Comparison between the conventional bacterial culture and the real time PCR in identifying the dairy cow mastitis causative agents

Mohammed Farah Mohammed Abdelhameed[1], Ahmed Eltigani Almansoori[2] R. H. Zaid[1]and Mohamed T. Ibrahim[1]

1 Department of Dairy Production, College of Animal Production Science and Technology, Sudan University of Science and Technology, Sudan, 2 Al Rawabi Dairy Company, Dubai, UAE.

Keywords: Mastitis; dairy; Bacterial culture; Real time PCR.

مقارنة بين الزرع البكتيري التقليدي وتقنية real time PCR في تحديد مسببات التهاب الضرع في الأبقار الحلوب

محمد فرح محمدعبدالحميد[1], أحمد التجاني المنصوري[2], رانيا حسن زايد[1], محمد تاج الدين إبراهيم[1]

جامعة السودان للعلوم والتكنلوجيا, كلية علوم وتكنولوجيا الإنتاج الحيواني, قسم إنتاج الإلبان

الملخص

يعد التهاب الضرع من الامراض الشائعة والمكلفة في مزارع الألبان. تُقارن هذه الدراسة بين الزراع البكتيري الروتيني وتفاعل البلمرة التسلسلي real time PCR (rt PCR) في تحديد العوامل المسببة لالتهاب الضرع. يعتمد تشخيص أي عدوى تصيب الضرع على تحديد العامل المسبب لها كما يلعب الوقت المستغرق في طريقة التحديد دورًا مهمًا في إدارة المرض. من بين 9000 بقرة حلوب من سلالة هولشتاين في القطيع، تم أخذ ثلاثة وثمانين عينة حليب بشكل معقم من الأبقار التي تم التحقق سريرياً من إصابتها بالتهاب الضرع في الأشهر الأولى من عام 2019. تم استخدام مجموعة CHROMagar™ Mastitis في زراعة كل من البكتيريا سالبة الجرام وموجبة الجرام، بالإضافة إلى وسط إدواردز HiVeg Base ، الوسط المعدل. من ناحية أخرى تم استخدام نظام التنقية King Fisher™ Duo Prime ومجموعة تنقية الأحماض النووية Mag MAXTM CORE ومجموعة VetMAX™ Masti Type لكل عينة لإجراء تجربة rtPCR واستخراج الحمض النووي. تم تحليل جميع البيانات باستخدام اختبارات كاي-تربيع. أظهرت منحنيات ROC أن اختبار rtPCR كان أكثر فعالية من طريقة الزراع البكتيري (P = 0.59) و (AUC = 0.653)بفضل دقته التحليلية العالية وإمكانية تطبيقه لتحديد العوامل المسببة لإلتهاب الضرع فى الابقار وبالتالي فإن اختبار rtPCR يعد اختبار واعد للغاية.

الكلمات المفتاحية: إلتهاب الضرع, الألبان, الزرع البكتيري, تفاعل البلمرة التسلسلي.

Abstract

Mastitis is a prevalent and costly disease in dairy farms. This study compares routine in-vitro microbial culture and real time PCR (rtPCR) in identifying mastitis causative agents. The diagnosis of any intramammary infection is based on the identification of the infectious agent and the time taken by the identification method plays an important role in disease management. Out of 9,000 Holstein milking cows in the herd, eighty-three milk samples were aseptically taken from cows with clinically verified mastitis in the first few months of 2019. The CHROMagar™ Mastitis kit was used in microbial culture for both gram-negative and gram-positive bacteria, along with Edwards Medium HiVeg Base, Modified Medium. On the other hand, the King Fisher™ Duo Prime Purification System, the Mag MAXTM CORE Nucleic Acid Purification Kit, and VetMAX™ Masti Type Kit were utilized for each sample to conduct the rtPCR experiment and DNA extraction. All data were analyzed by the Chi-square tests. The ROC curves showed that rtPCR was more effective than the bacterial culture approach in a comparative analysis (chi-square = 0.27, P = 0.59) and (AUC = 0.653). With its high analytical accuracy and potential for application in regular bovine intramammary infection testing services, the rtPCR assay is a highly promising test.

