Isolation and characterization of *streptococcus bovis* from rumencontent of awassi sheep in iraq

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Abstract

This is the first study in Iraq aimed to isolate and characterize *Streptococcus bovis* from rumen of Awassi sheep .Ten sheep with different ages fed on grain base diet for three days were used to collect 20 ruminal fluid samples twice at fourth and fifth days by rumenocentesis method , samples cultured on selective media (Modified membrane-bovis agar(M-BA) ,broth of basal medium and modified blood brain heart infusion) ,the isolates were identified according to their morphological, physiological ,biochemical tests and serological by Lancefield group.Cultural characteristic on the selective medium M-BA showed two types of streptococci :first type (23) comprised the majority of isolates ,this type characterized by high acid producing streptococci formed mucoid ,creamy ,orange –centered colonies and second type (4) characterize by low acid producing formed small white colonies .Morphologically the isolates were identified as gram positive ,the cells were oval or spherical , singles, pairs & short chains of 4 to 8 cells. The organisms were found to full under the Lancefield group D.All isolated bacteria grew on broth of basal medium post incubation at 45C˚ ,but not grew at 10C˚ and 50C˚. Adding of 2% NaCl permit the growth, while in 6.5% NaCl didn't grow. And did not grew on adding 0.04 % Potassium tellurite .All isolates produced lactic acid but ammonia production from arginine was negative, no hemolysis on blood agar . All isolates ferment starch, lactose, fructose, glucose, raffinose and cellobiose.The results of culturing and Physiological and biochemical tests showed that 27 isolates has the characterictices of *Streptococcus bovis* in 3 types (S_1,S_2 &S_3) ,first type of colony divided into two strain ($S_1$15)($S_2$8)according to difference In arabinose. And (S_3) called on the second type white pigmented isolates which did not ferment inulin

Introduction

Streptococcus bovis was recognized as an important ruminal bacterium in terms of carbohydrate fermentation in early 1950s (1) but was not classified as an active proteolytic bacterium until 30 years later. In different animals the population of this bacterium remains lower under normal feeding (forage diet) ,but its numbers can increases significantly following dietary change when large of starch or sugars were added to the diet (2,3,4).*Streptococcus bovis* is a gram- positive bacteria, it was a ruminal bacterium that can produce large amounts of lactate and its involved in the onset of ruminal acidosis (1,5) .During period of rapid growth , lactic acid production by *S.bovis* can cause decline in rumen pH to the point where growth rates of other rumen bacteria are inhibited (6,7,8).Kamra (9) showed that the anaerobic conditions in the rumen are maintained by gases generated during fermentation e.g. Carbone dioxide , methane and traces hydrogen ,and some of the oxygen with feed consumed by the animal is utilized by the facultative anaerobes present in the rumen and thus a perfect anaerobic condition is generated and maintained.Hungate (2) was developed technique "roll tube or hungate method"for cultivation of strict anaerobes in rumen, and this technique is very convenient for isolation of *S. bovis*.This study aimed to determine the presence of *S. bovis* in rumen of sheep fed with grain base diet and to characterize the isolates by different bacteriological, biochemical and serological methods.

Material and methods

1- **Animals:-** Ten Awassi sheep in different ages were run as a single flock on alfa alfa and green berseem grass pasture ,sheep were housed indoors in individual pens and fed on high grain-based diet with access to fresh water at all times for three days were used for isolation of *Streptococcus bovis* at fourth (10 samples) and fifth days(10 samples) from ruminal fluids of these animals.

