


## ORIGINAL ARTICLE

# Significant presence of biofilm-producing gut-derived bacteria in anal fistula of chronic duration

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## Abstract

Fistula-in-ano though not a life-threatening condition, yet its symptoms often significantly impact patients' social, intimate, and work lives. There is an established role of bacterial microflora in acute infections. However, we proposed that biofilm-forming organisms might be present in the microflora of anal fistula of prolonged duration. This aspect has rarely been studied earlier. Therefore, the study describes the microbiology of anal fistula and the biofilm-forming capacity of the isolated organisms. A total of 30 patients were included in the study as per the criteria. Anal fistula tissue sample, tissue fluid, and blood samples were collected from each individual. The collected specimens were detected for the presence of aerobic and anaerobic microflora through standard microbiological method and polymerase chain reaction. Furthermore, the role of biofilm formation by microtitre plate assay and serum matrix metalloproteinases-9 was also studied. The result showed significant predominance of gut-derived microflora with high-to-moderate biofilm-producing ability in anal fistulas of prolonged duration. The study emphasises the presence of biofilm-forming bacteria in chronic, non-healing fistula.

## KEYWORDS

anaerobic, *E. coli*, microtiter plate assay, MMP9, persistent

## 1 | INTRODUCTION

Anal fistulas are the acute and chronic complications of perianal infections. Usually one third of the patients with perianal abscess develop chronic anal fistulas. Infection in the fistulas is an integral part.<sup>1,2</sup> There have been studies suggesting the role of gut microflora in these fistulas as opposed to skin commensals.<sup>3</sup> The microbiology of acute anorectal fistulas is well established.<sup>4</sup> On the contrary, there is not only dearth of literature on microbiological details for chronic persistent fistulas but also

doubt on the relevance of infection.<sup>5</sup> The role of biofilm-forming microorganisms in this context as a cause of persistent infection has not been studied. Therefore, based on the "cryptoglandular hypothesis," we proposed that microorganisms with greater biofilm-producing capacities persist in the chronic anal fistulas.

Anal fistulas are troublesome complications of several bowel conditions like Crohn's disease where the matrix metalloproteinases (MMPs) have been implicated in tissue injury and matrix degradation. The role of specific MMPs such as MMP3 and MMP9 has been predicted to

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contribute to fistula formation in these cases. With this background, this study was performed to detect the microbial flora associated with anal fistulas with special reference to biofilm-forming ability of the isolated microorganisms along with MMP9 levels in the serum of the patients with anal fistulas.

## 2 | MATERIALS AND METHODS

### 2.1 | Study site and design

This was a prospective case series conducted in the Department of General Surgery and Microbiology, Institute of Medical Sciences, Varanasi, over a period of 1 year (August 2018–July 2019). The study involved patients of anal fistula attending the Outpatient Department of General Surgery. The study was approved by the institute's ethical committee, and informed consent was obtained from all the patients involved in the study.

### 2.2 | Study subjects

All consecutive cases of anal fistula over a period of 1 year who underwent surgical treatment within the age group 18–60 years were included in the study. Cases associated with pregnancy, recurrent, traumatic, and malignant fistula; chronic specific diseases such as tuberculosis, inflammatory bowel diseases, and sepsis; and those on immunosuppressive treatment were excluded. Demographic profile of the subjects was noted concerning age, gender, and body mass index (BMI, kg/m<sup>2</sup>).

### 2.3 | Microbiological study

Granulation tissue from the fistula tract and tissue fluid samples, wherever possible, were collected from the anal fistula by aseptic methods. Briefly, samples were collected by both sterile swabs and needle aspiration after initial cleaning with saline and 2% povidone iodine and were immediately transported to the laboratory. Tissues were finely ground with tissue mixer and were processed for both aerobic and anaerobic organisms. Isolation of microorganisms was performed by direct specimen subculture onto blood agar and MacConkey agar (Hi Media Laboratories Pvt. Ltd., India) following incubation for 24 to 48 hours. Positive growth was identified based on standard microbiological methods namely colony morphology, Gram's staining, and biochemical reactions according to standard protocol.<sup>6</sup> Due to unavailability of anaerobic culture facility, detection of anaerobic bacteria