1. Introduction

The most prevalent disease among dairy cows is intramammary inflammation, or mastitis. Although producers in contemporary dairy farms have made significant efforts to reduce its impact, mastitis is widely acknowledged to have negative consequences for animal welfare and the profitability of dairy farms [1]. When mastitis strikes lactating animals, it results in significant financial losses [2]. Bacterial infection, trauma, or injury can primarily cause the sickness in cows (3). One hundred and forty different pathogen species, subspecies, and serovars have been identified in mastitis milk samples [4]. These pathogens have been classified as opportunistic, environmental, and contagious mastitogens. An abundance of unfavourable factors, the most common of which are infections, can develop simultaneously and overlap, resulting in dairy cow mastitis. Climate-related differences in

species or breeds can affect the etiological agents of mastitis. This group encompasses a variety of bacteria, including both Gram-positive and Gram-negative species, mycoplasma, fungi, yeasts, algae, and viruses. We can categorize the pathogenic bacteria that cause mastitis most frequently into two groups based on their origin: contagious pathogens (*Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma* spp., and *Corynebacterium bovis*), and environmental pathogens (coliform bacteria and *Streptococcus* species other than *Streptococcus agalactiae*). While infected mammary glands are the source of infectious pathogens, the cow habitat is the source of ambient bacteria [5]. On the other hand, mastitis is inflammation of the tissues in the udder brought on by a bacterial infection, chemical irritation, or physical trauma [6].

Dairy cow mastitis is diagnosed based on clinical observations or direct or indirect measures of the inflammatory response to infection; an intramammary infection is diagnosed based on the identification of the infectious agent [7]. *In vitro* bacterial culture (BC) is a basic test for both the identification and isolation of infectious microorganisms. To identify particular bacteria, fresh milk swab samples are grown in a particular medium. The isolated organisms are then identified microbiologically and biochemically [8]. Furthermore, both polymerase chain reaction (PCR) and BC have benefits and drawbacks when it comes to diagnosing intramammary infections. Undoubtedly, identifying the bacterial agent responsible for the intramammary infection can aid in developing prevention and treatment plans for the farm, ultimately contributing to a decrease in the disease's incidence and prevalence [7]. On the other hand, rtPCR has more advantages than traditional PCR and bacterial culture. Not only is it more sensitive and quicker, but it's also safer for the environment and workers (no ethidium bromide is used), requires no post-reaction handling (agarose electrophoresis), and produces better results through visualization and digitization, allowing for data exchange and documentation with other teams. When it comes to diagnosing mastitis pathogens, rtPCR sensitivity and specificity can both approach 100%. Furthermore, by measuring the intensity of the fluorescence produced by the reaction, rtPCR can be used to quantify pathogens in infected milk [9, 10]. Additionally, it is more accurate than conventional PCR at detecting any nucleic acid, even at microconcentrations, and it can identify amounts above their limit. It is also possible to

determine the type and stage of the infection. Its speed, sensitivity, and specificity are therefore all greater than those of conventional PCR [11]. This indicates that it is the recommended option for both molecular characterization and laboratory diagnosis [12]. A comparison of the two approaches for detecting bovine mastitis bacteria revealed that the rtPCR assay is superior to the standard culture technique in terms of speed, objectiveness, result interpretation, and high sensitivity in bacterial identification [10]. Further, the rtPCR assay can detect all typical mastitis bacteria that can be found in high concentrations in clinical mastitis samples, including those that do not develop in traditional culture.