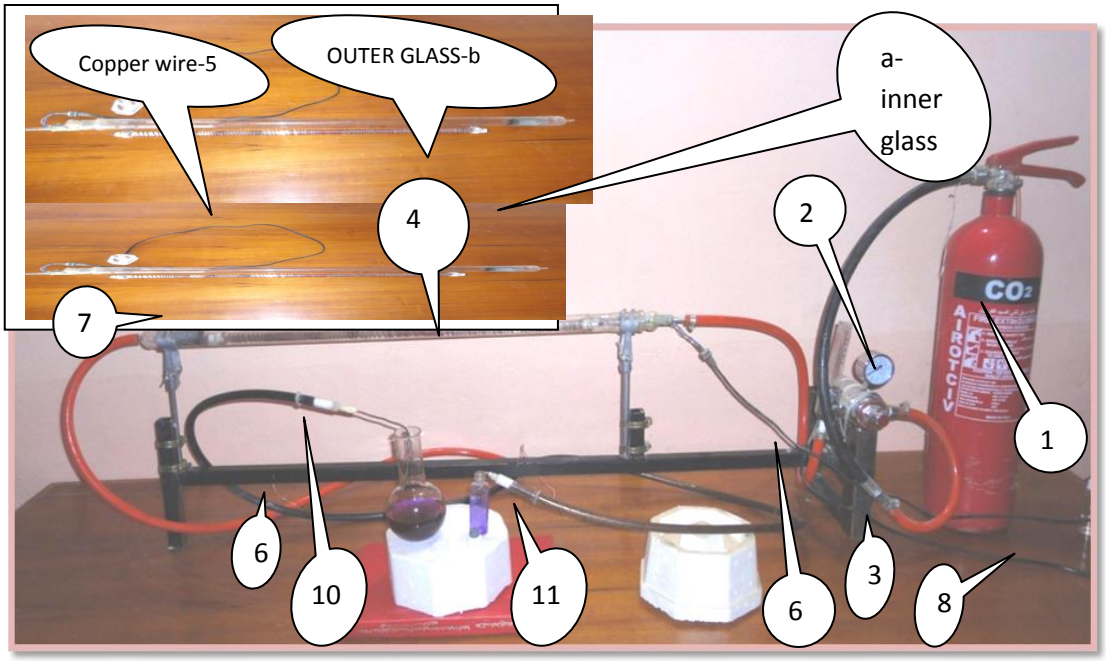
2- Local modified anaerobic apparatus

A local anaerobic apparatus was designed by As'ad jassim and Afaf Abdulrahman similar to hungate apparatus(2) with some modification and used for purification of commercial carbon dioxide gas by passage on heated copper column to forming copper oxide (Picture No1).

The apparatus consist from:-

1-CO_2 tube 5Kg (Aeroticiv ,Italy)2-CO_2 regulator3-Rubber conduit hose4-the pyrex glass column consist of an a-inner glass (65 x1.5cm) inserted with heater are used to attach the electrical supply

b-outer glass(75x 3cm) was provided by glass stopper was contain two opens for enter CO_2gas and electrical wire to apparatus.5-Copper wire 5 meter is wrapped around an inner glass6- Holder 7-filter 8-electeric supply 9-Conduit hose was divived into two leads(10,11) provided with needles gauge 20 at its end for flushing the flask and roll tubes at same time with O_2 free CO_2.



Picture No. (1) local anaerobe apparatus

3-Solution and media:-

a-Reducing solution prepared a freshly:- each 1 ml containing 0.026 g cysteine – HCl and 0.026 g $Na_2S.9H_2o$ after boiling then pre –reduced by bubbling with CO_2 gas on ice until cold . t This solution was used for preparation of media

b-Carbonate solution: - (8%Na_2CO_3 solution) , used for preparation of media .

c-Resazurin solution:-0.1g resazurin in 100 ml distilled water.

d- The anaerobic dilution solution(ADS) contained 7.5% mineral solution I (0.6% K_2HPO_4) and 7.5 % mineral solution II [1.2 % NaCl , 1.2 % $(NH_4)_2SO_4$, 0.6% KH_2PO_4 ,0.12 % $CaCl.2H_2O$,0.25% $MgSo_4.7H_2O$], 0.1% Resazurin solution. Make up to volume less 7% with distilled

water , autoclave at 121 C˚-15Ib pressure for 15 min. Gassing directly with CO2 then add 2% cysteine solution and 5% Na_2Co_3 solution ,continue gassing until reduced and sealed
(10).

e- Modified membrane- bovis agar (M-BA) medium was prepared according to (11) is highly selective medium for cultivate of S. bovis ,the medium contained (g/100ml):- 0.2 $(NH_4)_2SO_4$; 0.01 yeast extract ;0.4 inulin ;0.3 raffinose hydrate ;0.1 sodium beta-glycerophosphate ;0.005 sodium azide ;0.01 NaCl ;0.005bromocresol purple ;0.02 KCl ;0.02$MgSo_4.7H_2o$;0.01 K_2HPO_4 and 1.2 agar . After dissolving the ingredients, 7 ml of 8%$NaCo_3$ and 1ml of reducing solution were added .the solution was heated gently to dissolve all ingredients and the pH was adjusted to 6.8 . **f- Basal medium broth** was prepared refer to (12).

