RESEARCH ARTICLE

Comparative Diagnostic Study of *Streptococcus dysgalactiae* Isolated from Cow's Milk by using Media, Biochemical Tests, VITEK 2, and Polymerase Chain Reaction

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Received: 23rd July, 2021; Revised: 04th August, 2021; Accepted: 27th October, 2021; Available Online: 25th December, 2021

ABSTRACT

The current study aims to investigate compassion among the four most common diagnostic methods used for the detection and isolation of *Streptococcus dysgalactiae*. It included the media culture (Columbia Horse Blood Agar), classical identification (biochemical tests), VITEK 2 assay, and polymerase chain reaction (PCR) in the detection of *S. dysgalactiae* taken from bovine milk. Fifty samples of milk samples are collected from cows randomly (healthy and unhealthy cow), then kept in the sterile tubes and sent to the microbiology laboratory for detection by the above methods. All the used methods test all the samples in our study. These methods are included culture on Columbia horse blood agar, biochemical tests, compact bacterial identification system (VITEK 2) assay, and PCR. Our results showed that the percentage of *S. dysgalactiae* isolated from cow's milk using culture on Columbia Horse Blood Agar was 14/50 (28)%. The percentage of *S. dysgalactiae* isolated from cow's milk using the biochemical tests was 12/50 (24)%.

Furthermore, the percentage of *S. dysgalactiae* isolated from cow's milk using the VITEK 2 system was 11/50 (22)%. Finally, the percentage of *S. dysgalactiae* isolated from cow's milk using PCR was 10/50 (20)%. Besides that, sensitivity and the specificity of the culture on Columbia horse blood agar were (42.85) and (88.88), respectively. The sensitivity and the specificity of the biochemical tests were 58.33 and 92.1, respectively, and the sensitivity and the specificity of the VIITEK 2 assay was 81.81 and 97.43 respectively. However, the PCR method considered the gold test in the study. The current study considered the detection by PCR method are a standard golden test, and suggest the VITEK-2 assay is more sensitive compared with other methods, followed by the biochemical tests method and media culture on Columbia Horse Blood Agar methods.

Keywords: Biochemical tests, Cow, Milk, Polymerase chain reaction (PCR), *Streptococcus dysgalactiae*, VITEK 2.

International Journal of Drug Delivery Technology (2021); DOI: 10.25258/ijddt.11.4.47

How to cite this article: Neamah AA, Fahad KH, Sadeq JN, Al-Fatlawi MAA. Comparative Diagnostic Study of *Streptococcus dysgalactiae* Isolated from Cow's Milk by using Media, Biochemical Tests, VITEK 2, and Polymerase Chain Reaction (PCR). International Journal of Drug Delivery Technology. 2021;11(4):1399-1404.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

The milk is rich in nutrient sources, contain nearly on all the nutrient elements such as proteins (casein), sugar (lactose), and fat (glyceride and cholesterol) with the vitamins and minerals; therefore, the milk is considered ideal food and essential for the health in human and animals (Górska-Warsewicz, H, *et al.* 2019).[1] (Thorning TK, *et al*. 2016).[2] Milk is a media that allow the reproduction of germs because it consists of more than 200 nutrient substances (Nutritional Status During Pregnancy and Lactation, 1991).[3] (Laboratory Animal Nutrition.1995).[4]

Many methods were used for detection of the bacterial agent in the liquid samples, such as culture on rich media and selective media, biochemical test, AP20, VITEK-1, VITEK-2, PCR, MS, and MALDI-TOF.[5-7]

Identifying the microorganism that is isolated from infection is the key to confirmative diagnosis. Determining the sensitivity and specificity of many cultural techniques is an important thing to adopt for bacterial identification. Wherever, the type and composition of the media are very important for determining its sensitivity and specificity (Jordan R, *et al*. 2014)[8] (Tops S, *et al*. 2018).[9]