As a result, rtPCR is a promising non-conventional method for diagnosing mastitis that can be applied in addition to standard methods. The rtPCR assay was used to provide a rapid and precise method for the specific detection of *Staph. aureus* in cow's milk [12]. Consequently, multiplex PCR was found to be more effective when compared to the culture approach in a comparative analysis conducted utilizing milk samples from patients with subclinical mastitis. When used directly for the evaluation of bulk milk samples, the multiplex PCR subsequently demonstrated successful target bacterial detection [13]. Additionally, when comparing the pathogen isolation process with multiplex PCR, the latter was able to identify a greater number of strains from the DNA recovered from milk samples than was possible with traditional BC. It was discovered that the created multiplex PCR assay was easy to use, quick, accurate, and specific for species identification of up to ten bacteria at once. The test will be helpful in differentiating between species, detecting mastitis, and evaluating the bacteriological safety of milk [13]. The gold standard for diagnosing mastitis has long been BC [14]. The use of PCR-based assays, however, has been proposed as a potential supplement to or replacement of traditional bacteriological intramammary infection identification techniques [15, 16, 17]. Short throughput times, the capacity to identify bacteria and antibiotic resistance genes objectively and independently of the user, and the sensitive detection of growth-inhibited and dead bacteria are some of the main arguments in favor of PCR assays [18]. Since 50% of clinical mastitis samples do not exhibit bacterial growth in bacterial culture [19], and apart from the potential reduction of false-negative outcomes, the use of PCR-based assays in identifying nonviable bacteria would also improve dairy herd health [18].

In regular test laboratories, the identification of mastitis pathogens from milk using traditional culture methods may result in false positives for bacteria. For instance, the total rate of incorrectly identified bacteria varied between 9 and 37% across the several mastitis culture facilities, based on a European proficiency testing program [19]. The combination of this low specificity and up to 50% of findings showing no growth in BC [20] makes it extremely difficult to assess the genuine effectiveness of any new technology in comparison to the standard procedure now in use. When it comes to traditional BC, facultatively anaerobic species like *Arcanobacterium pyogenes* or *Corynebacterium bovis* grow slowly or could need extra growth factors, while anaerobic bacteria do not grow on standard media [19, 20]. This study was conducted to compare the conventional bacterial culture with the rt-PCR technique in bacterial identification.

2. Materials and methods

2.1 Sample collection

Out of 9,000 Holstein milking cows in the herd, 85 milk samples were aseptically taken from cows with clinically verified mastitis in the first few months of 2019. Three times a day, the cow produced an average of 37 L of milk. The three-step cleaning in place system (CIP) for the milk line started with a standard water rinse. Next, soda was added and heated to 65 ˚C to 85 ˚C for seven to ten min. Finally, regular water was added and left for an additional ten min. Next, a wash with VA4-nitric acid for 5 to 10 min at 40 ˚C to 60 ˚C, followed by a normal water rinse and paracetic acid sanitization at 35 ˚C to 55 ˚C. 10 ml of milk, from the infected udder quarter, were collected and placed in sterile plastic tubes. After that, they were immediately taken to the lab and kept in a refrigerator at 5 °C.

2.2 Bacterial culture

The CHROMagar™ Mastitis Kit is a commercial instrument designed to facilitate the quick and easy identification of the primary bacteria responsible for mastitis infections. Gram-positive bacteria, which include *Staph. agalactiae*, *Staph. uberis*, and *Staphy. aureus*, and gram-negative bacteria, which include *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Proteus*, *Morganella*, *Providencia*, *Pseudomona*s, and *Candida albicans*, are housed in separate mediums, which make up this system. When a sample is infused into

chromogenic media, distinct morphologies and color formations are produced for every kind of chosen organism. The exam has a minimum time requirement of 20 hours, which includes sample preparation [21]. On the other hand, *Strep. agalactiae* and other streptococci linked to bovine mastitis were quickly and selectively isolated using Edwards Medium HiVeg Base, Modified Medium [22]. All manufacturing instructions were followed during the experiment.

2.3 DNA extraction and amplification

The King Fisher™ Duo Prime Purification System, the Mag MAX™ CORE Nucleic Acid Purification Kit, and VetMAX™ MastiType Kit were utilized for each sample collected in order to conduct the PCR experiment and extract DNA. After pipetting up and down several times, 50 µl of Mag Max CORE Mastitis Panbacteria Solution was added to 200 µl of milk and mixed for 5 minutes at room temperature. Thirty seven minutes were spent running the automated extraction procedure routine after adding 10 µl of Mag Max CORE proteinase K (20 mg/ml). Once the plate is in the device, run the "Mag MAX_ CORE_ DUO_ Mastitis." script. About eight minutes into the script, it paused. At that point, the deep well plate was taken out of the apparatus, and after vortexing each well to allow the milk samples to be digested, 720 µl of lysis-bending-Bead-Mix (Mag Max TM core lysis solution 350 µl + Mag Max TM core binding solution 350 µl + Mag Max TM core magnetic beads 20 µl) was added. After that, the sample plate was put back into the device to carry out the run. Applied Biosystems VetMAX™ MastiType multiplex qPCR kits are used in conjunction with an Applied BiosystemsTM 7500 PCR apparatus, and all procedures are carried out in accordance with Thermo Fisher workflow recommendations. Every DNA sample is analyzed in a different well, and the DNAs of 4 pathogens and an internal amplification control (IAC) are uniquely identified in the same well.

