DOI Number: 10.37506/v20/i1/2020/mlu/194349

Iron Oxide Nanoparticles Reduced Biofilm Formation and Detection of *lmb Genes* in *Streptococcus agalactiae* Isolated From Patients with Diabetes Mellitus

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Abstract

Fifty eight isolates of *Streptococcus agalactiae* were obtained from wound and urine specimens among one hundred thirty six from patients with diabetes mellitus and identified through cultural, morphological, and biochemical examinations in addition to VITEK 2 Compact System. All of the isolates were tested regarding their sensitivity to 18 antibiotics and the results showed that all of the isolates were resist to clindamycin, and sensitive to teicoplanin. The current study was carried out to use two methods (i.e. microtiter biofilm and Congo red agar) so as to provide and extend insight into bacterial colonization and biofilm formation among diabetic patients. The two methods were compared and the results revealed that microtiter was the best method for biofilm detection, and two chemical materials (i.e. silver oxide, and iron oxide) were used as anti-biofilms for all isolates, one of the best concentration and kind of chemical materials was Iron oxide at 75 mg/ml for reducing biofilm formation. PCR system was employed in order to detect the virulence genes (i.e *lmb* gene), the results that 30(51.72%) were positive for *lmb* genes.

Keywords: *Biofilm, Diabetes mellitus, Nanoparticles, Streptococcus agalactiae , Virulence genes*

Introduction

The term diabetes commonly used by public refers to a series of metabolic disorders that involve increased levels of blood sugar for long periods of time, which are collectively labeled Diabetes mellitus (DM) [1].

It is a widely accepted phenomenon that diabetic patients are more likely to develop bacterial infections [2].

Proteases break the peptide bonds in protein chains through hydrolysis[3]. Microorganisms perform their hemolysis using a substance called hemolysin [4].

The VITEK 2 system has advanced the technology to the level where it can identify and perform Antibiotic Sensitivity Test (AST) on bacteria, once the initial inoculations are performed and brought to its standards[5].

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Microbial organisms have developed various mechanisms in order to survive against the stressful conditions of the environment. One of these defensive mechanisms is their capability of developing a layer of slimy bio-film, which can be defined as a self-secreted polysaccharide aggregate matrix attached to their surface in a certain direction [6].

The isolated bacteria colonies were grown on the polystyrene layer of the microtiter plates' flat bottomed wells [7].

The Congo Red Assay (CRA) is a simple qualitative test method that has the additional benefit of leaving the colonies open for further testing [8].

Forming antibiofilm through the use of nanoparticles like a silver oxide leads to a decrease in resistance within biofilm because silver oxide interferes with most bacterial cell structures and functions [9]. Various chemical techniques like co-precipitation, hydrothermal, and laser pyrolysis have proposed synthesis of iron oxide nanoparticles [10].

PCR products are usually analyzed within agarose gel electrophoresis, where the DNA products can be put into an order based on their size and electric charge [11]. Detection of the *lmb* genes using conventional PCR .

Materials and Method

Bacteriology

Fifty eight *S. agalactiae* isolates (urine and wound) were obtained from 136 diabetic mellitus patients were depending on cultural, morphological and biochemical tests in addition to Vitek 2 compact system. Sensitivity test kit for gram positive bacteria which included several antibiotics such as clindamycin , erythromycin , tetracycline , inducible clindamycin , vancomycine , ceftriaxcine , cefotaxime , benzylpenicillin , ampicillin , trimethoprim , linezolid , tigecycline , nitrofurantoin , moxifloxacin , ciprofloxacin , imipenem , levofloxacin , and teicoplanin and VITEK 2 Compact System were employed. Enzyme assays like protease test, and hemolysin, were used for all isolates, and biofilm formation was performed by two methods of the microtiter plate method and Congo red method.

In microtiter plate assay test, the strains are inoculated in 10 ml of LB broth for 18 hours at 37 ºC. Then a mixture of 50 µl inoculum and 50 µl fresh of LB broth is placed in a microplate (96 cells), and left for incubation overnight, once again at 37 ºC. The cells are then emptied and washed with PBS thoroughly. 100 µl of 1% crystal violet solution is then added to the cells, and the microplate is left to rest at room temperature for half an hour. The plate is then washed once again with PBS, then the cells are introduced 200 µl 95% ethyl alcohol each. Finally, 125 µl of this solution is collected from each cell to measure under 540 nm using a microplate reader [12].

The Congo red stain was obtained from Research lab fine chem. Industries, India, and was prepared as a 0.8 g/200 ml distilled water solution. Appearance of black dry crystalline colonies on the CRA plates indicated biofilm production while the colonies of biofilm non-producer remained pink or red colored [13].

MIC of nanoparticles on DM isolates

Two nanoparticles were used as antibiofilm agents against *S. agalactiae* including silver oxide, and iron oxide. The MIC Ago-np on 58(42.64%) isolates were 100 µg/ml from varies concentration (25, 50, 100, and 150 µg/ml), Also the MIC Io-np on 58 (42.64%) isolates were 75 mg/ml among different concentration were used as (20, 35, 50, 75, and 100 mg/ml).

