In vitro Salmonella typhi biofilm formation on gallstones and its disruption by Manuka honey

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Abstract: Biofilm is a complex community of single or different types of microorganisms (bacteria, viruses, fungi, protozoa) attached to a surface and stick to each other through production of extracellular matrix. *Salmonella typhi* forms biofilm on cholesterol gallstones resulting in carrier state. Once formed, biofilm is difficult to treat. To date cholecystectomy is the only cure for this condition. Manuka honey is known to have tremendous antibiofilm activity against various organisms. *S. typhi* biofilm was grown *in vitro* on clinical samples of human cholesterol gallstones by Gallstone tube assay method for 12 days. Biofilm mass was quantified on day 1, 5, 7, 9 and 12 by crystal violet assay and was also examined by scanning electron microscope. Three concentrations w/v of Manuka honey (40%, 60% and 80%) were used, each one at 24, 48 and 72 hours. The most effective concentration (80% w/v) was repeated on two sets of gallstones. Biofilm mass was re quantified by crystal violet assay and was examined by scanning electron microscope. *S. typhi* formed uniform biofilm on cholesterol gallstone surface. The optical density measurements exhibited a rising pattern with time thereby indicating an increase in biofilm mass. It was 0.2 on day 1 and 0.9 on day 12. With 80% w/v Manuka honey, biofilm mass decreased most effectively with 0.5 OD after 72 hours. Biofilm formation by *S, typhi* on gallstones is surface specific and bile dependant. Either increasing the duration (beyond 72 hours) of the effective concentration (80% w/v) of honey or increasing the concentration (above 80%) of honey for a specific duration (72 hour) may cause complete disruption of the *S. typhi* biofilm on gallstone. *S. typhi* forms biofilm on cholesterol gallstones surface *in vitro* and it can be visualized by scanning electron microscopy. Biofilm mass can be quantified using crystal violet assay. Among various concentrations 80% Manuka honey for 72 hours is most effective in disrupting *S. typhi* biofilm on gallstones *in vitro* as evident from crystal violet assay.

Keywords: *Salmonella typhi,* Biofilm, Manuka honey, cholesterol gallstones, scanning electron microscope.

INTRODUCTION

Biofilm is a complex community of single or different types of microorganisms (bacteria, viruses, fungi, protozoa) attached to a surface that stick to each other through production of extra cellular matrix (Richards and Melander, 2009). Biofilms lead to persistence of chronic infections as these films are highly resistant to antimicrobials, host defenses, stress, dehydration, antiseptics, disinfectants and UV radiation (Taraszkiewicz *et al.*, 2012).

Salmonella enterica serovar Typhi infections may lead to asymptomatic carrier state, possibly due to the formation of biofilms as a mechanism that contributes to the development of the "carrier" state (Reeve, 2010). A recent study reported that Pakistan has the highest incidence of typhoid fever, which is 451.7 per 100,000 persons each year and mean affected age is 7 year (Dewan *et al.*, 2013). About 5% of individuals suffering from typhoid fever become carrier. It has been shown that gallstones are the most significant risk factor for becoming a chronic carrier of *S. typhi* because the organism colonizes there by forming biofilm (Crawford *et al.*, 2008).

In patients carrying both Typhi and cholesterol gallstones in the gallbladder, clinically administered antibiotics are typically resistant against chronic bacterial infection (Hall-Stoodley *et al.*, 2006). It is impossible to eliminate carrier state of Typhoid disease with currently available antibiotics. To date cholecystectomy is the only procedure to overcome this condition (Raza *et al.*, 2011).

Individuals who become chronic carriers of Typhi may transmit bacteria for whole life by shedding the organisms through cystic duct. Invasion of gall bladder epithelium and formation of biofilms on gall stones may protect pathogen from bactericidal activities of bile salts (Bäumler *et al.*, 2011). Chronic cholecystitis is the most prevalent disease in many industrialized countries and is commonly associated with gall stones (Elwood, 2008).