The broth prepared(Per liter)from:- 292 mg $K_2H\ PO_4$; 292 mg KH_2PO_{44} ;480 mg $(NH_4)_2\ SO_4$;480 mg NaCl ; 100 mg$MgSo_4.7H_2o$;64mg $CaCl.2H_2o$;4g $NaCo_3$; 0.6 cysteine hydrochloride ,1 g trypticase ;0.5 yeast extract and 4 g glucose . The broth was adjusted to pH 6.7 and the final pH was never less than 6.5 .

g- Modified blood brain heart infusion. was modified by adding reducing solution to brain heart infusion ,after sterilization the tubes contained medium applied in water bath at 45C˚,then 5% of sheep blood was added (13) the medium was prepared anerobically same as above media

i- Modified MRS-agar medium :- A semi-selective MRS medium, (Hi-media) was modified by adding a freshly prepared reducing solution (1ml) per 100 ml of media (14) . the medium was prepared anerobically same as above

4- **Removal of oxygen from the media and other solutions**

The way to remove oxygen from heat – stable solution is to boil it vigorously for about one minute .then add reducing agent After boiling the media and solutions leave to cool and flushing with purified CO_2 gas which is prepared in our anaerobe apparatus. Adequate amount from the media in the flask are transferred to roll tubes(this tubes sealed with rubber stopper and closed with screw in cap) that it contain agar these procedure under CO_2 gas . These tubes containing medium can be sterilized in autoclave at 105C˚ for 45 minutes .after sterilization tor rolling tubes containing sterile medium with agar are rolling on ice or cold water which allowed the agar medium to distribute as a thin layer over the internal surface of test tubes ,and a continuous flushing of CO_2 during this procedure.

4-Culturing of ruminal fluid on prepared media.

Twenty ruminal fluid samples were collected from sheep fed on grain base diet at fourth and fifth days by rumenocentesis method, the fluids were emptied into plastic container , flushed with purified CO_2 and transported immediately to the laboratory . At laboratory the fluids were mixed well and a subsample of 5 ml of rumen fluids were mixed with 45 ml of anaerobic dilution solution (ADS) in a plastic container provide with rubber stopper ,flushed with CO_2

The homogenized samples serially diluted 10 –fold in ADS to a final dilution of 10^7. Three dilutions ,10^5 ,10^6 and 10^7 ,were used to inoculate M-BA medium in hungate roll tubes in triplicate for each dilution .the tubes were incubated at 39C^o for 48hours .after incubation single colonies were picked from the roll tubes by using Pasteur pipette and inoculated into basal medium (BM) broth dispensing in hungate tubes were incubated at 39C˚ for 6-10 hours , this procedure was repeated at least twice to increase purification.

4- Stains:- Gram□s stain, Hiss capsular stain (15).

5-Physiological and biochemical tests were performed to insure the diagnosis of *Streptococcus bovis* include:-

Growth at different temperatures (10C°, 45C°, 50 C°), Growth in 2% and 6.5 % NaCl,
Catalase test, Carbohydrates fermentation (16) .Growth on potassium tellurite (0.04 %) (17).All previous testes were showed on a broth of basal medium which prepared anaerobically. Determination of ammonia produced from arginine (18) , Measuring of lactic acid accor according to (19), Hemolysis is detected in anaerobic roll tubes contains modified blood brain heart infusion agar (13) .

6- Lancefield grouping of streptococcus:-

The streptococcal isolates were also tested serologically for Lancefield group identification, using a rapid latex test system. (Massas Virginia, USA)

