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ANTIMICROBIAL ACTIVITY OF COW URINE DISTILLATE; GOW-ARK AGAINST 3 PERIODONTAL PATHOGENS-AN IN-VITRO STUDY

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ABSTRACT

Cow Urine Distillate (CUD) has been a well-established alternate treatment modality in the field of Ayurveda for a while now, with decades of research evidence. There is evidence to suggest its antimicrobial activity against some clinical pathogen. This in-vitro study aims to explore the antimicrobial activity of CUD against a few oral pathogens, specifically periodontal pathogens namely, aggregatibacter actenomycetemcomitans (Aa), porphyromonas gingivalis (Pg) and prevotella intermedia (Pi). These are organisms that are present in plaque (a soft but tenacious substance that accumulates on our teeth every day) and can cause periodontitis. The in-vitro study attempted to determine the minimum inhibitory and bactericidal concentrations of CUD as against chlorhexidine which is an established gold standard in oral antimicrobial agents and is routinely used in pre-procedural rinsing, routine oral hygiene regimens, post-operative maintenance regimes etc. The distillate was satisfactorily effective against Pg and Pi but showed little or no antimicrobial activity against Aa. If CUD is to be even considered a potential antimicrobial agent for oral hygiene, a full battery of animal models, clinical trials and ethical issues need to be addressed.

Keywords: cow urine distillate, periodontal pathogens, antimicrobial agent, complementary medicine, alternate therapy.

INTRODUCTION

Complementary, Traditional and Alternative Medicine are fast becoming popular not only in Asia but on a global scale. They are set of loosely clustered disciplines that can range from Ayurveda, Unani to Korean and Irani medicine etc. to name just a few. It is built on the premise of religious beliefs, natural products and indigenous concepts.[1]

It is documented that a quarter of the populations who forego conventional care opt for alternative forms of healing in the US.[2] Ayurveda are one such discipline. Even though Ayurveda has been irrevocably commercialized in the current scenario,

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its origins were nobler to begin with than we are aware of.[3] The popularity probably lies in the fact that "Ayurveda does not treat the disease, it treats the individual who has the disease."[4]

There may be many reasons why complementary/alternative medicine may have come to the forefront. It begins to make more sense if we begin to look at the shift in treatment beliefs towards holistic health, where disease is multifactorial and involves the whole person, or the use of CAM could be associated with beliefs that natural remedies are safer and more effective than prescription medications. It is also possible that CAM users prefer an active or collaborative role in treatment decision making when compared to conventional medicine users [5]. Antibiotic resistance may not have directly contributed the apparent rise of alternative therapies, but it is an established reality and rising threat to the success of conventional medicine. [6]

"*dhenunaamasmi kamadhuk*" - Bhagawad Geetha Chapter 10

Lord Krishna says – Amongst the cows, I am *Kama Dhenu* – the proverbial cow that fulfills your wishes.

The cow or "bos indicus" as it is scientifically christened, holds a sacred place in the hearts and religious psyches of Hindus in India. Its virtues have been extolled next only to Gods in the sacred Hindu texts of the yore. *Vishnu Smriti, Atharva Veda, Charaka Samhita* are but a few of such examples. It is safe to say that, there are no ancient Indian scriptures which do not speak about the cow or its products. The Panchagavya is an amalgamation of various products from the cow. It consists of cow urine, dung, milk, curd & ghee has been extensively used in Ayurveda to boost immunity, to enhance the spirit and to nurture the sick back to health. Cow urine, popularly known as *Gowmutra* is well known for its anti-ageing, anti-glycemic and weight loss benefits.[7]

A highly purified and impeccably distilled form of cow urine (CUD) has been patented for its anti-fungal[8] and antibacterial effect by the Council of Scientific and Industrial Research, one of India's largest Research and Development organizations. China has granted the distillate a patent as a DNA protector. The distillate of cow urine (CUD) has been used to treat various skin diseases.It has also been used to treat hyperglycemia. The CUD has profound antioxidant and antibacterial effects.[7,8]

That explains its significant presence in anti-cancer therapy too. It has been patented for its property of enhancing and facilitating the activity and availability of bioactive molecules including anti-infective and anticancer agents (US Patent no: 6410 059/2002).[9] Distillation is the process of heating a liquid until it boils, then condensing and collecting the resultant hot vapors. Mankind has applied the principles of distillation for thousands of years.[10] Likewise, fresh cow urine is obtained and contained in an earthen pot. It is heated and the vapor arising from the pot is collected in a tube. The pot after a critical point is cooled for the vapor to condense. The water under the pot is changed at appropriate intervals to keep the pot cool and collect condensed vapor. The tube is transparent. Thus collected cow urine is vastly dissimilar to fresh undistilled cow urine, in that it is not offensive to smell

and not all components are present, some get left behind in the residue.[11]

This distillate as mentioned a while earlier has a number of medicinal properties and uses. Two in particular, antimicrobial and anti-oxidant properties have come into sharp focus as of late.