For the last two to three decades there has been an increasing interest in traditional medicine throughout the world. Manuka honey has a very high level of

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nonperoxide antibacterial activity. The pronounced antibacterial activity of Manuka honey is an important medical & commercial property which is referred for marketing purposes as the so called 'Unique Manuka Factor" (UMF). The antibacterial properties of many honeys are associated with hydrogen peroxide but Methylglyoxal (MGO) has been shown to be an important antibacterial agent in Manuka honey (Mavric *et al.*, 2008). Manuka honey has better antibacterial activity than other types of honey. It is a standardized FDA approved honey (Visavadia *et al.*, 2008) (Atrott and Henle, 2009) (Alnaimat *et al.*, 2012). It is capable of disrupting the biofilm produced by different strains of *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans* and other pathogens *in vitro* which are otherwise resistant to antibiotics(Alandejani *et al*., 2009) (Majtan *et al.*, 2014) (Maddocks *et al.*, 2012) (Nassar *et al.*, 2012). It is being used effectively as a dressing for wounds, burns, and skin ulcers (M. D. Mandal and Mandal, 2011).

The aim of this study is to establish biofilm of clinical isolates of *Salmonella typhi* strains on gallstones *in vitro* by Tube Assay method, to examine the structure of biofilm on gall stones by Scanning electron microscopy and to identify the role of Manuka honey in gall stone biofilm disruption by using Micro broth dilution method. To the best of our knowledge, no work has been done on *S, typhi* gallstone biofilm disruption till date.

MATERIALS AND METHODS

Setting

The study was conducted in the Department of Microbiology, University of Health Sciences, Lahore, Centre of Excellence for Solid State Physics, University of the Punjab, Lahore and the Department of Immunology, University of Health Sciences, Lahore.

Study design

This is a laboratory based Experimental study. It was completed in three weeks.

Sample size

Human gallstones (n=34) of uniform size were used.

Materials

Honey sample

Manuka honey (UMF20 +) FDA approved was obtained from Comvita Newzealand. The sterility was checked by spreading a loopful quantity on blood agar medium and incubating at 37^{o}C aerobically overnight. After overnight incubation no growth was observed on blood agar plate. Manuka honey was stored at 4º to 6^{o}C.

Bacterial isolates

A strain of *Salmonella typhi* isolated from a patient was used in this study while *Pseudomonas aeruginosa* (ATCC 27853) used as a standard control strain. The isolates had been stored in microbanks at -70^{o}C in the Department of Microbiology, University of Health Sciences Lahore, Pakistan**.**

Identification of bacterial isolate

The isolate was cultured on blood and MacConkeys agar. The plates were incubated at 37^{o}C aerobically. The identification was done by their morphological and cultural characteristics and confirmed by using biochemical profile (API 20E). The serological identification of *S. typhi* was performed by BD DifcoTM antisera. AST susceptibility was performed by applying panel of 6 drugs which were ampicillin, amikacin, tetracycline, ceftriaxone, co-trimoxazole and chloramphenicol. The isolate was characterized as a Non-MDR strain.

Scanning electron microscope

The formation and disruption of biofilm on gallstones was visualized by scanning electron microscope at 5000 to 8000x.

Gall stones

A total of 34 gallstones from a single patient were used. Chemical analysis of gallstone was got done by Agha Khan University laboratories, Karachi. According to the report, the size was 0.2cm and the colour was yellow with amorphous consistency. These were composed of cholesterol as the major component. The stones were disinfected with 90% ethanol (90 ml ethanol in 10ml distilled water) and their sterility was checked by incubating in nutrient broth for 18-24 hours at 37C. No growth was observed after 24 hours of incubation aerobically. Out of 34 gallstones, specimen 1 was taken as negative control, specimen 2 was taken as positive control of biofilm for *P. aeruginosa*, and the remaining 32 gall stones were taken as test stones for *S. typhi* biofilm.