#### Key messages

- biofilm-producing gut-derived bacteria constitute the major microflora of anal fistula of prolonged duration
- MMP9 could be a factor facilitating fistula formation
- high/medium biofilm-producing *Escherichia coli* (commonest isolate) were significantly associated with fistulas of >6 months duration

was performed by conventional PCR targeting common anaerobes such as *Bacteroides*, *Peptococcus*, *Prevotella*, and *Clostridium* spp. as evident from previous literature.<sup>7</sup> For PCR reaction, DNA extraction was performed by QIAGEN mini DNA extraction kit (Qiagen Pvt. Ltd., Germany). Furthermore, the extracted DNA was used as template for PCR.<sup>8</sup>

### 2.4 | Biofilm formation

To assess biofilm formation, bacterial isolates were subcultured onto nutrient agar media (Hi Media Laboratories Pvt. Ltd., India) and kept for overnight incubation at 37°C. The isolated pure colonies were suspended into normal saline and the suspension was adjusted according to 0.5 McFarland ( $\sim 1.5 \times 10^8$  CFU/mL). Biofilm formation was performed in 96-well microtitre plate with 0.1% crystal violet staining method. The optical density at 578 nm (OD<sub>578</sub>) of each well was measured using a LisaScan EM microtitre plate reader. The experiment was carried out in triplicates. Isolates were categorised as non-biofilm producer and weak/moderate/strong biofilm producers based on their OD values. Reference OD range for each category was taken by calculating three SDs above of the mean OD of clean microtitre plate containing only media and stained by the explained method.<sup>9</sup>

### 2.5 | Detection of MMP-9

Whole blood was collected in a serum separator tube and serum was allowed to clot at room temperature for about 4 hours, followed by centrifugation at 1000g for 15 minutes. Tissue fluid collected during sampling in those with active discharge from the external opening of the fistula was also considered for MMP-9 estimation. All samples were stored at –20°C.

Concentration of MMP-9 in serum and tissue fluid was tested using Human MMP-9 ELISA Kit PicoKine (Boster Biological Technology, Pleasanton, California, Catalogue #EK0465) as per manufacturer's instructions. The kit works on tetramethylbenzidine substrate (TMB) colorimetric sandwich ELISA principle, which detects human MMP-9 with <5 pg/mL sensitivity. It contains

96-well plate with removable strips and required reagents for the reaction. The result was interpreted by taking OD absorbance at 450 nm within 30 minutes of the reaction with the help of LisaScan EM microtitre plate reader.

## 2.6 | Statistical analysis

Several parameters relating to the fistula such as type of fistula, microbial presence, and presence of biofilm-forming organisms were studied in relation to the values of serum MMP-9 by Pearson's correlation coefficient. Duration of illness was compared with the degree of biofilm production by chi-square test. All statistical analysis was performed by SPSS version 23.0 (Armonk, New York; IBM Corp).

