Isolation, Transportation and Culture Methods of Tannerella forsythia - A Review

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INTRODUCTION:

The oral microbiology comprises of a complex bacterial community which is made up of more than 700 different bacterial species. [Paster et al., 2001, 2006; Aas et al., 2005] In a healthy individual, the oral bacteria exist in a natural balance with their host. However, different factors such as smoking, diabetes, genetic predisposition, or poor dental hygiene can cause the community to become dysbiotic, thus enabling potentially pathogenic bacteria to increase in numbers and cause persistent infections, such as periodontitis and dental caries [1,2].
 Majority of these organisms are associated with the formation of dental plaque by the formation of a highly organized microbiological community. These organisms exhibit diverse interactions within themselves while forming organized biofilm structures and carry out sophisticated physiological functions and thus induce microbial pathogenesis [Kolenbrander et.al, 2002; Kuramitsu et al., 2007] [3]. The oral micro flora has been found to be majorly involved in many diseases like dental caries and in periodontitis which are multifactorial in origin.

Periodontal disease is a destructive inflammatory disease characterized initially by connective tissue destruction, followed by the involvement of alveolar bone and ultimately ending in the loss of the tooth, which are manifested clinically as the various stages in periodontitis. Many microbial organisms are implicated in the pathogenesis and the progression of the periodontal disease, and primarily characterized by a shift in the microbial composition and promotion of growth of Gram-negative anaerobes. It has been found that the stimulus for the the initiation of periodontal destruction is attributed to the presence of complex microbial biofilms which occupy the deepened sulcular regions between the tooth surface and the gingival margin and is dominated by Gram negative anaerobic rods and spirochetes [4]. The current concept is that, it is not merely the presence of a single virulent organism in the biofilm community which is responsible for the properties of the micro flora, but it is the interactions between the organisms present that is crucial for the pathogenicity. The key organisms involved in the etiology of periodontal disease include *Porphyromonas gingivalis, Tannerella intermedia, Tannerella forsythia, Treponema denticola* which are strictly anaerobic and other bacteria like *Aggregatibacter actinomycetemcomitans* and *Campylobacter* [5].

Periodontal disease is preceded by the accumulation of sub gingival plaque which is thriving in a variety of microbial organisms. One such organism is *Tannerella forsythia*, which is abundantly found in the biofilm where periodontitis is manifested [6]. *Tannerella* meets the criteria required for it to be classified as a periodontal pathogen. The criteria given by Socransky and Tannerella meets the criteria is as follows: 1) the bacteria is present in increased levels in periodontitis. 2) there is evidence of host response to antigens 3) it causes the disease in animal models 4) it expresses virulence factors which may contribute to the disease process [7].

Tannerella forsythia belongs to the Cytophaga- Bacteriodes family and was first described around the mid 1970s by Tanner et. al. as 'Fusiform Bacteroides' because it did not resemble characteristics of oral or enteric or Gram negative anaerobic rods. [7] It was first isolated from patients characteristic of aggressive periodontitis. It was later classified under the genus *Tannerella* as *Tannerella forsythensis* and then reclassified as *Tannerella forsythia* by Sakamoto et al. based on 16SrRNA phylogenetic analysis [8].

Tannerella forsythia is a member of the red complex, along with *P. gingivalis* and *T. denticola*. *Tannerella forsythia* is a gram negative, strictly anaerobic fusiform organism with fastidious growth requirements and anaerobic growth requirements for culture which makes it difficult to study. It is proposed to be in an increased frequency as the periodontal condition deteriorates, and it has a positive relationship with pocket depth and bleeding on probing [9]. A preliminary rapid identification T. forsythia strains obtained from periodontitis patients can be based on the following eight criteria: positive activity for (i) □alpha-glucosidase; (ii)beta-glucosidase; (iii) sialidase; (iv) trypsin-like enzyme; (v) negative indole production; (vi) requirement for N-acetylmuramic acid; (vii) colonial morphology; and (viii) Gram-stain morphology from blood agar medium deficient in N-acetylmuramic acid.

 Periodontal pathogens colonize not only sub gingival sites, but also in supra gingival sites and appears in saliva also. Microbiological culture, loop amplification, and immunofluorescence assays are some methods which can be used to detect and evaluate the presence of Tannerella forsythia. The aim of this article is to review the methods which are ideal for the isolation, transport and culture of *Tannerella forsythia*.