Method

Bacterial suspension for biofilm formation

Four well isolated colonies from overnight culture of the test strain (*S. typhi*) and ATCC strain (*Pseudomonas aeruginosa*) were suspended separately in 5 ml nutrient broth tubes. After overnight incubation each culture was diluted (1:10) in nutrient broth.

Formation of biofilm on gall stones by tube assay method

A total of 350ml of biofilm forming medium was prepared by adding 340ml nutrient broth, ox bile 10.5 grams (3%) and 480ul of tetracycline (10mg/ml) to prevent contamination. A 10ml diluted (1:10) Typhi suspension was added to the medium. Then 10 ml of the prepared medium simulated with the test organism was added in each of the 34 universal (28ml vol) bottles containing one human gall stone and the bottles were

incubated in a shaking waterbath at 37°C for 24 hours aerobically. After every 24 hours gall stones were washed with nutrient broth and fresh medium without bacterial suspension was added. The procedure was carried out for 12 consecutive days (optimal time period for biofilm development) (Crawford *et al.*, 2008). One negative control, one positive control of biofilm with *P.aeruginosa* and sterility controls of medium were also run parallel.

Crystal voilet assay for quantification of biofilms

Crystal violet assay was done on specific days i.e, day 1,5,7,9 and 12 on specimen no. 3,4,5,6 and 7 respectively. Gall stones were washed three times in nutrient broth. Biofilm on gall stones was fixed with 95% ethanol for 10 min. A solution of 1% crystal violet was then added to stain cells for 30 minutes at room temperature. Specimens were washed thoroughly with nutrient broth until the liquid ran clear. The dye was removed using 95% ethanol for 60 minutes at room temperature and then quantified at OD_{595} by spectrophotometer to determine the amount of dye retained by the biofilm cells (Hammond *et al.*, 2010).

Visualization of biofilm under scanning electron microscope

Specimen no. 8, 9 and 10 were air dried overnight and then fixed with 2% glutaraldehyde for 4 hours. Fixed samples were dehydrated in electrical furnace. Formation of biofilm was examined by scanning electron microscopy at 5000 to 8000x (Crawford *et al.*, 2008).

Application of manuka honey on biofilm

Manuka honey UMF 20+ was applied on eighteen stones (11 to 24). Three working concentrations w/v of Manuka honey i.e, 40%, 60% and 80% were tested to determine its efficacy in disrupting Typhi gallstone biofilm. A total of 15 ml of each working concentration was prepared and 5 ml of each concentration was applied for each duration i.e, 24, 48 and 72 hours separately. After application of manuka honey, eighteen stones were divided into two groups. Nine out of eighteen gallstones were subjected to crystal violet assay and the other nine were visualized by scanning electron microscopy to observe biofilm disruption.

40% w/v honey was applied on specimens 11, 12 for 24 hours, specimens 13, 14 for 48 hours, specimens 15, 16 for 72 hours. 60% w/v honey was applied on specimens 17, 18 for 24 hours, specimens 19, 20 for 48 hours, specimens 21, 22 for 72 hours. 80% w/v honey was applied on specimens 23, 24 for 24 hours, specimens 25, 26 for 48 hours, specimens 27, 28 for 72 hours. Specimens 11, 13, 15, 17, 19, 21, 23, 25 and 27 were examined under electron microscope to visualize the efficacy of Manuka honey [5]. Crystal violet assay was done on specimens 12, 14, 16, 18, 20, 22, 24, 26, 28 to observe biofilm disruption and OD_{595} was measured by spectrophotometer (Hammond *et al.*, 2010).

Then the most effective concentration was retested for reproducibility of results. It was applied on specimens 29 and 30 for 24 hours, specimen 31 and 32 for 48 hours, 33 and 34 for 72 hours and crystal violet assay was done again (Hammond *et al.*, 2010).