**TABLE 1** Microflora identified in anal fistula samples

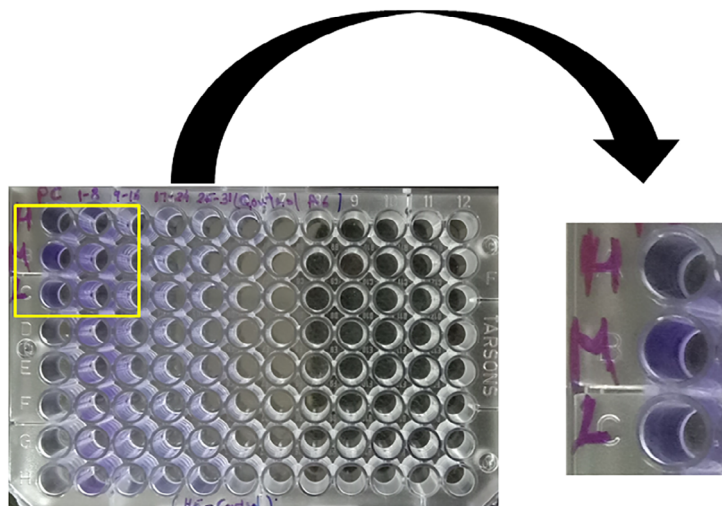
Microbial burden	Microorganisms	N (%)
Monomicrobial (n = 15)	<i>Escherichia coli</i>	11 (37)
	<i>Klebsiella pneumoniae</i>	2 (7)
	<i>Acinetobacter baumannii</i>	1 (3)
	<i>Enterococcus</i> spp.	1 (3)
Polymicrobial (n = 14)	<i>E coli</i> + <i>K pneumoniae</i> + <i>Prevotella</i>	1 (3)
	<i>Enterococcus</i> spp. + <i>Peptococcus</i> + <i>Prevotella</i>	1 (3)
	<i>E coli</i> + <i>K pneumoniae</i>	2 (7)
	<i>E coli</i> + <i>Acinetobacter baumannii</i>	1 (3)
	<i>E coli</i> + <i>Citrobacter</i> spp.	1 (3)
	<i>E coli</i> + <i>Proteus mirabilis</i>	1 (3)
	<i>E coli</i> + <i>Staphylococcus</i> spp.	1 (3)
	<i>E coli</i> + <i>Prevotella</i> spp.	3 (10)
	<i>E coli</i> + <i>Peptococcus</i> spp.	1 (3)
	<i>Citrobacter</i> spp. + <i>Clostridium</i> spp.	1 (3)
	<i>Staphylococcus</i> spp. + <i>Prevotella</i> spp.	1 (3)

## 3 | RESULTS

A total of 30 cases of anal fistula in 30 different patients were studied. The mean age of the patients was  $32.9 \pm 12.43$  years. All the patients were male. The mean body mass index (BMI) was  $23.75 \pm 3.76$  kg/m<sup>2</sup>. Of the total, 15 patients had complaints for  $\leq 6$  months while other 15 had complaints for  $> 6$  months. Overall, 19 patients had intersphincteric type of fistula and in 29 patients fistulas had a single external opening.

### 3.1 | Description of microflora

A total of 36 facultative aerobes and 9 anaerobic isolates were recovered from 30 anal fistula tissue specimen (1.2 aerobes and 0.3 anaerobes per specimen), while 1 case



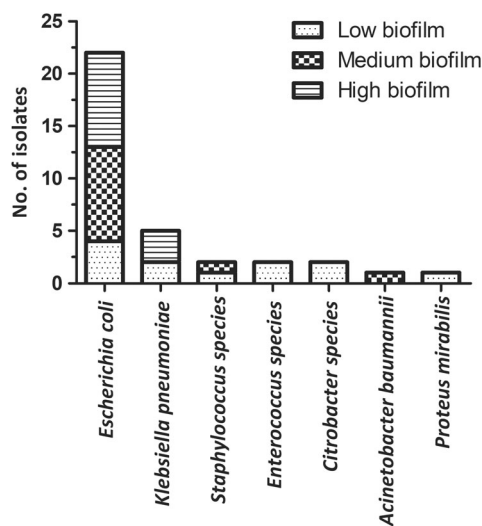
**FIGURE 1** Microtitre plate assay for detection of biofilm formation in microorganisms

Representative wells showing high (H), medium (M) and low (L) biofilm formation in the wells

did not show any growth. Of the 30 patients, aerobic/facultative bacteria were isolated by culture in 20 patients while both aerobic and anaerobic bacteria were seen in 8 patients. Gut-derived bacteria were detected predominantly as monomicrobial flora with *Escherichia coli* 50% (n = 15), *Klebsiella pneumoniae* 6.6% (n = 2), *Enterococcus* spp. 6.6% (n = 2), as against skin-derived flora with *Staphylococcus* spp. 3.3% (n = 1). *Prevotella* spp. 16.7% (n = 5) was the commonest anaerobe. The microbiological profile of the fistulas has been shown in Table 1.