ISOLATION AND TRANSPORTATION METHODS FOR *TANNERELLA FORSYTHIA*:

The method of sampling employed may also affect the outcome of the microbiological diagnostics. The periodontal bacteria in the sub gingival plaque are continuously washed out from the gingival crevicular fluid into the saliva. The possible sample collection for the detection of *Tannerlla forsythia* include sub gingival plaque, whole saliva, and supra gingival plaque [10]. Gingival swabs are unacceptable for the culture of *T. forsythia*. The complexity of the sampling technique employed commonly for collection of sub gingival plaque samples has limited application in routine diagnosis [11].

Sub gingival plaque collection can be done by isolation of the desired site with cotton rolls and then gently air- drying them. Ideally he deepest pocket has to be selected and after removal of plaque, Sterile paper- points should then be inserted into the gingival pocket up to the apical portion for 10 seconds and the sub gingival fluid is collected. The paper points should be immediately placed in Eppendorf vials containing 500 microliters of TE buffer (10mM Tris- HCL, pH 8.0, 1 mM EDTA, pH 8.0) and stored at -20^0 C till the culture is carried out. The bacteria are then grown anaerobically on Tryptone menadiona (1microgram/ ml) at 37^0 C in 85% N_2, 5% CO_2, and 10% H_2 in an anaerobic chamber [12].

Saliva can be collected in calibrated medical cups followed stimulation by paraffin or chewing gums with no fragrance or flavouring agents. This loosens still-attached microorganisms or clumps of micro organisms from oral biofilms into salivary sediment. According to Umeda et al., whole saliva was even superior to pooled subgingival samples to detect *P. gingivalis*, *P. intermedia*, and *T. denticola* in the oral cavity, and reasonably good detection rates were obtained also for *A. actinomycetemcomitans* and *T. forsythia.*

Supra gingival plaque can be collected by tooth brushing for a total of 2 minutes, followed by immersion of the toothbrush in phosphate- buffered saline. The sample thus collected must be centrifuges at 3,000 rpm for 5 min at 4^0 C and then cultured. Another method is the pocket- of collection method, where a sterile foam tipped applicator is used to apply a light pressure in the surface of the masticatory mucosa of the tooth in the study, and immediately transferred into the Eppendorf tubes containing TE buffer. Another method is the Curette collection method where the supra gingival plaque is removed without disturbing the sub gingival environment and using a sterile curette, the sample is collected with a single stroke sample where in the curette tip is extended to the base of the pocket and dram coronally along the root surface. The plaque sample has to be immediately transferred to a separate Eppendorf tube containing TE buffer. (I22) Reduced transport medium like Bowden and Hardie (1971), Reduced transport medium VGMA-III of Moller(I29) can be used to suspend the sample obtained.

CULTURE METHOD AND IDENTIFICATION OF *TANNERELLA FORSYTHIA:*

Despite significant advances in recent years in culture independent molecular biology methods, detailed study of the individual bacterial species still relies on culture in a laboratory. However,
Some bacteria which are metabolically dependent on others may be impossible to grow in pure culture. Contrary to this, bacteria which are always associated with a biofilm or microbial community can be isolted when grown in in- vitro culture methods. (Vartoukian et. al, 2010; Tanaka and Benno 2015). Such microbial communities depend on one another for for metabolic cooperation and intercellular signals which have to be met in culture. (Vartoukian et. al, 2020, Stewart 2012, Mihai et. al, 2015). It has been suggested that the unculturable bacteria may have lost the capacity to produce siderophores (Lewis et. al, 2010) and so depend on external environment or neighboring bacteria for it. Adding siderophores to culture media will stimulate the growth of previously unculturable organisms. (Guan and Kamino 2001; D'Onofrio et. al, 2010; Vartoukian et al., 2016)

It has been found in a study done by Sumita Roy et. al that Sialic acid, Glycolyl Sialic cid, and siallillactose stimulates the growth of *T. forsythia* biofilms, and that sialidase activity is key to utilization of sialoconjugate sugars and is involved in host–pathogen interactions in vitro. Also the study tells that oseltamivir or siastatin B might be used as alternative drug therapies for reduction of dental plaque biofilms [13, 14, 15].

T. forsythia is an organism with fastidious growth requirements. It is the only sulturable organism from the genus *Tannerella*. As stated by Wyss in 1989, *Tannerella forsythia* requires an exogenous source of N- acetyl muramic acid to grow. (I14) T. forsythia can be cultivated under anaerobic conditions at 37 °C in tryptic soy broth, supplemented with yeast extract (5 g/L), phytone peptone (5 g/L), cysteine (0.2 g/L), horse serum (20 mL/L), hemin (2.5 µg/mL), menadione (2 g/mL), and N-acetylmuramic acid (10 µg/mL) (M12-28)

It can also be grown in brain heart infusion (BHI) medium containing calf serum (CS) which is heat inactivated, N-acetly muramic acid (10 mg/ litre), hemin (5 mg/ litre), L- cysteine (1 gm/ litre) TF medium for 3-7 days at 37^0 C under anaerobic conditions containing H_2, CO_2, N_2.