A biochemical examination is a group of tests that are used for the identification of the bacterial agent. It is used to detect mycoplasma in the cell culture wherever the sensitivity and specificity of the biochemical test have more sensitivity than selective media (Molla, *et al*. 2014).[10] VITEK 2 is widely used in the detection of bacterial agents. Comparison of results of Vitek 2 with the rest of other Laboratory methods at 98.3% to 99% (Bobenchik A, *et al.* 2014).[11]

The performance of the new VITEK 2 system in the identification of Enterobacteriaceae is high in the detection of Enterobacteriaceae at sensitivity 98.1% and specificity 99.7%

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(Spanu T, *et al*. 2006).[12] Besides, PCR is a rapid test for use in the detection of Bactria; it has high sensitivity reach to 98%. Levels of sensitivity also ranged from low to high based on the type of detected causative agent (Noordhoek, G. *et al*. 1994).[13]

Aims of the current study are included determining the percentage of *S. dysgalactiae* that isolated from cow milk by examination it by many diagnostic laboratory methods such as selective media (Columbia horse blood agar), biochemical tests, VITEK-2 assay, and polymerase chain reaction, And the calculation of sensitivity and specificity of each method.

MATERIAL AND METHODS

Sample Collection

Fifty milk samples 50 are collected from 21 cows (note: each cow has four quarter) randomly from clinical mastitic cows and non-clinical mastitic cows (healthy) from different areas of Al-Diwanyia province. After cleaning of the teat by warm water and soap, disinfectant of the teats by cotton contain with alcohol 70% and left first three strep to prevent interference with environment bacteria that come from the ground, All the milk samples were kept at 4°C and sent to the lab for centrifuge and culture.

The Centrifuge

The milk samples are submitted in the lab to centrifuging at 3000 rpm for five minutes (Olympus Company, japan), for separation of the milk to two layers, the supernatant layer consists of the fat the sediment layer consists from the milk serum.

Table 1: Composition of the Columbia Horse Blood Agar that used in the study

No.	*Ingredients*	*Grams / Liter*
1	Special peptone of Animal Tissue	25
2	Pancreatic Digest of Casein	12
3	Starch	1
4	Sodium chloride	5
5	Beef Extract	3
6	Yeast extract	3.5
7	Agar	13.25
8	pH	7.3 ± 0.2
9	Adding: Defibrinated horse blood	50 ml (5) %

Media Culture

Sterile swap (after putting it in the sediment layer by circular movement) used for culture on Columbia horse blood agar (selective media) (Dickinson company, Germany) that used for isolation and culture of *S. dysgalactiae*. Composition of the BD™ Columbia agar with 5% horse blood as listed in Table 1.

Preparation Columbia Blood Agar Base is done by adding (41.25) gran on one liter of the water then sterilize by autoclave at 121°C for 15 minutes then cool for 45ºC then adding horse blood 5%.

Bacterial Culture

All milk samples were collected from cows were culture on Columbia horse blood agar by streaking method then incubated 37ºC for one day. The bacterial colony on Columbia Horse Blood Agar examined morphology (shape; size; color) and examined by gram stain. The colonies are small brown colonies spread on the media. The gram stain showed the bacteria cocci chain has a violet color (gram-positive).

Biochemical Tests

Several biochemical tests test all the milk samples and confirm the detection of the *S. dysgalactiae,* biochemically. It was agreed with the results of the Table 2.

VITEK® 2 Assay

VITEK 2 System (bioMérieuxis company, France) techniques used for rapid Identification of suspected cases. The positive gram card was used in the study because our bacterial samples are *S. dysagalctia.* After sealing it into the VITEK 2 at 35.5°C, the filled card is put in the device. The results were collected from the database. All used cards were discarded after isolation of the bacteria.