First, we determined the number of required reactions in the mix preparation and scaled the reaction components based on the quantities of each individual reaction before adding the 10% overage. Secondly, utilizing the master mix and primer mixes in adequately sized microcentrifuge tubes, four separate PCR reaction mixes were generated (Table 1). Thirdly, the tubes are sealed first, and then the solutions are combined by vortexing and rapidly centrifuged to push the PCR reaction mixtures to the bottom of the tubes and eliminate air

bubbles. To prepare the PCR plates, 15 µL of each PCR reaction mix were added into the corresponding wells of an optical reaction plate. Then, in accordance with Table 2, the sample and controls were added to the well. At this stage, optically transparent covers were used to seal the plate, the contents were rapidly centrifuged to eliminate air bubbles and settle the contents into the well's bottom, and the real-time PCR was carried out in compliance with Thermo Fisher workflow instructions.

Statistical analysis: The Chi-square test and the ROC curve were used to analyze the data by using SPSS software.

3. Results

From the milk samples (n = 83) tested using the rtPCR test, 80.72% showed positive bacterial infection; 52 samples (62.65%) had multiple infections, while the other 15 samples (18.07%) showed a single organism. On the other hand, using the BC test, only 5 samples (6.02%) had multiple infections, while 31 samples (37.35%) contained a single organism, with a total of 43.37% positive infection (table 3). Table 4 shows that a maximum of 6 isolates per sample were identified by rtPCR out of 200 isolates, with an average number of isolates from the positive PCR samples of 2.99±1.5. A maximum of 2 of 41 isolates per sample were identified by bacterial culture, with an average number of isolates from the positive BC samples of 1.14± 0.4. When comparing the two tests, the BC yielded a positive predicted value (PPV) of 83.3% and a very low negative predicted value (NPV) of 21.3%, with a sensitivity of 44.8% and a specificity of 62.5% (table 5) while the ROC curve analysis (Figure 1) indicated an AUC of 0.653. Although the PCR kits included 16 primers, only 12 tested positive (75%). These were *Streptococcus* spp. (50.6%). Of these, 94.7% of them were *Strep. agalactiae* (47.6%). They were followed by E. *coli* (44.6%), *Staph*. spp. (39.8%), Corynebacterium bovis (7.2%), *Enterococcus* (6.0%), *Strep. dysagalactiae* (4.8%), *Klebsiella*, *Serratia*, *Staph. aureus*, and *Strep. uberis* (3.6% each), and *Trueperella pyogenes* (2.4%). Almost about one-quarter of these isolates carry the β-lactamase gene (26.5%). On the other hand, BC tested positive for both E. *coli* and *Streptococcus* spp. (19.2% each), followed by *Streptococcus agalactiae* (4.8%), Klebsiella (2.4%), and lastly *Enterococcus*, *Staph. aureus*, and *Strep. uberis* (1.2% each). *Mycoplasma bovis*, *Mycoplasma, prototheca*, and yeast were not detected by rtPCR nor by BC. The

sensitivity and specificity of BC in identifying *Strep*. spp. were 23.8 and 85.4% (table 6) and E. coli were 37.8 and 95.6% (table 7).

4. Discussion

In contrast to the BC test, the rtPCR result revealed that the following bacteria had higher isolation frequencies: *Strep.* spp., *Strept. agalctiae*, *E. coli*, *Staph.* spp., β- lactamase gene, *Corynebacterium bovis*, *Enterococcus*, *Streptococcus dysagalactiae*, *Klebsiella*, *Serratia*, *Staph. aureus, Strep. uberis*, and *Trueperella pyogenes*. This result may be due to the rtPCR ability to detect even micro-nucleic acid concentrations. Further, rtPCR has a unique method of detection, that helps to identify the β-lactamase gene, which is not applicable for the BC. Additionally, out of 200 isolates, a maximum of six isolates per sample were detected by real-time PCR, whereas only a maximum of two isolates per sample were detected out of 41 isolates by bacterial culture. This finding is similar to (9, 10, 11, 13), who described PCR in terms of ease, quickness, accuracy, sensitivity, and specificity better than the microbial culture.