Studies on Antibacterial effects of CUD have revealed it to be toxic to and inhibitive to growth of Staphylococcus aureus, E.coli, Pseudomonas, Bacillus Subtilis, Proteus Vulgaris, Steptococcus species, Klebsiella pneumonia and Salmonella typhi in culture. Antifungal activity on Aspegillus species was also observed. [12]

Despite the fact that periodontitis is multifactorial, by definition it is still an infection of the periodontal tissues caused by pathogenic microflora like aggregatibacter actenomycetemcomitans, Porphyromonas gingivalis and prevotella intermedia for instance. These are often referred to as primary periodontal pathogens. These organisms evade the host response in a number of ways and invade the tissue to cause attachment loss. One amongst the various therapeutic modalities for periodontitis is antimicrobial therapy - in local or systemic form. However fear of development of antibiotic resistance or adverse effects from antibiotics has paved way for research and development of novel biological drugs.

To that effect, Cow Urine Distillate becomes a legitimate candidate, it is an alternative form of therapy, has proven antimicrobial properties against various organisms like Staphylococcus aureus, E.coli, Pseudomonas, Bacillus Subtilis, Proteus Vulgaris, Streptococcus species, Klebsiella pneumonia and Salmonella typhi.[12] However, till date there is no documented data on the efficacy of CUD against periodontal pathogens. The present study was planned to address the possibility that CUD could be used as an adjunctive periodontal therapy. But first we need to determine its efficacy against known periodontal pathogens.

Aims and objectives:

The aims and objectives of the present study were

1) To determine the antimicrobial activity of CUD on Aa, Pg, and Pi.
2) To compare the antimicrobial profile of CUD with the gold standard 0.2% CHX
3) To determine the MIC of CUD against the Periodontal pathogens
4) To evaluate the inhibitory effect of CUD on cultures of Aa, Pg, Pi
5) To evaluate the time dependent growth kill curve of CUD verses CHX

MATERIAL AND METHODS:

The present in-vitro study was carried out after obtaining clearance from Ethical Clearance Committee of Sharavathi Dental College and Hospital, Shimoga. For the purpose of this study, stock cultures of aggregatibacter actenomycetemcomitans (ATCC no :- 43718, porphymonas gingivalis (ATCC No – 33277, 53978) prevotella intermedia (ATCC No :- 25611) were obtained from the Department of Microbiology Nathaji Rao G Halgekar's Institute of Dental Sciences and Research Center, Belgaum. Purified form of Cow Urine Distillate by the trade name Go Ark, and 0.2% Chlorhexidine mouthrinse was used for the present study.

Minimum inhibitory concentration (MIC) procedure:

1. 9 dilutions of each drug were done with brain-heart infusion (BHI) for MIC.

2. In the initial tube 20microliter of drug was added into the 380microliter of BHI broth.
3. For dilutions 200microliter of BHI broth was added into the next 9 tubes separately.
4. Then from the initial tube 200microliter was transferred to the first tube containing 200microliter of BHI broth. This was considered as 10^{-1} dilution.
5. From 10^{-1} diluted tube 200microliter was transferred to second tube to make 10^{-2} dilution.
6. The serial dilution was repeated up to 10^{-9} dilution for each drug.
7. From the maintained stock cultures of required organisms, 5microliter was taken and added into 2millilitersof BHI broth.
8. In each serially diluted tube 200microliter of above culture suspension was added.
9. The tubes were incubated for 24 hours and observed for turbidity

Minimum bactericidal concentration (MBC) procedure:

To determine bactericidal concentration (MBC), the MIC dilution tubes, with no visible growth (no turbidity) and the control tube were subcultured (Fig.1 and 2) onto the respective media (Blood agar for Pg and Pi, BHI agar for Aa.) and incubated for 24 hours anaerobically at 37◦C and the colonies were counted on the next day.

The organisms' growths from the control tube were then compared with the organism grown from the MIC test tubes.

The test was read as follows:

a) Similar number of colonies – indicates bacteriostatic activity only
b) Reduced number of colonies – indicates a partial or slow bactericidal activity
c) No growth – if the whole inoculum has been killed

Agar diffusion procedure:

Inoculum preparations:

The colonies were transferred from the plates to the BHI broth (fig.3) with a sterilized straight nichrome wire. The turbidity was visually adjusted with BHI broth to equal that of a 0.5 McFarland unit turbidity standard that has been freshly prepared. Alternatively, the suspension was standardized with a photometric device.