The various anaerobic systems which can be used for the culture of Tannerella forsythia include the following:
(i) Coy anaerobic chamber. The Coy anaerobic chamber consists of a flexible glove box filled with 85% N_2–10% H_2–5% CO_2 and heated palladium catalyst pellets. Anaerobiosis of the chamber must be monitored by using a BBL disposable anaerobic indicator strip (Becton Dickinson).

(ii) BBL Gas Pak system. The GasPak system includes a 2.5-liter jar with palladium catalyst pellets and a Gas Pak anaerobic envelope. Pellets should be heated in a 125°C oven for 2 h before each use. Prior to the incubation of the blood agar plates, the Gas

Pak anaerobic envelope is activated by adding 10 ml of water to the envelope. The final CO_2 concentration must be 4 to 10%. The anaerobic conditions have to be monitored after 60 min of incubation by using the BBL disposable anaerobic indicator strip.

(iii) Anaero Pack system. The Anaero Pack system includes a rectangular container (9.5 by 6.75 by 3.25 in.; 2.5 liters) and one Anaero Pack sachet. The sachet must be opened and placed into the container along with inoculated blood agar plates and a BBL disposable anaerobic indicator strip. After 60 min of incubation, the oxygen concentration will be less than 1% and the CO_2 concentration will be approximately 18% [16].

Out of the above mentioned anaerobic culture methods, the Coy anaerobic chamber system and the GasPak system were more efficient than the Anaero Pack system in the growth of organisms like Tannerella forsythia and Campylobacter species. Downes et al. evaluated the Anaerobe Systems (San Jose, Calif.) anaerobic chamber, the Anaerobic Pouch System Catalyst-Free (Dif- co Laboratories, Detroit, Mich.), and the Bio-Bag Environ- mental Chamber Type A (Marion Scientific, Division of Marion Laboratories, Inc., Kansas City, Mo.) for the cultivation of anaerobic bacteria. They concluded that the anaerobic chamber was more efficient than the pouch systems in recovering fastidious anaerobes. The results of a study done by Nguyen Doan et. al regarding the efficiency of three anaerobic culture systems in recovering anaerobic periodontal pathogens state that for oral microbiology laboratories that process a limited number of anaerobic samples, the GasPak anaerobic culture system seems to offer a convenient and effective method for recovering periodontal pathogens [16].

Tannerella forsythia shows satelliteism beside *Propionibacterium* acnes which proves that *Propionibacterium* acnes influences and promotes or accelerates the growth of Tannerella forsythia. Also, better results were obtained when Tannerella is cultured over using a lawn culture of P. acnes than by culturing directly on agar with P. acnes. This indicates that greater amount of factors which promote growth as provided by a larger surface area of lawn culture is better than a narrow streak [17].

The growth of *T. forsythia* is stimulated by many factors out of which one is N-acetyl- muramic acid. Cultural detection of *T. forsythia* is compromised if N-acetyl muramic acid is not included in the isolation medium and too short (<10 days) an incubation time is used for primary isolation [18]. The shape of T. forsythia cells and colonies varies depending on the growth conditions. Cells of cultures grown on agar media without N- acetyl muramic acid are large, filamentous, and pleomorphic, with tapered (fusiform) ends, also with spheroids. Colonies are tiny and opaque. Colony morphology changes in the presence of either N-acetyl-muramic acid or a growth-stimulating species, for example *F. nucleatum.* Colonies become pale pink and speckled, circular, entire, slightly convex, and may have a depressed center (donut-shaped). In the presence of N-acetylmuramic acid, cells become regularly shaped, short, gram-negative rods. Since *T. forsythia* lacks a metabolic pathway to synthesize N-acetyl muramic acid, the bacteria may possess unique systems to scavenge peptidoglycan degradation products released during cell-wall recycling of oral biofilm bacteria or it derives this compound from sialylated glycoproteins like salivary mucins and fibronectin present in the oral cavity.

T. forsythia has the ability to metabolize a range of substrates and, in common with many enteric Bacteroides species, to hydrolyze esculin. However, T. forsythia is not resistant to bile, which is in contrast to the enteric species and hence it does not grow on the routine Bacteroides medium for enteric species which is the Bile Esculin Agar. While *Porphyromonas gingivalis* and *T. forsythia* will grow on media supplemented with vitamin K (menadione) for *P. gingivalis*, and N-acetyl muramic acid for *T. forsythia,* each species is inhibited by the growth requirement of the other, so compromising primary isolation. On primary isolation from oral samples, however, strict anaerobe condition favors isolation of *T. forsythia* and other subgingival anaerobes.