### 3.2 | Biofilm formation

The plate assay for detection of biofilm producers has been shown in Figure 1. Among the 28 fistulas that showed microbial growth, 11 (39.28%) harboured high biofilm-producing bacteria followed by 8 (28.57%) medium biofilm producers. Low biofilm producers were seen in 9 cases (32.14%). Among the gut-derived bacteria,



**FIGURE 2** Degree of biofilm formation among the gut-derived bacteria

majority were high biofilm producers as shown in Figure 2. When bacterial biofilm producers were compared with duration of illness, medium and high biofilm-producing bacteria were significantly isolated from those fistulas, which were of >6 months duration ( $P = .054$ ). Among the *E coli* isolates, high/medium biofilm-producing strains were significantly more ( $P = .012$ ) in the abovementioned group as shown in Table 2.

### 3.3 | Serum MMP-9 estimation

The mean serum HMMP-9 value of all 30 patients was 2570.7 ( $\pm 1143.71$ ) pg/mL. It was slightly higher [2773.54 ( $\pm 1293.19$ ) pg/mL] for the patients having total duration of illness of  $\leq 6$  months as against those of >6 months [2367.85 ( $\pm 974.19$ ) pg/mL].

Of the 5 cases with active discharge and in which tissue fluid could be obtained, mean tissue fluid MMP-9 levels were significantly higher ( $6612.32 \pm 1062.87$  pg/mL) than mean serum MMP-9 levels ( $2090.95 \pm 863.81$  pg/mL,  $P = .004$ ). However, there was no significant association of MMP-9 levels with the other features as shown in Table 3.

## 4 | DISCUSSION

This study revealed dominance of moderate to high biofilm producing gut-derived microflora in patients with anal fistula of prolonged duration (>6 months). To the best of our knowledge, this microbiological aspect in prolonged anal fistulas has not been yet studied. Fistula-in-ano is a condition where patients typically suffers with pain, drainage of pus or stool, pruritus, and excoriation of adjacent tissue.<sup>10</sup> According to reports, the prevalence of fistula is 8.6 cases per 100 000 of the population. In men, the prevalence is 12.3 cases per 100 000 population and in women 5.6 cases per 100 000 population with mean patient age of 38.3 years.<sup>11</sup> It has been reported that

Biofilm formation	Total duration of illness		P value
	$\leq 6$ months (n = 15)	>6 months (n = 15)	
All cases			
Low	7	2	.05*
Medium/high	7	12	
<i>Escherichia coli</i> isolates			
Low	4	0	.01*
Medium/high	2	16	

**TABLE 2** Bacterial biofilm formation in comparison with duration of illness

Note: \*P value < .05.

**TABLE 3** Comparison of mean serum MMP9 with various parameters

Parameters	Serum MMP9 value (mean ± SD)	P value
Duration of fistula		
≤6 months	2773.54 ± 1293.19	.34
>6 months	2367.85 ± 974.19	
Nature of fistula tract		
Single non-branching linear	2329.52 ± 912.66	.29
Single curved	3484.94 ± 1753.53	
Single low anal	2860.53 ± 1485.33	
Irregular branching	2319.16 ± 719.23	
Type of fistula		
Intersphincteric	2639.27 ± 1294.55	.71
Transsphincteric	2159.26 ± 996.99	
Suprasphincteric	2166.49 ± 756.38	
Extrasphincteric	2961.39 ± 670.11	
Microbial growth		
Present	2540.20 ± 1175.37	.59
Absent	2997.68 ± 492.74	

men are more likely to be affected with 4:1 preponderance of male-to-female ratio.<sup>12</sup> In our study, all the 30 cases were in men. The mean age of the patients recorded were 32.9 years.