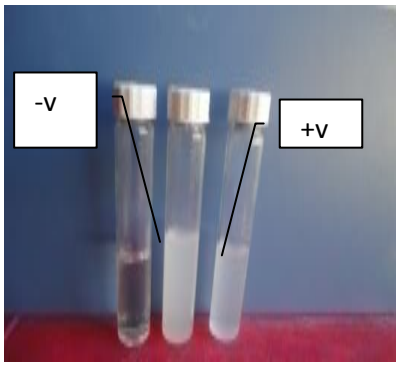
Results

Culture characteristic

Twenty seven isolates of streptococcus bovis were identified from the 20 ruminal fluid samples of sheep. Cultural characteristic on the selective medium M-BA showed two types of streptococci : type 1(23) comprised the majority of isolates which characterized by high acid producing *S.bovis* formed mucoid ,creamy ,orange – centered colonies (picture 2) and were similar in all respects except for the arabinose fermentation .while the second type(four isolates) characterize by Low acid producing formed small white colonies (picture 3).In basal broth the bacterium grew rapidly during 6 hours and change medium from clear colour into milky color (picture 4). All isolates showed no hemolysis on modified blood brain heart infusion (picture 5). Morphologically the organism was identified as gram positive and the stain more concentrated in the capsule ,the cells were oval or spherical , pairs , short chains of 4 to 8 cells and singles.(Picture 6)

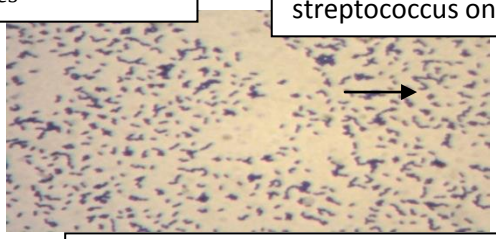
Picture (2)High acid producing S. bovis colonies

Picture (3)Low acid producing S. bovis colonies



Picture (4)Growing of streptococcus on basal broth

Picture .(5) Colonies of streptococcus on Modified blood brain heart infusion



Picture (6). Gram stain of *Streptococcus*

Results of Physiological and biochemical tests:-All isolated bacteria grew on basal broth post incubation at 45C˚ ,but not grew on incubation at 10C˚ and 50C˚ . Adding of 2% NaCl to the broth of basal medium permit the growth while in 6.5% NaCl didn't grow. And did not grew on 0.04 % Potassium tellurite .All isolates produced lactic acid but ammonia production from arginine was negative.Each isolates fermented starch ,lactose ,fructose ,inulin ,glucose ,raffinose and cellobiose and none of isolates ferminted of xylose ,mannitol, glycerol or sorbitol but some isolates gives positive result to arabinose . White pigmented isolates did not ferment inulin.The results of culturing and Physiological and biochemical tests showed that27 isolates has the characterictices of *Streptococcus bovis* in 3 types (S_1,S_2 &S_3) as showed in table (1)

Serological typing:-All isolates were belong to group D by using Lancefield grouping

Table -1. Showed the charactization of *Streptococcus bovis*

Characterization	S1 (15 isolates)	S2 (8 isolates)	S3 (4 isolates)
Colony on M-BA	Yellow,large,,creamy orange in the center	Yellow large ,creamy orange in the center	White small pinpoint
Catalase	Negative	Negative	Negative
NaCl 2%	Growth	Growth	Growth
NaCl 6.5%	no growth	No growth	No growth
Growth at 45C˚	Growth	Growth	Growth
Growth at 10C˚ ,50C˚	no growth	No growth	No growth
Potassium tellurite 0.04%	no growth	No growth	no growth
Production of NH3 from arginine	-ve	-ve	-ve
Production of lactic acid	High	High	Low
Blood hemolysis	No hemolysis	No hemolysis	No hemolysis
starch ,lactose ,fructose, glucose,raffinose&cellobiose	+ve	+ve	+ve
Inulin	+ve	+ve	-ve
xylose ,mannitol, glycerol and sorbitol	-ve	-ve	-ve
Arabinose	-ve	+ve	-ve

Discussion

Streptococcus bovis is an indigenous resident in the gastrointestinal tracts of both humans and animals; it is one of the major causes of bacterial endocarditis and has been implicated in the incidence of human colon cancer (20). Certain feeding regimen in ruminants can lead to overgrowth of *S. bovis* in the rumen resulting in the over-production of lactate and capsular polysaccharide causing acute ruminal acidosis and bloat (21,22, 9).In our study the change of diet from roughage and alfa alfa to high grain base diet cause an increase in the number of *S. bovis* isolated from ruminal fluid of ten awassi sheep. this compatible with (1,2)they found that *S. bovis* is a normal inhabitant of the rumen ecosystem are rarely greater than 10^7/ml ,but its numbers can increase to 10^{10} when large quantities of starch or highly fermentable carbohydrates are added to the diet. Allison *et al*,(3) found that the rapidly switching from a forage to a grain diet effect