STATISTICAL ANALYSIS

The data was entered and analyzed by using SPSS version 20.0. Descriptive statistics i.e. Mean ± SD was given for Optical Density. Paired sample t-test was applied to compare mean of three optical densities of the most effective concentration of honey at each interval, with baseline reading. ANOVA was applied for comparison among three mean optical densities of the most effective concentration. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Crystal violet assay before application of Manuka honey

Crystal violet assay done on day 1, 5, 7, 9 and 12 on specimens 3,4,5,6, and 7 showed optical density readings 0.2, 0.3, 0.5, 0.7 and 0.9 respectively. A linear relationship was observed between days and optical density readings showing a sequential rise in optical density readings as shown in the fig. 1.

Table 1: Effect of 40%, 60% and 80% w/v concentrations of Manuka honey on gallstone biofilm after 24, 48 and 72 hours treatment.

Concentration w/v %	OD_{595}after24 hrs	OD_{595} after 48 hrs	OD_{595} after 72 hrs
40%	1	0.9	0.8
60%	0.9	0.8	0.7
80%	0.7	0.6	0.5

Table 2: Effectiveness of 80% w/v Manuka honey.

80% concentration w/v	24 hours (OD)	48 hours (OD)	72 hours (OD)
1st reading	0.7	0.6	0.5
2nd reading	0.76	0.68	0.62
3rd reading	0.81	0.72	0.57
Mean OD_{595}	0.75	0.66	0.56

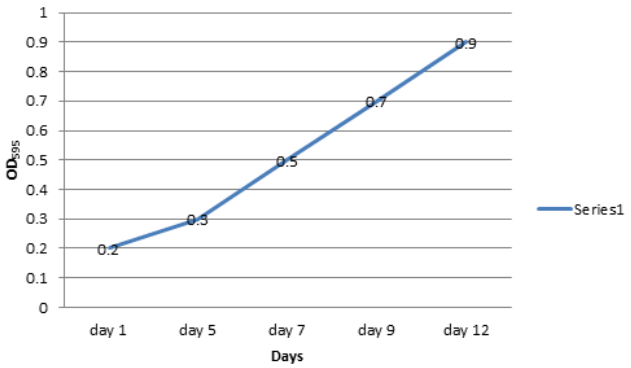

Fig. 1: Quantification of biofilm density showing a growth curve

Crystal violet assay before application of manuka honey

After treatment of Manuka honey on gallstone biofilm, a sequential decrease in optical density reading from 24 hours to 72 hours was observed as shown in the fig. 2. After 24 hours, 40% and 60% w/v honey did not decrease optical density but 80% w/v honey decreased OD to 0.7. After 48 hours, 40% w/v honey did not decrease OD, 60% w/v honey decreased OD to 0.8 and 80% w/v honey decreased OD to 0.6. After 72 hours, 40% w/v honey changed OD to 0.8, 60% decreased OD to 0.7 and 80% decreased OD to 0.5 as shown in table 1.

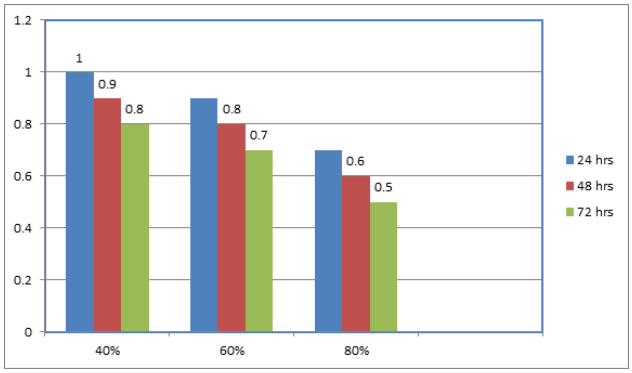

Fig. 2: Comparison between 40%, 60% and 80% w/v concentrations of Manuka honey.

Based on these findings it is evident that 80% w/v Manuka honey effectively disrupted the biofilm. To further validate its effectiveness, 80% w/v Manuka honey was applied in duplicate on specimens 29, 30 for 24 hours with OD of 0.76 and 0.81 respectively. Specimens 31, 32 were treated for 48 hours and OD was 0.68 and 0.72 respectively. Specimens 33, 34 were treated for 72 hours and OD was 0.62 and 0.57 respectively. The mean optical density readings were calculated and are shown in table 2.