Microflora of these fistulas showed *E coli* as the major microorganism, a finding backed by many previous studies.<sup>3,12-15</sup> Very few skin-originated microflora like *Staphylococcus* spp. (3.3%) was detected. In comparison with one of the recent studies, where 42 samples from anal abscesses were assessed, *E coli* was present in the highest number (53.8%) in the fistulised abscesses while anaerobic bacteria were present in 100% samples.<sup>3</sup> However, the study had not mentioned the distribution of the anaerobic flora other than *Bacteroides fragilis* (16.7%) and *Peptococcus* (9.5%). A similar aerobe/anaerobe ratio (1.2:0.3) has also been reported in another study on 81 patients of perianal abscesses.<sup>16</sup> The predominance of anaerobic flora on the other hand has been reported in perirectal abscesses vis-à-vis anal abscesses probably owing to proximity to the colon.<sup>7</sup> The presence of anaerobes consisting of *Bacteroides*, *Peptostreptococcus*, *Clostridium*, and *Prevotella* has been reported in 15% of the perianal infections in another study.<sup>17</sup> Therefore, even without anaerobic culture facility, which could have been one of the limitations in this study, the detection of anaerobes by targeting the common microbes by

molecular methods is justified due to comparability of the results. Although the study does not rule out any pathogenic role of the bacterial population detected, the synergistic attributes of polymicrobial infection have been well established in other chronic infections.<sup>18</sup>

There are only handful of reports suggesting that the chronicity of persistent of bacterial infections can be because of bacterial biofilm formation.<sup>19</sup> Biofilm is a complex organised exogenous matrix, which provides microorganism protection and susceptibility towards various agents. There is lack of report regarding biofilm-producing bacteria in anal fistula. In a study where bacterial biofilm in chronic anal fissures (CAF) was studied, it was found that bacteria isolated from CAF in the form of monoculture (*E coli* or *Pseudomonas aeruginosa*) formed thickest biofilm in 100% cases, whereas mixed bacterial population (*E coli*, *P aeruginosa*, *Enterococcus* spp., and *Staphylococcus aureus*) formed moderate and high biofilms in 30% and 70% cases, respectively. The authors suggested that the microorganisms with the ability to produce biofilm biomass complicates the antimicrobial therapy and defines its chronic development.<sup>20</sup> The present study also proposed a similar concept as significant number of isolated organisms showed high and medium biofilm production that belonged to those patients with fistulas for >6 months duration.

The structure of biofilms in situ is best studied by advanced microscopy such as electron microscopy, which not only requires huge infrastructural setup and expertise, but also is not feasible in a majority of clinical settings. In this context, the microplate assay for determining the biofilm-forming ability of the organisms in vitro is a viable indirect alternative.<sup>21</sup> Therefore, we used this method to assess whether biofilm-producing isolates were associated with anal fistulas of prolonged duration.

The role of MMP9 has been well studied and implicated as a biomarker in Crohn's disease.<sup>22,23</sup> Although MMP-9 has been strongly linked in tissue injury in Crohn's disease, their role in fistula formation is unknown. We quantified the level of serum MMP-9 and compared them. Interestingly, cases of acute presentation of fistula were having mean serum MMP-9 level higher than that of chronic anal fistula. When mean serum MMP-9 level was compared with the mean tissue fluid MMP-9 level, it was found that MMP-9 level was significantly higher than MMP-9 level in serum. It is known that matrix metalloproteinases are markedly upregulated in intestinal fistulae and may contribute to fistula formation through degradation of the extracellular matrix, irrespective of the underlying disease process.<sup>24</sup> Though we could not associate the presence of intestinal conditions with anal fistula, MMP-9 could be a factor facilitating

fistula formation, especially, in acute conditions. However, further studies are required to validate these findings.

The study was not without limitations. Being a resource-limited setting, we could not use better culture facility and advanced methods for detection of biofilms. The contributions of the biofilm-producing organisms towards progression of the infection in the fistulas could not be directly revealed. However, despite limitations, this study clearly showed the significant role of biofilm-producing gut bacteria in anal fistulas of prolonged duration.

## 5 | CONCLUSION

This study highlighted the predominance of medium- to high-level biofilm-producing gut microflora in cases of anal fistula with tendency for chronicity. Further studies can elucidate whether biofilm formation is one of the primary reasons for chronicity and non-healing anal fistula.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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