showed higher degree of resistance than lactose-negative *Salmonella* Typhimurium standard strain, which reflect the importance of isolation and identification of lac+ strains of *Salmonella*. Bismuth sulfite agar or combinations of XLD and lysine iron agar are especially useful in the isolation and identification of lactose-positive *Salmonella*. Our future work will deal with understanding the genetics of the lac gene and the resistance determinants found in lac+ strains of *Salmonella.*

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السالمونيلا الإيجابية لتخمرسكر اللاكتوز
(تيفميوريم ، ستراتفورد و بليجدام)

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المفهوم العام بندرة عترات ميكروب السالمونيلا القادرة علي تخمر سكر اللاكتوز قد يكون صحيحا ولكن هذه العترات قد تصبح مستوطنة في منطقة جغرافية معينة وعندما لايتم التعرف البكتريولوجي لهذه العترات فذلك قد يمثل مشكلة تهدد الصحة العامة .

سجل هذا البحث وجود عترات سالمونيلا ايجابيه لتخمر سكر اللاكتوز وهم أربعة عترات سالمونيلا تيفميوريم عترتان سالمونيلا ستراتفورد معزولين من عجول مصابة باسهال وعتره واحدة سالمونيلا بليجدام معزوله من طيور مصابه. هذه العترات تتماثل مع الميكروب القولوني في العزل المبدئي وذلك باستخدام الميديا الإنتقائية الأولية وباستخدام الأجار ثلاثي السكر والحديد .

لذلك كان الغرض الأساسي من هذا البحث هي المقارنة بين قدرة أربعة أوساط منابت بيئية انتقائية وهي: Xylose lysine Desoxycholate (XLD), Novobiocin-Brilliant Green-glucose agar (NBG)_Modified Semisolid Rappaport-Vassiliadis agar (MSRV) and Bismuth sulphite agar (BS) للتعرف علي عترات السالمونيلا تيفميوريم ، سالمونيلا ستراتفورد ، سالمونيلا بليجدام التي تم زرعها في بدائل ألبان وذلك عن طريق الزرع مباشرة أو بعد التحضين في ميديا انتقائية selenite -F broth .

أوضحت النتائج أن BS هي أفضل وسط منبت بيئي يليه استخدام XLD مع LIG للتعرف علي عترات السالمونيلا الإيجابيه لتخمر سكر اللاكتوز في حين نمو عترتان فقط علي MSRV وعترة واحدة استطاعت أن تنمو بشكل متحرك. في حالة استخدام NBG تبين حدوث نمو بكتيري ضعيف جدا بعد مرور 48 ساعه من التحضين .

أوضحت النتائج أن نمط اختبار الحساسيه يختلف بين عترات السالمونيلا الإيجابية والسلبية (ATCC14028) لتخمر سكر الاكتوز، فكانت درجه المقاومة للمضادات الحيوية المستخدمة أعلي في عترات سالمونيلا ايجابية لتخمر سكر الاكتوز عن ميكروب السالمونيلا السلبي (ATCC14028) ولوحظ هذا الاختلاف باستخدام الإريثروميسين والكلورمفينكول وستربتوميسين وتتراسيكلين .

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