It is quite evident from the readings that 80% honey is the most effective concentration in disrupting biofilm (fig. 3). Statistical analysis of mean O.D values of 80% Manuka honey at each interval shows, ± SD is 0.06 and p-value is 0.046 at 24 hours, ± SD is 0.06 and p-value is 0.022 at 48 hours, and ± SD is 0.06 and p-value is 0.011 at 72 hours. This shows that the results are significant after all three durations of treatment with 80% w/v Manuka honey. The cumulative p-value of all three mean O.D values is 0.02 which is significant.

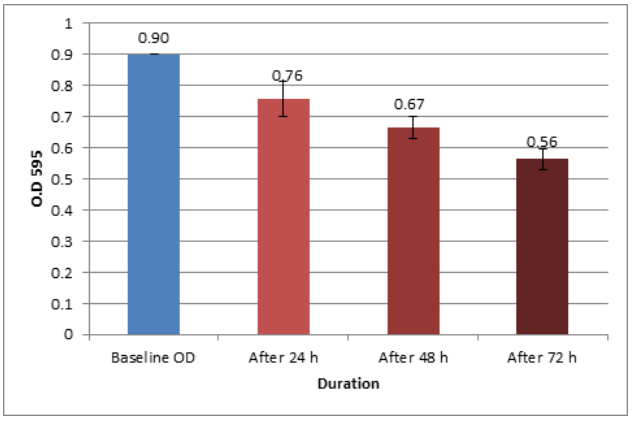

Fig. 3: Comparison between baseline OD_{595} and mean OD_{595} readings after treatment with 80% w/v Manuka honey.

Scanning electron microscopy of gallstone samples

Scanning electron microscopy was used to visualize the developed biofilm and its disruption by Manuka honey at different concentrations. A total of twelve specimens, three before treatment with honey and nine after treatment with Manuka honey were subjected to Scanning electron microscopy. Specimens 8, 9 and 10 were subjected to SEM to visualize developed biofilm. It showed web like strands indicating a developed biofilm as shown in fig. 4.

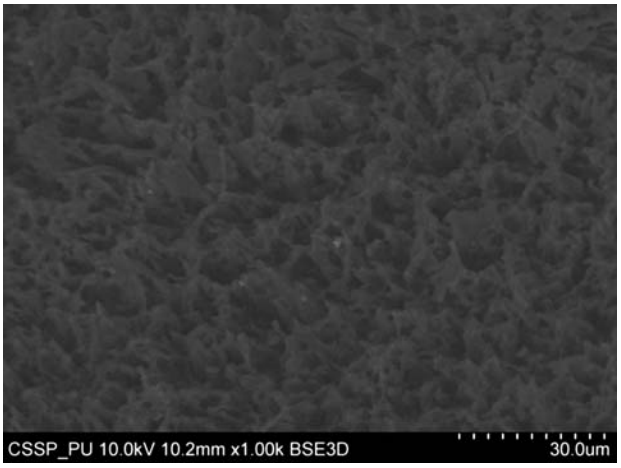

Fig. 4: Specimen 12. Presence of biofilm in the form of web like strands.

Specimens treated with 80% Manuka honey were visualized at 24, 48 and 72 hour by SEM which showed disruption of biofilm by 80% Manuka honey as shown in fig. 5, 6 and 7. Visualization of 40% and 60% Manuka honey treated biofilm after 24, 48 and 72 hours did not have a considerable effect on biofilm mass and did not show any noticeable reduction in surface biofilm mass.