This finding illustrates how accurate rtPCR is at identifying many organisms that cause mastitis when compared to microbial culture. Furthermore, rtPCR using the VetMAX™ MastiType Multi Kit requires only four hours to get a result, whereas BC requires twenty hours, which highlights its speed as well.

The 16 PCR negative results may be due to chemical irritation or physical trauma [6, 2] or it may be due to other organisms not present in the used rtPCR kit [4].

It has been noted that all common mastitis bacteria are present in high concentration in clinical mastitis samples that fail to grow in traditional culture and that the rtPCR technique is a useful tool for bacteriologically diagnosing these milk samples, despite the frequent hypothesis that low bacterial concentrations account for the no-growth milk samples [11]. This finding may explain the results of 15 samples (18.7%) containing a single organism and 52 samples (62.65%) containing multiple infections that were detected by real-time PCR testing, while only 5 samples (6.02%) had multiple infections, 31 samples (37.35%)

contained a single organism, and 47 samples (56.63%) tested negative, which was detected by BC.

On the other hand, according to a European proficiency testing program, the overall rate of incorrectly identified bacteria varied between 9 and 37% across the several mastitis culture facilities [19]. This could potentially account for the results of E. *coli*, *Staphylococcus aureus*, and *Streptococcus* spp. that tested negative by rtPCR but positive by BC and reflect the role of rtPCR in false-positive detection.

Anaerobic bacteria do not grow on standard media, whereas facultatively anaerobic species such as *A. pyogenes* or *C. bovis* grow slowly or may require additional growth factors [19, 20]. Also, this can explain the non-growth of *Corynebacterium bovis, Staphylococcus* spp., *Trueperella pyogenes,* and *Serratia* in bacterial culture while it is detected by real-time PCR.

The outcomes for the identification of the β-lactamase gene were also favorable, as rtPCR identified 22 positive samples that BC was unable to identify.

5. Conclusion

This result showed that the real-time PCR assay has a high degree of analytical accuracy and can be used as a highly promising diagnostic tool for routine testing for bovine intra-mammary infections.

Acknowledgement

For their continuous support during the course of the project, I would like to express sincere thanks to the management and laboratory staff of the Public Health Services Department at Dubai Municipality, the management and laboratory of AL Rawabi Dairy Co., and Integrated Gulf Biosystems Co.

References

1. Ruegg, P.L. A 100-Year Review: Mastitis detection, management, and prevention. *Journal of Dairy Science*, 2017, https://doi.org/10.3168/jds.2017-13023.

2. Romero, J., Benavides, E. and Meza, C. Assessing Financial Impacts of Subclinical Mastitis on Colombian Dairy Farms. *Frontiers in Veterinary Science*, 2018, **5**, Article 273.

3. Anita, T. Bovine Mastitis: An Important Dairy Cattle Disease. *Indian Dairyman*, 2014, **66(4)**, 62-65.

4. Patterson, C. Veterinary Medicine: *A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats*. 2017, **(1&2)**.

5. Watts, J.L. Etiological agents of bovine mastitis. *Veterinary Microbiology*, 1988, **16(1)**, 41-66. https://doi.org/10.1016/0378-1135(88)90126-5.

6. Ashraf, A. and Imran, M. Causes, types, etiological agents, prevalence, diagnosis, treatment, prevention, effects on human health and future aspects of bovine mastitis. *Animal Health Research Reviews*, 2020, 1-14. https://doi.org/10.1017/S1466252319000094.

7. Adkins, P.R.F. and Middleton, J.R. Methods for Diagnosing Mastitis. *Veterinary Clinics of North America: Food Animal Practice*, 2018, https://doi.org/10.1016/j.cvfa.2018.07.003

8. Pyorala, S. Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research*, 2003, **34**, 565-578. https://doi.org/10.1051/vetres:2003026.