DNA extraction

To obtain a pure culture, all of the *S.agalactiae* strains were streaked twice on blood agar (Oxoid, UK), and the single colony was inoculated into a 50 ml flask containing 10 ml Luria Bertani (LB) broth made of 10 g(w/v) NaCl2 5g (w/v) yeast extract (Oxoid, the UK) and 1 L distilled water. The flasks were kept in the incubator shaking at 110 rpm for a night. Isolation of DNA from bacterial cells was performed by using Presto™ Mini gDNA bacterial kit.

The DNA were extracted from the bacteria that had grown in the pure culture.

PCR amplification

Following the instruction of the manufacture of the PCR primers (Gene work, Australia). The primer sets were utilized to promote the invasion of the damaged epithelium(*lmb*) gene codes for lmb (laminine-binding protein). (Table 1).

Table 1. Primer sets used for the detection of virulence genes in *Streptococcus agalactiae*

Putative function	Target gene	Primer Sequence (5-3)	Ampli-con size (bp)	Annealing temp. (C)	Reference
Promotes invasion of the damaged epitheli-um	*lmb*	ACCGTCTGAAATGATGTGG GATTGACGTTGTCTTCTGC	572	54	Spellerberg *et al.*, (1999)

AccuPower PCR PreMix is the powerful technology and easy to perform DNA amplification. It contains DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet. Primer (1.3 µl) of each forward and reverse, (2.5µl) of DNA template were added to AccuPower PCR tube then 20µl of distilled water added to AccuPower PCR tubes. After that lyophilized blue pellet dissolved by vortexing. PCR performed for samples, procees in the thermal cycler for 30 cycles as mentioned in table (2).

Table 2. PCR protocol and thermocycling conditions.

Gene name	Initial denaturation	Cycles	Denaturation	Annealing	Elongation	Final elongation
lmb	95°C/5min	30	94°C/1min	54°C/1min	72°C/2min	72°C/5min then 4°C→∞

The most common way to separate DNA molecules according to size is electrophoresis technique in agarose gel [14].

a. **Agarose preparation**

A 1.5% was made by adding 1.5 gm agarose to 1X TBE buffer. The agarose solution was boiled until all the agarose was dissolved in a microwave oven for 1 minutes and left to cool at 50°C then an appropriate comb was placed in a sealed mould, and agarose was poured into the mould. The gel was allowed to cool for at least 20 minutes before the seal and the comb was removed [14].

b. **Preparation of sample**

Before electrophoresis each well was loaded with 5µl of PCR product and 5 µl of ladder DNA marker (100bp) was loaded into the well flanking the samples.

Result

In current study 58 isolates of *S. agalactiae* were obtained from 136 diabetic mellitus patients. According to the results of enzyme assay, it was observed that all isolates produced hemolysin about 100% except for the protease test which indicated a percentage of about 60%.

Fifty eight isolates of *S. agalactiae* were tested for their sensitivity and resistance to 18 antimicrobial agents by using VITEK 2 Compact System, which revealed that their resistance to the antibiotics varied. The results indicated that the highest percentages of resistance of the isolates were respectively related to clindamycin 58 (100%), erythromycin 42 (72.4%), tetracycline 40 (68.9%), inducible clindamycin Resistance 38 (65.5%), vancomycine 36 (62.0%), ceftriaxcine 34 (58.6%), Cefotaxime 30 (51.7%), Benzylpenicillin 27 (46.5%), Ampicillin 25(43.1%), trimethoprim 20 (34.4%), linezolid 15 (25.8%), tigecycline 13 (22.4%), nitrofurantoin 11 (18.9%), moxifloxacin 9 (15.5%), ciprofloxacin 6 (10.3%), imipenem 5 (8.6%), and levofloxacin 4 (6.8%), and all isolates were susceptible to teicoplanin (Table 3).

Table 3. Antibiotic susceptibility test and degree of resistance of all isolates

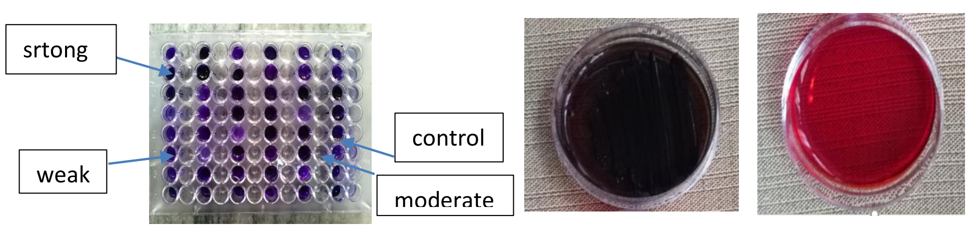
No. of antibiotics	Antimicrobial agent	Symbol	R	S	Percentage (%)
1	Clindamycin	Clind.	58	0	100%
2	Erythromycin	Eryth.	42	16	72.4%
3	Tetracycline	Tetra.	40	18	68.9%
4	Inducible clindamycin Resistance	I.C.R.	38	20	65.5%
5	Vancomycine	Vanc.	36	22	62.0%
6	Ceftriaxcine	Ceft.	34	24	58.6%
7	Cefotaxime	Cefo.	30	28	51.7%
8	Benzylpenicillin	Benzy.	27	31	46.5%
9	Ampicillin	Amp.	25	33	43.1%

