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Original Research Article

Isolation of *Streptococcus Mutans* and its Bacteriophage from Human Plaque Samples

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Abstract

Background*: Streptococcus mutans* (*S. mutans*) is one of the main agents of caries formation, mainly because of the ability to form biofilms on the tooth surface. Bacteriophage of *S. mutans* are viruses that can attack and limit the pathogenic activity of *S. mutans*, hence limiting their cariogenic effect and preventing dental caries. There is a deficiency in the literature on the successful isolation of phage against *S. mutans*. ***Aims:*** The purpose of this study is to isolate *S. mutans* strains from clinical plaque samples, screen those samples for phage and test them against laboratory type cultures for phage. ***Methods****:* Thirty-eight clinical plaque samples were collected from participants using ESwab (Copan Italia, Brescia, Italy) and cultured on Brain Heart Infusion (BHI) and Tryptone-yeast-cysteine-sucrose-bacitracin (TYCSB) agars to isolate *S. mutans* strains. Following isolation and identification by Gram stain and PCR, phage screening by spot assay against laboratory type cultures was carried out. Six NCTC *S. mutans* strains (10832, 10919, 10920, 10923, 11060, 11061) and twelve type strains provided by Newcastle University (*S. mutans* UA159, 10449, UA140, Ingbritt, GS5, *sobrinus* 12279, *gordonii* DL1, *sanguinis* SK36, *oralis* 34, *tigurinus* JP1BV1, *oligofermentans* LR11BV4 and *Actinomyces oris* MG1) were all used for spotting. ***Results***: The isolation of *S. mutans* strains from the clinical samples was successful. TYCSB agar showed to be selective for *S. mutans* while BHI media showed rich growth of different colonies. Gram stain was performed on the suspected colonies and confirmed later by PCR for *S. mutans*. On spot assay, no evidence of phage lysis was found within pooled filtrate samples against NCTC type strains and Newcastle type strains. ***Conclusion***: The isolation of *S. mutans* from clinical samples was achieved using TYCSB media. Phage isolation was unsuccessful from the 38 clinical plaque samples probably due to low frequency of their natural occurrence. Isolation of *Streptococcus mutans* and bacteriophage from human plaque samples

Keywords: Streptococcus mutans, dental caries, bacteriophage, phage, oral cavity, dental plaque.

INTRODUCTION

The human oral cavity is densely populated by microorganisms including viruses, bacteria, protozoa, fungi and archaea. Dental caries is the most prevailing disease of the oral cavity arising from an ecological imbalance of the metabolic activities in the oral microbiome. *Streptococcus mutans* considered one of the most important cariogenic bacteria, is a Gram-positive, coccus shaped, facultative anaerobic bacterium naturally present in the oral cavity. *S mutans* produce abundant exopolysaccharides thus contributing to the formation of the biofilm matrix and has acidogenic activity in the presence of dietary sucrose. Identifying streptococci to the species level can be challenging, especially between *S. mutans* and *S. sobrinus* within the mutans streptococci group. There are various methods currently used for the identification of *S. mutans*, include morphological differentiation on selective agars and PCR identification [2].

The study of the human microbiome has focused predominantly on bacterial flora, although there are numerous reports of viral communities inhabiting different body sites. The majority of these viruses identified are bacteriophages [2]. Bacteriophages are viruses that attack and kill bacteria within a narrow host range. They are self-replicating and are increasingly considered potential replacement or alternative to antibiotics. Several studies have been carried out in this field to investigate and isolate bacteriophages from

human saliva and dental plaque for the control of oral diseases such as dental caries and periodontitis. However, there is a limited number of reports and knowledge in the field of oral microbiology regarding the isolation and use of phage to control *S. mutans* growth and cariogenic activity. The difficulty in isolating phage from clinical samples is most probably due to the low frequency of those phages naturally or due to the narrow host range [3]. The aim of this study is to isolate *Streptococcus mutans* and detect the presence of bacteriophage in human plaque samples.

MATERIALS AND METHODS

Patient enrolment and sample collection

Subject enrolment was approved by the East of Scotland Research Ethics Service of Ninewells Hospital and Medical School (protocol no. AC16095). All subjects completed a consent form demonstrating their approval to participate in the study. All subjects were healthy adults with clinically diagnosed dental caries. Plaque samples were obtained using ESwab™ Liquid Amies Collection and Transport System (catalog no. 481CE, Copan Italia, Brescia, Italy). The dental plaque samples were obtained by swabbing the surfaces of the teeth of each participant, using ESwab™ which is a sterile applicator swab with flocked nylon fibre tip. Following that, the swab was placed into a screw-cap tube with internal conical shape filled with 1 ml of Liquid Amies Medium. The samples were collected in the morning and processed within 3 hours after collection. A total of 38 samples were collected over five separate days.