Polymerase Chain Reaction (PCR)

It is an assay used for confirmative detection of *S. dysgalactiae* by using special primers designed at National Center for Biotechnology Information (NCBI) website for detection of the (16SrRNA gene) according to the method described by the company instructions. The company instructions are included several steps as following:

Extraction of the DNA

The DNA of *S. dysgalactiae* was extracted using the Genomic DNA Mini Kit by following the company directions for the preparation of pure sediment DNA in the tube then stored at

Table 2: The used biochemical tests in our study for the detection of *S. dysgalactiae*

N	*The test*	*Results*	*N*	*The test*	*Results*
1	Gram	Positive	7	Catalase	Negative
2	Blood analysis	beta	8	Voges proskauer	Negative
3	Shape	Cocci	9	NaCl	6.5%
4	Environment	facultative anaerobic	10	Hippurate Hydrolysis	Negative
5	Temperature	37 °	11	Starch hydrolysis	Negative
6	movement	Non-motile	12	Lactose Fermentation	Positive

4ºC. The extracted DNA was checked electrophoresis using by adding 1.5% agarose gel. The preparation of the PCR master mix is done by following the direction of (AccuPower PCR PreMix) as the Table 3.

The primers that used in the study are designed by the website (www.primeplus3.com) depending on the partial genome of the *S. dysgalactiae* (16srRNA) gene that has Accession number of representative sequences (AB159678) (NCBI) (https://www.ncbi.nlm.nih.gov), the used primers are listed in the Table 4.

These primers were made in Korea (Bioneer Company, Korea). Then (PCR mix master combine) was done by treat with the mixture (AccuPower® multiplex PCR mixture kit).

Thermo-cycling Stage

All the extracted genome DNA samples are put in the mini-tube for thermocycler apparatus, running of the device at several steps as shown in Table 5.

The Electrophoresis

The electrophoresis is done using a 1.5% agarose gel consisting of Agarose gel and 1X TBE at 100°C for 15 minutes, then cooling the mixture for 50°C. Adding Ethidium bromide stain, then poured in a tray after fixed the comb, then left to be sold at 25ºC then removing the comb. Adding the extracted DNA 10 µL in all the wells. Fixing of the gel in the electrophoresis chamber and adding buffer. Then required electric current was 100 V, 80 AM for 60 minutes, then were visualized by UV light system.

Incidence of *Streptococcus dysgalactiae*

According to the Table 6, the percentage of *S. dysgalactiae* isolated from cows by using Columbia horse blood agar from milk was 14/50 (28%. The percentage of S.treptococcus *dysgalactiae* that isolated from cow's by using Classical identification (biochemical tests) was 12/50 (24) %, percentage of *S. dysgalactiae* that isolated from cow's milk by using VIITEK 2 assay was 11/50 (22%). Finally, the percentage of *S. dysgalactiae* that was isolated from cow's milk by using PCR assay was 10/50 (20%), as Figure 1.

The Sensitivity and the Specificity

According to our results in Table 7, sensitivity and specificity of the media culture methods (Columbia horse blood agar) in our study were 42.85 and 88.88, respectively, as shown in Table 7 and Figure 2.

According to our results, Sensitivity and Specificity of the biochemical test methods in our study were 58.33 and 92.1, respectively as shown in Table 8 and Figure 2.

According to our results, sensitivity and specificity of VITEK 2 methods used in our study were 81.81 and 97.43, respectively as shown in Table 9 and Figure 3.

Table 3: The used compositions of the PCR master mix and the volumes

PCR master mix		*Volume*
DNA template		5 µl
Printers	F printers	5 µ
	R Printer	5 µ
PCR water		12 µ
Total volume		20 µ

DISCUSSION

S. dysgalactiae is a beta-hemolytic bacteria, coccal bacterium, it belongs to the C and K groups according to landfilled classification, causes many diseases in animals and humans. In the veterinary world, *S. dysgalactiae* causes the most common mastitis in cows (Alves-Barroco, *et al*. 2019).[1]

Incidence of *Streptococcus dysgalactiae*:

Our results showed the percentage of *S. dysgalactiae* isolated from cow's milk by using Columbia horse blood agar culture, the biochemical examination tests, VITEK 2 assay, and the polymerase chain reaction were 28, 24, 22, and 20%, respectively.