Inoculation of agar plate*:*

After adjusting the inoculum to a 0.5 McFarland unit turbidity standard, a sterile cotton swab was dipped into the inoculum and rotated against the wall of the tube above the liquid to remove excess inoculum. Entire surface of kanamycin blood agar plate was swabbed three times, rotating plates approximately 60° between streaking to ensure even distribution. The inoculated plate was allowed to stand for at least 3 min but no longer than 15 min before punching the wells in agar plate.

A hollow tube of 5mm diameter was taken and heated. It was pressed on the inoculated agar plate and removed immediately after making a well in the plate. Likewise, three wells were made on each plate.

75µl, 50µl, 25µl of the 500µl/ml CUD were added into the respective wells on each plate. The plates were incubated within 15 min of compound application for 18-24 hr at 37°c anaerobically. The plates were read only if the lawn of growth was confluent or nearly confluent. The diameter

of the inhibition zone was measured to nearest whole millimeter by holding the calipers.

RESULTS AND OBSERVATIONS

Minimum Inhibitory concentration: CUD

MIC is the lowest concentration at which the drug will inhibit the growth of a micro-organism after incubation for 24 hours. CUD showed an MIC of 0.2 against P.gingivalis and 0.4 against P. intermedia, while A.actenomycetemcomitans was resistant at all concentrations of CUD. (Fig. 4,5 and 6) (Table. 1)

Anti-bacterial activity of CUD on P.gingivalis, P.intermedia and A.actenomycetemcomitans by Agar Well diffusion Method

The antibacterial activity of CUD on P. gingivalis, P.intermedia and A. actinomycetemcomitans was assessed by well diffusion method. 75µl of CUD showed a zone of inhibition of 11 mm for P.gingivalis (Fig.7) and 10 mm inhibition zone for P.intermedia (Fig 8). At 50,25,10 and 5µl of CUD it did not exhibit any zone of inhibition for either Pg or Pi. There was no zone of inhibition for Aa at any concentration (Fig.9). Chlorhexidine demonstrated a zone of inhibition of 16mm, 18 mm and 25 mm respectively for Pg, Pi and Aa. (Table.3)

Minimum Bactericidal Concentration

The MBC is the minimal concentration of antibiotic that kills the inoculum and is determining by subculturing samples from broth dilution tests onto an agar plate without antibiotics. P.gingivalis showed growth at every concentration except 1.6(Fig.10), which is in accordance with the fact that MBC is usually more than 4 times its MIC[13] (MIC of CUD for P. gingivalis being 0.4) P.intermedia showed no growth at 1.6(Fig 11) and colonies were formed at every other lower concentration. Aa showed growth at every concentration of CUD (Fig.12) and showed no growth at every other concentration of chlorhexidine. (Table.2)

DISCUSSION

The cow urine distillate has grown extremely popular as an alternative therapy in the field of Ayurveda for a variety of diseases. Urine therapy is not only used in India, but in several countries around the world. There have been instances where child urine, camel urine have been used as mouthrinses for the treatment of pyorrhea[14].

Chlorhexidine a bisbiguanide is used as a chemical plaque control agent or antimicrobial agent in the treatment of gingivitis and periodontitis. Often research studies, compare any novel drug or agent with CHX to evaluate its efficacy. A vast amount of circumstantial evidence implicates free radicals as the mediator for a wide range of diseases including diabetes, periodontitis, ageing and cancer.[15]

CUD has been proven to possess antioxidant activity. The mechanism of this is attributed to the free radical scavenging activity of the urine component and these components may prevent the progression of periodontitis.

Studies on Antimicrobial activity of CUD have proven it to be on par with Ofloxacin and Streptomycin[12.]Numerous bacteria have been found to be sensitive to CUD. For instance an in-vitro study was conducted to find the anti-bacterial effect of CUD against a few gram positive and gram negative organisms. The test organisms used in the study were E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa,

Staphylococcus aureus, Coagulase negative staphylococci, Streptococcus pyogens and Bacillus subtilis.[16]

Studies have shown fresh but photo activated cow urine to possess better antimicrobial activity than CUD. However, usage of fresh cow urine poses a health hazard, because of the fear of disease transmission. The lowered antimicrobial activity in CUD may be due to loss of volatile component during the process of distillation, or due to presence of more cations and formation of nitrosamines.