9. Koskinen, M.T., Holopainen, J., Pyörälä, S., Bredbacka, P., Pitkälä, A., Barkema, H. W., Bexiga, R., Roberson, J., Sølverød, L., Piccinini, R., Kelton, D., Lehmusto, H., Niskala, S. and Salmikivi, L. Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. *Journal of Dairy Science*, 2009, **92**, 952-959. doi:10.3168/jds.2008-1549.

10. Koskinen, M.T., Wellenberg G.J., Sampimon O.C., Holopainen J., Rothkamp A., Salmikivi, L., van Haeringen, W.A., Lam, T.J. and Pyörälä, S. Field comparison of

real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. *Journal of Dairy Science*, 2010, **93**, 5707-5715. https://doi.org/10.3168/jds.2010-3167.

11. Taponen, S., Salmikivi, L., Simojoki, H., Koskinen, M.T. and Pyörälä, S. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *Journal of Dairy Science*, 2009, **92**, 2610-2617. https://doi.org/10.3168/jds.2008-1729.

12. Zakary, E.M., Nassif, M.Z. and Mohammed G.M.O. Detection of Staphylococcus aureus in bovine milk and its product by Real-time PCR assay. *Global Journal of Biotechnology and Biochemistry*, 2011, **6**, 171-177. https://www.semanticscholar.org/paper/7dca93681ab2b4ce91.

13. Shome, B.R., Das Mitra, S., Bhuvana, M., Krithiga, N., Velu, D., Shome, R., Isloor, S., Barbuddhe, S.B. and Rahman, H. Multiplex PCR assay for species identification of bovine mastitis pathogens. *Journal of Applied Microbiology*, 2011, 1349-1356. https://doi:10.1111/j.1365-2672.2011. 05169.x

14. National Mastitis Council. *Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality*. 4th ed. National Mastitis Council, Madison, WI, 2004.

15. Riffon, R., Sayasith, K., Khalil, H., Dubreuil, P., Drolet, M. and Lagace, J. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *Journal of Clininical Microbiology*, 2001, **39**, 2584-2589.

16. Phuektes, P., Browning, G.F., Anderson, G. and Mansell, P.D. Multiplex polymerase chain reaction as a mastitis screening test for Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysagalactiae and Streptococcus uberis in bulk milk samples. *Journal of Dairy Research*, 2003, **70**, 149-155.

17. Gillespie, B.E. and Oliver, P. Simultaneous detection of mastitis pathogens, Staphylococcus aureus, Streptococcus uberis, and Streptococcus agalactiae by multiplex real-time polymerase chain reaction. *Journal of Dairy Science*, 2005, **88**, 3510-3518.

18. Bustin, S.A. *Quantification of nucleic acids by PCR. An A-Z Manual of Quantitative PCR*. P. 5-46 in IUL Press, La Jolla, CA, 2004.

19. Pitkälä, A., Gindonis, V., Wallin, H. and Honkanen-Buzalski, T. Interlaboratory proficiency testing as a tool for improving performance in laboratories diagnosing bovine mastitis. *Journal of Dairy Science*, 2005, **88**, 553-559.

20. Makovec, J.A. and Ruegg, P.L. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *Journal of Dairy Science*, 2003, **86**, 3466-3472.

21. http://www.chromagar.com/publication.php

22. https://www.himedialabs.com/us/mv748-edwards-medium-hiveg-base-modified.html

Table 1. PCR Amplification Component

Component	Volume	
	1 Well	N (1) Wells
PCR Reaction Mix Multi-1		
Masti Type Master Mix	10 µL	N× 10 µL
Masti Type Multi Primer Mix 1	5 µL	N× 5 µL
PCR Reaction Mix Multi-2		
Masti Type Master Mix	10 µL	N× 10 µL
Masti Type Multi Primer Mix 2	5 µL	N× 5 µL
PCR Reaction Mix Multi-3		
Masti Type Master Mix	10 µL	N× 10 µL
Masti Type Multi Primer Mix 3	5 µL	N× 5 µL
PCR Reaction Mix Multi-4		
Masti Type Master Mix	10 µL	N× 10 µL
Masti Type Multi Primer Mix 4	5 µL	N× 5 µL