Many reports found that there are increasing incidence rates of *S. dysgalactiae* over worldwide during the last years (Oppegaard, O., *et al*. 2017).[10] *S. dysgalactiae* is increasingly recognized as animal and human pathogens, although it Trans by indirect pathways (McDonald, M., *et al*. 2007).[14]

S. dysgalactiae causes bacteremia in adults in the skin and soft-tissue infections at 43 and 26%, respectively, and were the most frequent clinical manifestation, there are similarities and differences among the studies (Ruppen Corinneab, *et al*. 2017).[15]

The prevalence of *S. dysagalctia* was 2.9% in pregnant women (Jaalama, M. *et al*. 2018),[16] and that represents less than our rates and the study considered that *S. dysgalactiae* is an opportunist agent that causes nosocomial infections, wherever It was isolated from 32.37% from the wounds. The prevalence of this microorganism increased from 26.19% in 2002 (21.54%) in 2003 to 42.00% in 2004 in feces of the animals (horses and sheep). This increase was supported and explained the differences among the reports (Torres, *et al*. 2007)[17] (Ishihara, H, *et al*. 2020).[18]

S. dysgalactiae was isolated from many cows herds during the outbreak and was 71%, which is considered more than our results (Lundberg, Å., *et al.* 2014).[19] Infection of *S. dysgalactiae* was detected in some geographic regions worldwide (Oppegaard, O; *et al.* 2015).[20]

S. agalactiae caused mastitis in cows herds worldwide at 36/59 (61) % and more than our rate.[7] *S. agalactiae* is caused mastitis in cows at a percentage reach from 11% to 47% because vary contract is a normal thing (Keefe G. 1997).[21]

Table 4: Sequence and size of the used primers of 16srRNA gene in the study

16S rRNA gene	*Sequence*	*Primer size*	*The accession number of representative sequences*
Left Primer	GGGAATCTTCGGCAATGGAC	152 bp	AB159678
Right Primer	TTAGCCGTCCCTTTCTGGTT		(NCBI)

Table 5: Stage name, temperature, and time of each thermo-cycling stage

N.	*Stage*	*Temperature*	*Time*	
1	Denaturation start	(95)ºC	(2) minute	
2	Denaturation	(95)ºC	(30) second	
3	Annealing	58.3ºC	(30) second	*Repeated* 29 times
4	Elongation	(72) ºC	(20) second	
5	Final	(72)ºC	(5) minute	
6	Store	(4)ºC	forever	

Table 6: The number and percentage of *S. dysgalactiae* on media, classical identification, VITEK 2, and PCR

	The results				
The used method	*Positive*	*Percentage (%)*	*Negative*	*Percentage*	*Total*
Media culture	14	28	36	72	50
Biochemical tests	12	24	38	76	50
VITEK 2	11	22	39	78	50
PCR	10	20	40	80	50
	47				

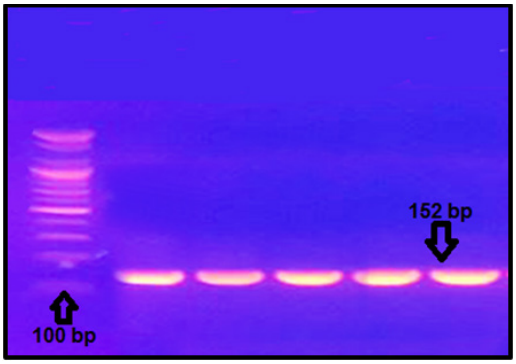

Figure 1: The band of 16SrRNA gene of *S. dysgalactiae* on gel agarose on electrophoresis, wherever the vertical lines mean the leader, while the horizontal lines are the mean band of 16SrRNA (152) bp