The ability of T. forsythia to hydrolyze the trypsin- like benzyol-DL-arginine-2-naphthylamide, (BANA), has been incorporated in the test for periodontal pathogens given by Loesche. Trypsin-like activity is common to both *P. gingivalis* and *T. denticola* of the the red complex organisms. Other sub gingival species are also BANA-positive, but not as strongly as *P. gingivalis, T. denticola,* and *T. forsythia*, thus making this biochemical marker a test for the presence of these species [18].

VIRULENCE OF TANNERELLA FORSYTHIA:

Virulence is the ability of an organism to cause disease or to interfere with a metabolic or physiological function of its host. Understanding the virulence factors may help in development of techniques to target specific micro organisms. These so-called "red complex" bacteria are able to subvert host immune responses, modulate the infection process within the sub gingival pocket, and promote dysbiosis through the expression of virulence factors [19]. *Tannerella* forsythia has been shown to express several putative virulence factors; among them is its characteristic two-dimensional (2D) crystalline cell surface (S-) layer. *Tannerella forsythia* is the only member of the "red complex" group of organisms that possesses an S-layer fully covering the bacterial cells; this is formed by self- assembly of the two S-layer proteins TfsA and TfsB [20, 21]. The surface glycosylation affects the physicochemical properties of the bacterial cell surface through the introduction of charged sugar residues and modulates bacterial cell hydrophobicity. The prominent location and the presence of the *O*-glycan in abundance as well as the S-layer matrix itself make them ideal candidates for influencing inter bacterial or bacterial interactions with the hostas may occur in oral biofilms. The *T. forsythia* S-layer has been described to facilitate adhesion to and invasion of gingival epithelial cells, suppress pro-inflammatory cytokine production, and inhibit monospecies biofilm formation; however, without dissecting any potential contribution of the *O*-glycan attached to the S-layer [22, 23, 24].

Cell surface glycosylation, specifically glycosylation of proteins, might serve specific functions in infection and interaction with

host tissues as well as modulation of immune responses during pathogenesis. The levels of IgG antibody against the S-layer of T. forsythia are low in healthy individuals, they are significantly elevated in adult and early-onset periodontitis patients. These results do not only indicate that this major surface protein is antigenic in humans, but also suggest that an increased interaction between host adaptive immune mechanisms and this pathogen occurs during periodontal disease progression. Also, *Porphyromonas gingivalis* outer membrane vesicles enhance attachment to and invasion of epithelial cells by *T. forsythia*, co-infection of *T. forsythia* and *P. gingivalis* increases abscess formation in a mouse model, and *T. forsythia* cell extracts have been shown to have a growth-promoting effect on *P. gingivalis* [25, 26, 27].

The S-layer of *Tannerella forsythia* represents the first glycosylated S-layer of a Gram-negative organism and it is structurally unique due to the simultaneous presence of two S-layer proteins [M138]. Interestingly, in periodontal lesions, another S-layer-carrying bacterium, namely *Campylobacter rectus* is found. This organism is thought to be capable of inducing pro-inflammatory cytokines and its S-layer may temper this response to facilitate the survival of *C. rectus* at the site of infection [28]. Also, flagella with intact hook regions have been identified by freeze-etching which somehow challenges the description of T. forsythia as a non-motile species. However, their role in bacterial motility and a possible impact on co-aggregation of T. forsythia with other species in the biofilm is still unclear. In this context it should also be mentioned that according to a recent partial annotation of the open reading frames of the T. forsythia genome, homologous genes for hook and other pilus/flagella forming units have not been identified. LPS is an intrinsic feature of Gram-negative bacteria where it is located in the outer leaflet of the outer membrane. So far, structure-function studies on the LPS from T. forsythia do not exist but are considered essential to further understand the bacterium's pathogenesis.

CONCLUSION:

There are many developments coming up in the field of microbiological and molecular diagnosis. However, it has been found that there is a loss of quantitative information because of the internal technical limitations of anaerobic bacterial culture methods and at least 10^3 pathogens are required for detection. The main advantage of culture methods are that antibiotic susceptibility test can be done which will be useful in treatment. More advanced technologies like real time PCR technique can detect the organism if a lesser number of organisms are present in the sample. PCR is more sensitive than traditional culture methods. However, for routine detection tests, it is ideal to do bacterial culture and for quantification puproses it is ideal to do PCR studies.

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