Table 2. Volumes of the Reaction

Sample Type	Component	Volume Per Reaction
Test Sample	Sample DNA	5 µL
PC	1 Masti Type Positive Control	5 µL
MNC	Nuclease Free Water	5 µL

Table 3. Sample distribution according to the number of isolates using PCR and BC

	PCR		BC	
	frequency	%	frequency	%
Negative samples	16	19.28	47	56.63
Single organism	15	18.07	31	37.35
Multiple infection	52	62.65	5	06.02

Total	83	100.0	83	100.0

Table 4. The number of isolates per sample using PCR and BC (N= 83)

	Minimum	**Maximum**	**Sum**	**Mean±SD**
PCR	0.0	6.0	200.0	2.41±1.8
Bacterial culture	0.0	2.0	41.0	0.49±0.6

Table 5. Cross-tabulation between PCR and BC ($\chi^2 = 0.27$, P= 0.59)

			PCR		**Total**
			+ve	**-ve**	
Bacterial culture (BC)	**+ve**	**Count**	30	6	36
		% within BC	83.3	16.7	100.0
		% within PCR	44.8	37.5	43.4
	-ve	**Count**	37	10	47
		% within BC	78.7	21.3	100.0
		% within PCR	55.2	62.5	56.6
Total		**Count**	67	16	83

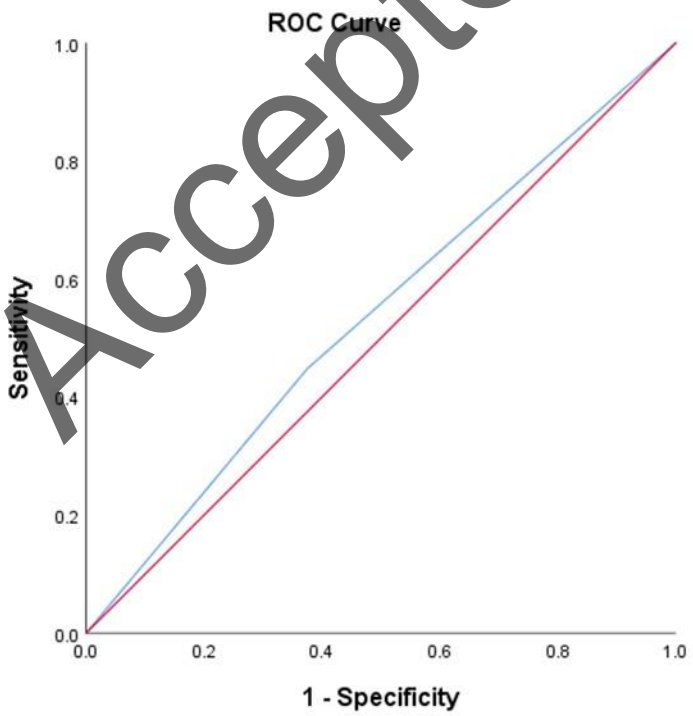
Table 6. Strep. Spp. crosstabulation between PCR and BC

			Strep spp. PCR		**Total**
			+Ve	**-Ve**	
Strep spp. BC	**+Ve**	**Count**	10	6	16
		% within Strep spp. BC	62.5	37.5	100.0
		% within Strep spp. PCR	23.8	14.6	19.3
	-Ve	**Count**	32	35	67
		% within Strep spp. BC	47.8	52.2	100.0

		% within Strep spp. PCR	76.2	85.4	80.7
Total		**Count**	42	41	83

Table 7. E. coli. cross-tabulation between PCR and BC

			E. coli. PCR		**Total**
			+Ve	**-Ve**	
E. coli. BC	**+Ve**	**Count**	14	2	16
		% within E. coli. BC	87.5	12.5	100.0
		% within E. coli. PCR	37.8	4.4	19.5
	-Ve	**Count**	23	43	66
		% within E. coli. BC	34.8	65.2	100.0
		% within E. coli. PCR	62.2	95.6	80.5
Total		**Count**	37	45	82



ROC curve comparing bacterial culture test with the PCR test (AUC =0.563)

Figure 1