Table 7: The sensitivity and the specificity of the Media (Columbia Horse Blood Agar)

	Media		
PCR	*Positive*	*Negative*	*Total*
Positive	6 (42.85)	4 (11.11)	10 (20)
Negative	8 (57.14)	32 (88.88)	40 (80)
Total	14 (28)	36 (72)	50
Sensitivity	42.85		
Specificity	88.88		

Table 8: The sensitivity and the specificity of the classical methods (biochemical tests)

	Biochemical tests		
PCR	*Positive*	*Negative*	*Total*
Positive	7 (58.33)	3 (7.89)	10 (20)
Negative	5 (41.66)	35 (92.1)	40 (80)
Total	12 (24)	38 (76)	50 (100)
Sensitivity	58.33		
Specificity	92.1		

Table 9: The sensitivity and the specificity of the VITEK 2 apparatus

	VITEK 2		
PCR	*Positive*	*Negative*	***Total***
Positive	9 (81.81)	1 (2.56)	10 (20)
Negative	2 (18.18)	38 (97.43)	40 (80)
Total	11 (22)	39 (78)	50 (100)
Sensitivity	81.81		
Specificity	97.43		

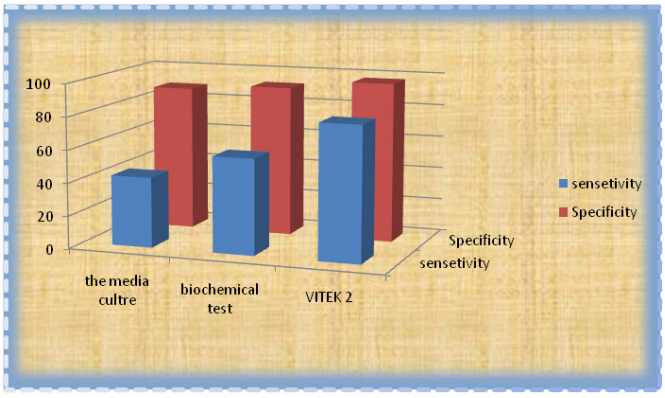

Figure 3: Diagram showed the value of sensitivity and specificity of the media culture, biochemical test, and PCR

As known, *S. dysgalactiae* causes summer mastitis in sumer, and that is disease-related with seasons, because it becomes more in the summer and less in the winter (Jousimies-Somer, *et al*., 1996).[22] The geographic determinant, season, hygienic level, immunity level, individual difference, and other factors related to *S. dysgalactiae's* epidemiology result in differences in spreading rates (Chirico J, *et al.,* 1997)[23] (Sentitula, *et al*., 2012).[24]

Sensitivity and Specificity

Based on our results, using the VIITEK 2 assay to detect *S. dysgalactiae* is more sensitive and accurate than detecting *S. dysgalactiae* by using the biochemical method and the media culture. VITEK-2 is applied to detect gram-positive bacteria. The VITEK-2 is detected at the level of the species. It's sensitivity percentage reach (91–99) %, and that close and agree to our results (Marco L, *et al*. 2002).[25]

Many bacteria that multiplicities in the bloodstream, such as *S. spp* are identified by accurate diagnosis method (VITEK-2 system) wherever identify these organisms rapidly and with high accuracy. The sensitivity reach from 95.6% to (99.2%), and that have in agreement with our finding (Teresa Spanu, *et al*. 2003).[26] The VITEK-2 (bioMérieux) was used for an identification card for gram-negative bacilli, wherever the results showed that VITEK 2 has high performance, simple, rapid manual tests (Funke, G., *et al.* 1998).[27]

If VITEK 2 is compared with reference methods, it identified 95% of isolates, and its sensitivity and specificity were 99% and 96%, respectively (Nonhoff, C. *et al*. 2005).[28]

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