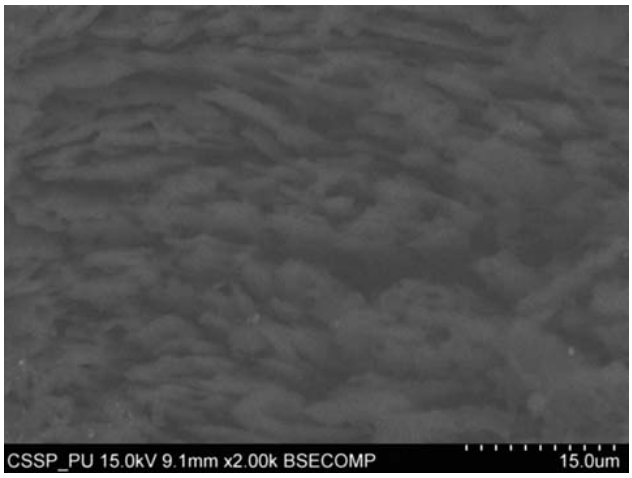

Fig. 5: Specimen 26. 80% w/v Manuka honey treated biofilm after 24 hours with obvious decrease in surface biofilm mass.

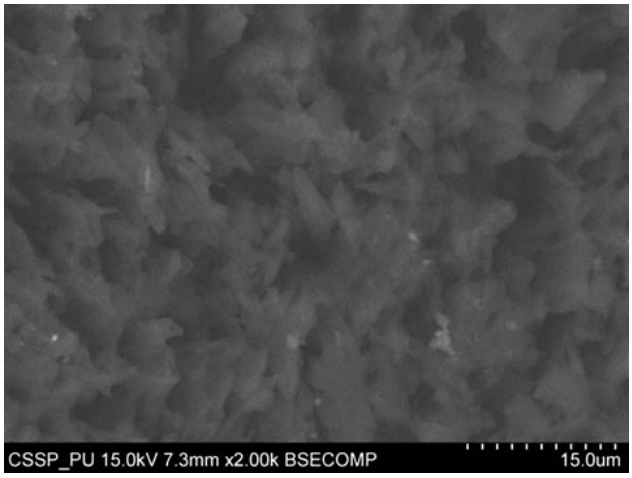

Fig. 6: Specimen 28. 80% w/v Manuka honey treated biofilm after 48 hours with considerably less organisms on the surface of gallstone.

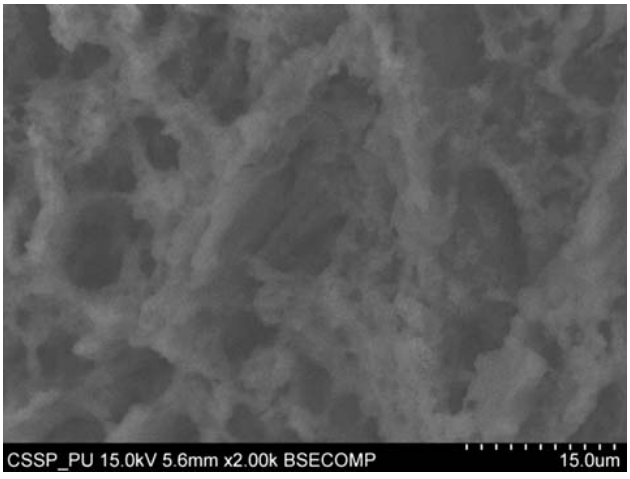

Fig. 7: Specimen 30. 80% w/v Manuka honey treated biofilm after 72 hours with noticeable reduction in surface biofilm mass.

DISCUSSION

The findings of the present study indicate clearly that 80% w/v Manuka honey has the ability to disrupt *Salmonella typhi* biofilm. Growth curve obtained after crystal violet assay showed that biomass increases as the time increases as shown in fig. 1.

Similar growth curve was obtained in the study of A.M Prouty in 2002 who quantified biofilm formed by *Salmonella* by growing it on glass slides. He described it as difficult to quantify the biofilm on gallstone due to brittle nature of gallstones, variations among gallstones, and the changing or uneven release of planktonic cells from the surface of gallstone by chemical or mechanical methods (Prouty *et al.*, 2002).