the ruminal fermentation and can become unbalanced ,resulting in digestive disorders which may include lactate acidosis.Many researcher explained different methods for isolation of *S.bovis* under strict anaerobe culture by a roll-tube (2,4,23). So this study need to design anaerobe apparatus for increase purity of commercial CO_2 gas , which give O_2 free CO_2 used in all steps of preparing media and culturing of organism.Results of culturing showed three types of streptococcus on a selective media MBA medium, two types colonies showed large yellow colonies with orange center and that were predominant in sheep fed with grain base diet and the third type showed small white pinpoint colonies , this similar as mentioned previously by (14,24).All isolated *S. bovis* produce lactic acid(two type produce high amount and the third type produce low amount) this compatible with study of (5) they found that the main L-lactate producers were those isolates closely related to *S. bovis*, *S. ruminantium* and *Lactococcus garvieae*. Xu and Ding (25) have reported the importance of physiology, biochemistry and pathophysiology of fermentative acidosis in ruminant which is related to many factors and not due to the level of lactic acid production only. Gozho et al ,(26) found that the drop of pH lead to proliferate of lactic acid producing bacteria such as *S.bovis* and lactobacillus's spp, leading to accumulation of lactic acid, which is known as lactic acidosis.In conclusion, our experimental study showed that the change of diet from roughage and alfa alfa to concentrate lead to an increase in the number of *S. bovis* isolated from ruminal fluid of sheep used, by different methods of culturing with importance of design a modified anaerobe apparatus for increase purity of commercial CO_2 gas.

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عزل وتوصيف بكتريا *Streptococcus bovis* من محتويات الكرش من الاغنام العواسي في العراق

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الخلاصة

هدفت الدراسة الاولى في العراق الى عزل وتوصيف بكتريا *Streptococcus bovis*من محتويات الكرش في الاغنام العواسي .استخدمت عشرة اغنام بمختلف الاعمار وغذيت بالحبوب المركزة لثلاثة ايام لجمع 20 نموذج لسائل الكرش مرتين في اليوم الرابع والخامس بعد التغذية،زرعت النماذج على الميديا الخاصة (Modified membrane –bovis agar,(M-BA) الميديا السائلة (BBM,basal broth medium,) واكار الدم والقلب والدماغ المطور،وصفت العزلات على اساس الشكل والاختبارات الفيزيائية والكيميائية والسيرولوجية باستخدام اللانسفيلد . اظهر الزرع على الميديا الخاصة ام-بي-اي نوعين من المستعمرات :النوع الاول (23) شكلت اغلبية العزلات،وهذا النوع يتميز بانتاج عالي للحامض وكونت مستعمرات مخاطية لونها كريمي او برتقالي ولكن النوع الثاني (4) تميزت بانتاج واطي او قليل للحامض وكونت مستعمرات بيضاء صغيرة ،شكليا جميع العزلات موجبة لصبغة جرام ذات شكل بيضوى اودائري تكون مفردة او زوجية او على شكل سلاسل مؤلفة من 4-8 خلايا.صنفت الجراثيم تحت مجموعة اللانسفيلد د. نمت جميع العزلات على الميديا السائلة (BBM) بعد الحضن بدرجة 45 درجة مئوية ولكنها لاتنمو بدرجة 10 و50 مئوية .اضافة 2% من ملح كلوريد الصوديوم يساعد على النمو ولكن التركيز 6.5 %يمنع النمو .لاتنمو باضافة بوتاسيزم توليريت 0.04%.جميع العزلات انتجت حامض الاكتيك ولنها اعطت نتيجة سالبة لانتاج الامونيا من الارجنين .لم يحدث تحلل على اكار الدم المطور.خمرت جميع العزلات البروتين وسكر اللاكتوز والفركتوز والكلوكوز والرافينوس والسيللوبوس.اظهرت نتائج الاختبارات الفيزيائية والكيميائية 27 عزلة تحمل صفات *Streptococcus bovis* وبثلاثة انواع س1،س2،س3 . النوع الاول من المستعمرات شمل نوعين س1 (15) وس2 (8)تبعا للاختلاف في تخمير الارابنوز .وس3 اطلق على النوع الثاني للمستعمرات البيضاء والتي لم تخمر الانيولين.