Cont... **Table 3. Antibiotic susceptibility test and degree of resistance of all isolates**

10	Trimethoprim	Trim.	20	38	34.4%
11	Linezolid	Line.	15	43	25.8%
12	Tigecycline	Tig.	13	45	22.4%
13	Nitrofurantoin	Nitr.	11	47	18.9%
14	Moxifloxacin	Moxi.	9	49	15.5%
15	Ciprofloxacin	Cip.	6	52	10.3%
16	Imipenem	Imip.	5	53	8.6%
17	Levofloxacin	Levo.	4	54	6.8%
18	Teicoplanin	Teico.	0	58	0%

Biofilm was carried out for all isolates by two methods including microtiter plate method and Congo red method, and it was observed that microtiter plate method led to the highest level of biofilm formation of 53 (91.37%) , and the least biofilm formation was related to the congo red agar method 25(43.10%). Moreover, (figure 1a) indicates the results of biofilm formation by using microtiter plate assay and the control without bacteria isolate. While , figure (1b) presents the results of biofilm formation by Congo red method.

Two nanoparticles of iron oxide, and silver oxide were utilized to reduce biofilm formation from all of the isolates, and the results revealed that the Iron oxide nanoparticles lead to a reduction of 43(74.13%) in the biofilm formation, and silver oxide 35(60.34%) (figure 2).



Strong(black)

control(red)

Fig. 1. Biofilm formation with microtiter plate assay (a). Biofilm formation by using Congo red method(b)

(a) **(b)**

Fig. 2. Reducing biofilm formation using (a) Nanopartecles of Iron Oxide, and (b) Nanoparticles of Silver oxide.

The detection of virulence genes was performed by using PCR technique, which led to detection of *lmb* gene with product size 572bp. Indeed, the results of PCR found that the *lmb* genes (Figure 3) .

Discussion

Out of the 136 specimens obtained from the patients with diabetes mellitus, 58 isolates were diagnosed to be *S. agalactiae*, this results is similar with those reported by Farely[15] who stated that *S. agalactiae* is more prevalent among diabetic patients and the increased number of *S. agalactiae* infections is associated with diabetes. Moreover, Batista and Ferreira[16] also concluded that *S. agalactiae* is quite common among diabetic patients.

The results obtained from VITEK 2 Compact System and Gram positive Kit indicated that all obtained isolates of *S. agalactiae* were 100% resistant to clindamycin, this finding is in line with the reports of the studies conducted by wang *et al.,*[17] who concluded that clindamycin resistance rate is extremely high among pregnant women, also Rawat *et al*.,[18] reported that resistance to clindamycin is high among diabetic patients. However, it was also observed that other antibiotics were used for *S. agalactiae* isolates gave variable resistant, while teicoplanin was susceptible for all isolates. This results similar with the results reported by Barberis *et al.,* [19].

The current results indicated that the highest and lowest amounts of biofilm production were respectively related to microtiter plate method (91.37%) and congo red agar method (51.72%). This finding was almost in line with those of the studies reported by Azeredo *et al.,* [20].

Fifty three (91.37%) isolates were biofilm producers by using microtiter plates assay, and this method was the better screening test for biofilm production than Congo red agar because it was easy to perform both qualitatively and quantitatively [21]. Also, Mathur *et al*.,[12] reported that the microtiter assay was an accurate and reproducible method than Congo red agar, and this method can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of microorganism [22].

With regard to the effect of the studied nanoparticles on reduction of biofilm, our results found that the Iron oxide nanoparticles resulted in a decrease of 43 (74.13%) in biofilm formation, and also, silver oxide nanoparticles were found to reduce biofilm formation by 35 (60.34%). This finding is closed to results of Ueno *et al*.,[23].

The *lmb* gene (laminin binding protein) plays an important role in the adherence of *S.agalactiae* 30 (51.72%) isolates were found to contain the *lmb* gene in the present study. According to Spellerberg *et al*., [24], the *lmb* gene was presenting the common serotypes of *S agalactiae*.

Conclusion

In current work it was indicated that *S. agalactiae* was quite common among the patients with diabetes mellitus, which could be reduced through, iron, and silver oxide nanoparticles. Since *S. agalactiae* can lead to result in severe invasive diseases, diabetic patients and medical experts are recommended to utilize such nanoparticle in order to inhibit or reduce biofilm formation as a result of S. agalactiae.

Conflict of Interest: Nill

Ethical Clearance: The study was approved by the ethical committee of the Salahaddin University University of Sulaimani/ College of Education

Source of Funding: Not

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