Agar Plating

The BHI agar plates were prepared using 35g/L Oxoid™ Brain Heart Infusion solids and 15g/L Oxoid™ Agar (Oxoid Limited, Hampshire, UK). While the TYCSB plates were prepared as described by Wan *et al*. [4] using dehydrated versions of the following: 0.2g L-cysteine HCL monohydrate (Sigma Chemical, St Louis, MO, USA); 15g bacteriological peptone (Oxoid Limited); 5g yeast extract (Oxoid Limited); 0.1g Sodium Sulphite (Sigma); 0.1g Sodium Chloride (Fisher Scientific); 1.0g Sodium Phosphate (Sigma); 2.0g Sodium bicarbonate (Fisher Scientific); 20g Sodium acetate GPR (VWR International Ltd, Leicestershire, England); 20% w/v sucrose (Sigma); 15g granulated agar (Oxoid Limited); 0.1U/ml bacitracin (Sigma); distilled water. Poured agar plates were sealed and stored at 4°C.

A 100 µl volume of each serial dilution of the sample filtrates were spread onto BHI and TYCSB agar plates. In addition, neat filtrates were streaked using plastic sterile inoculation loops and all plates were incubated at 37°C under anaerobic conditions for 48 hours.

Isolation and Identification of Streptococcus mutans

Catalase test was carried out by placing a small amount of the growth from the culture onto a clean microscopic slide and adding a few drops of H_2O_2. A positive result is the rapid evolution of O_2 as evidenced by bubbling, while a negative result is no bubbles or a few scattered bubbles. *S. mutans* are known to be catalase negative; therefore colonies showing negative result with catalase test were selected for Gram staining. The Gram stain is extremely useful and widely used. The described protocol was followed: the microscopic slide with the selected colonies were labelled and prepared by adding 2 loop-fulls of the bacterial culture onto the slide forming a smear layer. This is left to dry and then heat-fixed. Crystal violet stain, Iodine stain, absolute alcohol and Safranin stain were used then the microscopic slides were examined using OLYMPUS BX51 microscope under x100 power. Gram positive organisms will be dark blue/purple and gram negative organisms will be pink.

Culture collection and spot test for bacteriophage isolation

The following culture collection strains were used in this study was NCTC10832, NCTC10919, NCTC10920, NCTC10923, NCTC11060, and NCTC1106. Two strains were used in addition to the previously mentioned: DSM 20523 *Streptococcus mutans* and DSM 20742 *Streptococcus sobrinus.* The last two strains were used for reference Gram stain showing Gram positive cocci in clusters or pairs.

Table-1: Other type strains used were kindly donated by Newcastle University are the following:

	Genus	Species	Strain
1	*Actinomyces*	*oris*	MG1
2	*Streptococcus*	*gordonii*	DL1
3	*Streptococcus*	*sanguinis*	SK36
4	*Streptococcus*	*mutans*	UA159
5	*Streptococcus*	*mutans*	10449
6	*Streptococcus*	*mutans*	UA140
7	*Streptococcus*	*mutans*	Ingbritt
8	*Streptococcus*	*mutans*	GS5
9	*Streptococcus*	*oralis*	34
10	*Streptococcus*	*sobrinus*	12279
11	*Streptococcus*	*tigurinus*	JP1BV1
12	*Streptococcus*	*oligofermentans*	LR11BV4

A 10 µl of the pooled bacterial filtrates is spotted onto labelled plates poured with prepared sloppies. The sloppy is prepared by melting sloppy agar then adding 50 µl CaCl, 50 µl MgCl and 800 µl of the host bacteria (type strains). The plates are left to dry and incubated overnight at 37°C.

RESULTS

Isolation of Streptococcus mutans from human plaque samples

Unlike the results found by Wan *et al*. [4], the TYCSB plates showed poor bacterial growth. Better

bacterial growth was found with BHI plates. Random well-isolated colonies were selected from the plated clinical samples at various dilutions for Catalase test and Gram staining. Suspected colonies were picked up from TYCSB plates and grown into BHI liquid broth overnight, using the serial dilution technique of the overnight broth, the bacteria were sub-cultured on TYCSB plates. These plates showed good growth of white crystal-like small colonies which were then sub-cultured for the second time in BHI liquid broth overnight, and plated onto TYCSB plates by serial dilution and spread technique to form the 2nd sub-cultured colonies.

Six isolates tentatively identified as *S. mutans* were isolated from thirty-eight clinical plaque samples. Most TYCSB agar plates showed variable amount of growth of transparent crystal-like colonies, while BHI plates showed a wider variety of colony types. The putative *S. mutans* colonies were catalase negative and Gram positive cocci in pairs and clusters which was comparable to the microscopic morphological appearance of the reference prepared earlier from DSM 20523 *S. mutans* type strain.