Our study showed that optimal time period for the growth of biofilm was 12 to 14 days. This time period was also described as the best duration by A.M Prouty in his study. After 12 to 14 days, biofilm mass starts decreasing due to initiation of autolysis (Prouty *et al.*, 2002).

It was also observed in our study that addition of ox bile and selection of cholesterol surface was necessary for biofilm formation by *Salmonella*. Bile induces bacteria to produce an exopolysaccharide (EPS), the O-antigen capsule, which is necessary for biofilm formation and cholesterol surface provides distinct receptor site recognized by the bacterium for attachment (Crawford *et al.*, 2008) (Prouty *et al.*, 2002).

After honey treatment, it was found that 80% concentration of Manuka honey reduced the biofilm density to quite an extent after 72 hours as compared to 40% and 60% concentrations. Based on this finding , it can be deduced that either increasing the duration (more than 72 hours) of a specific effective concentration (80% w/v) of Manuka honey or increasing the concentration (80%) of manuka honey for a specific duration (72 hour) may cause complete disruption of Typhi gallstone biofilm.

Comparing all three intervals, mean difference between 24 and 48 hrs is insignificant and p-value is 0.22 , mean difference between 24 and 72 hours is significant and p-value is 0.01, mean difference between 48 and 72 hours, is insignificant and p-value is 0.16. This indicates that significant difference exists between 24 and 72 hours interval. It would be better to treat the biofilm for 72 hours rather than 24 hours.

One of the important aspects is to find out the mechanism of disruption of biofilm by Manuka honey. A mechanism described in one study for inhibition of *Pseudomonas aeruginosa* biofilm is by cell lysis and altered expression of oprF and algD genes involved in biofilm microcolonies formation (Roberts *et al.*, 2012). Manuka honey interferes cell division process in methicillin resistant

Staphylococcus aureus strains, thus inhibiting biofilm formation (Jenkins *et al.*, 2011). In case of *Streptococcus pyogenes*, Manuka honey reduces the expression of two fibronectin binding proteins resulting in decreasing the adhesiveness of the organism (Maddocks *et al*., 2012).

The mechanism of biofilm disruption by Manuka honey for *Salmonella typhi* is so far unknown and further research is needed. The overall antibacterial activity of Manuka honey is synergistic effect of methylglyoxal, hydrogen peroxide, sugar components, phenolic compounds including flavonoids, acids and minerals. Methylglyoxal and carbohydrate content are important factors which interfere with biofilm formation (Merckoll *et al.*, 2009).

To the best of our knowledge, this is the first ever study done on the formation and disruption of biofilm by Typhi on gallstones. However, we would like to discuss some of the limitations. Some advanced methods should also be used to quantify biofilms which we could not carry out due to limited resources (Peeters *et al.*, 2008). Biofilm mass could not be quantified by Scanning electron microscopy because of smaller size and amorphous nature of gallstones due to which cross sections could not be done. Sample size is limited in this study. One of the requirements for this study was that gallstones from a single source should be used so that it could be assumed that each stone is behaving same in the context of biofilm formation. The same stone could not be resused for different steps of the experiment. From a single source maximum 34 gallstones could be retrieved (Crawford *et al.*, 2008) (Prouty *et al.*, 2002).

Further *in vivo* trials are required to establish the antibiofilm role of Manuka honey against Typhi carrier state. The pharmacokinetics of Manuka honey has yet to be established for *in vivo* eradication of Typhi carrier state. Trials can be carried out on Animal models. Genetic factors involved in Typhi biofilm formation on gallstone should also be explored in future work.

CONCLUSION

S. typhi forms biofilm on cholesterol gallstones surface *in vitro* and it can be visualized by scanning electron microscopy. Biofilm mass can be quantified using crystal violet assay. Manuka honey possesses significant antibiofilm activity for gallstones *in vitro* against Typhi. Among various concentrations 80% w/v Manuka honey for 72 hours is most effective in disrupting Typhi biofilm on gallstones *in vitro* as evident from crystal violet assay.

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