Table-2: The findings in this study are summarised in the following table:

Sample ID/Date	Colony sample	Colony morphology	Catalase test	Gram stain	PCR	Species ID
NH 09.02.2017		Small hard granular crystal-like round colonies	-ve	+ve	+	0902171
S01 13.02.17	01a	Very small hard granular crystal-like round colonies	-ve	+ve	+	1302171
S01 13.02.17	01b	Very small hard granular crystal-like round colonies	-ve	+ve	+	1302172
S02 13.02.17		Very small hard granular crystal-like round colonies	-ve	+ve	+	1302173
S09 03.03.2017	09a	Small golden brown granular round colonies	-ve	+ve	+	0303171
S09 03.03.2017	09b	Very small hard granular crystal-like round colonies	-ve	+ve	+	0303172
S10 03.03.2017		Small hard granular crystal-like round colonies	-ve	+ve	+	0303173

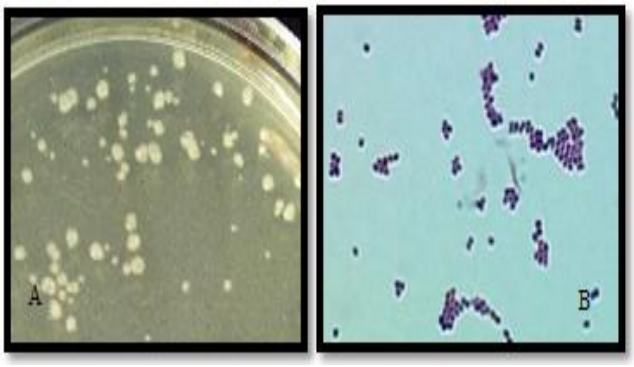

Fig-1: Putative colonies of S. mutans on TYCSB plate. (A) Crystal-like colonies, small and well isolated. (B) Gram stain microscopic view

Spot test against culture collection

The purpose for obtaining laboratory strains was to use them for phage typing and to determine the host range.

No zones of lysis were evident on spot assay of pooled filtrates against laboratory type bacterial strains. The control plates mostly showed good lawns. A positive finding was observed with the pooled filtrate of samples (04-07) spotted onto host *S. mutans* NCTC10832 based on a clear zone of lysis in a lawn of bacterial growth. Spotting of the pooled filtrate (04-07) on *S. mutans* NCTC10832 was repeated, a good lawn of bacterial growth was obtained on the control plate but no lysis zone was observed on the test plate. However, a medium white flat round colony was found on the test plate suggestive of possible contamination.

DISCUSSION

The present study showed that TYCSB and BHI agar plates allowed good growth of *S mutans* from clinical plaque samples. Five different media suggested by Wan *et al*. [4] for the selective isolation of *S. mutans* from clinical samples: MSB, MSKB, GSTB, TYS20B and TYCSB. In this study BHI and TYCSB agar plates

were used and the suspect colonies were well isolated. Wan *et al.* [4] reported that TYCSB allows the visual differentiation of *S. mutans* as tiny white colonies within a clear zone which was a similar finding in this study. This extends to dental plaque samples used although with a lower sensitivity with TYCSB than reported by Wan *et al*. [4]. This can be due to the variation in methodology, formulation of the media tested and strains of *S. mutans* used. Furthermore, TYCSB is reported to be the least supportive of non-*S mutans*, which enhances the accuracy and enumeration of *S. mutans*. Another advantage of TYCSB is that it allows the visual differentiation of *S mutans* as (tiny white colonies within a clear outer zone) from *S sobrinus* as (white-yellow colonies with or without a hazy outer zone) [4]. Presumed colonies of *S. mutans* were small, transparent, crystal-like and showing a granular surface, which is similar to the morphology described by Estela *et al*. [5] as ''colonies of *S. mutans* showed a granular surface, similar to ground glass, with or without a scintillant polysaccharide drop on the surface''.

Bacteriophage can limit bacterial abundance and pathogenicity in the oral cavity. A bacteriophage infecting *Lactobacillus casei* has been obtained from the oral material [6], bacteriophages specific for species of *Veillonella* species were isolated by Hiroki *et al*. [7]. Also, phages lytic for *Actinomyces* spp were isolated from dental plaque specimens and virus specific for *Actinobacillus actinomycetomcomitans* have been described [8]. Delisle & Rostkowski [9] have described bacteriophage lytic for *Streptococcus mutans*. and Bachrach *et al*. [10] tried to isolate bacteriophages for Gram positive oral pathogens such as *Streptococcus sobrinus*, *Streptococcus mutans* and *Streptococcus salivarius* from human saliva but found only bacteriophage for *Enterococcus faecalis*. Hitch *et al*. [11] isolated bacteriophages from oral cavity but they obtained phages specific for non-oral bacteria such as *Proteus mirabilis* but did not find any phage specific for oral pathogenic bacteria [6]. This study aimed to isolate bacteriophage against *Streptococcus mutans* by testing clinical samples against laboratory type strains of bacterial host but yielded unsuccessful results.

Phage therapy is being increasingly considered as a treatment option for pathogenic bacteria. As suggested in the literature, phage therapy can be used to help reducing the colonisation of the oral cavity or more specifically teeth surface by *S. mutans* hence lowering caries rate. Dalmasso *et al*. [3] suggested the combination of different phages to permit broadening the host range of a phage cocktail to target *S. mutans.*

In conclusion, phage therapy is a rich field for research although limited number of phage for *S. mutans* has been isolated from oral samples. We were unsuccessful to isolate any phage from 38 clinical samples probably due to the low frequency of natural presence as suggested in the literature.

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