Porphymonas gingivalis

P. gingivalis plays a significant role in the progression of chronic periodontitis[17]. This gram negative, black-pigmented anaerobe has even been classified as a bona fide periodontal pathogen[18] In the present study, it was found that CUD exhibited a high level of antibacterial action against P.gingivalis even at the lowest concentration. This result was on par with that exhibited by CHX. Other alternative antimicrobial agents that performed similarly was an undiluted sample of aloe vera gel which inhibited the growth of P.gingivalis considerably[19] , while a methanol extract of neem also showed significant antibacterial action against P.gingivalis[20] Probably the bactericidal activity of CUD can be explained on the basis of its high phenolic content. Phenolic mouthwashes are known for their plaque inhibitory and anti-inflammatory actions, possibly due to their antioxidative properties[21]. A decreased growth and an increased inhibition or bacterial cell lysis of P. gingivalis and P. intermedia is an encouraging factor for further studies. Phenol acts specifically on the cell membrane and inactivates intracytoplasmic enzymes by forming unstable complexes. The lipophilic molecules are trapped by the membrane phospholipids.[22] If one considers phenolic compounds from a purely clinical point of view, phenolic mouthrinses like Listerine have an additional effect of being anti-inflammatory as well.[23] However, that aspect of CUD if at all it is anti-inflammatory, can only be brought to light in the next level in experimental gingivitis studies etc.

A.actenomycetemcomitans

Growth of A.actenomycetemcomitans was not inhibited at any concentration. Also, there was no bactericidal activity exhibited against Aa. This could be attributed to their extreme virulence, or a tough cell membrane acting as a barrier to many environmental molecules.In contrast, green tea extracts like catechins are known to have a modest bactericidal effect against Aa,where the MIC of aqueous green tea extract that inhibits A.actinomycetemcomitans growth was 70% and the aqueous extract was effective at 80%[24] Aa happens to be mildly sensitive to aloe vera extracts as well[25]

Prevotella Intermedia

Prevotella intermedia is a Gram-negative, obligate anaerobic pathogenic micro-organism. CUD exhibited a zone of inhibition of 10 mm at 75µl, this was less than what was observed with P.gingivalis and displayed an MBC of 1.6. However, CUD did have a low MIC for P. intermedia at 0.4mg/ml, which is highly encouraging when compared to an MIC 12.5mg/ml for green tea catechin extract.[26] P.intermedia seems to be sensitive to a few other alternative plaque control agents too, for instance a

herbal mouthrinse containing marigold extract (calendula officinalis) and aloe vera extracts showed a greater than two-fold difference in MICs when compared to Listerine. If viewed from a point of view of steering plaque control away from conventional chemical plaque control agents, this seems to be encouraging.

Broth dilution test is carried out to determine the MIC of a drug, and the tubes with MIC are then subcultured onto another medium without antibiotics, to determine whether the antibiotic is bactericidal or bacteriostatic as well as the Minimum bactericidal Concentration of the test drug.

It would seem preferable for a drug possessing antibacterial properties to not just inhibit the growth of a pathogen but to kill it as well. The in-vitro bactericidal activity of an agent proving superior to bacteriostatic agents clinically has not been documented. Inadequate penetration of the infection site is one of the principal factors related to failure of antibacterial therapy. The active drug needs to reach the bacteria in appropriate bodyfluids and tissues at concentrations necessary to kill or suppress the pathogen's growth. A particular agent at a certain concentration maybe inhibitory to begin with, but may exhibit bactericidal effects at higher concentrations albeit against a select set of susceptible pathogens. Although, these are ambiguous areas that relate to efficacy of antibiotics, the same principles may apply to antimicrobial activity of CUD as well.[27] Currently, we are beginning to garner data to illustrate that CUD is active against human clinical strains of E. coli, klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococci,Bacillus subtilis, Streptococcus pyogenes etc. Yet, exact mode of action of CUD against these organisms is not yet clear.

But it is necessary to remember at this point that we do not have enough data to elucidate the high performance liquid chromatography details of CUD as such,[28] so there is a considerable knowledge gap regarding the active molecules, their pharmacokinetics and pharmacodynamics properties etc. Without such data it becomes rather difficult to pinpoint the mode of action by which CUD exhibits antimicrobial properties. But it is known to be bactericidal from time immemorial, the ancient texts of Indian Ayurveda claim that cow urine is bactericidal among other qualities.[29] In addition it is also documented to be slightly sweet and alkaline and known to restore pH as and can be used for topical application for itching etc. These are only a few properties that are listed along with its distinctly bactericidal property.

Despite the fact that cow urine is revered from the ancient times, there are a lot of hoops that CUD research needs to jump through before we can consider it going mainstream. The current research has only concentrated on its efficacy against a few microbes in-vitro, both systemic and oral. We have scant laboratory data regarding active molecules, virtually no HPLC data. It is no doubt used in various applications for treating certain diseases in animals, but that is far from being qualified to be called as controlled animal testing. We need actual animal models designed to test its antimicrobial efficacy, specifically oral pathogens. Only then can we contemplate human clinical trials, but even then there are full possi-

bilities of ethical contesting from different parties, who may take offence at being told that CUD is a therapeutic agent. Despite a few patents that CUD product has got in its kitty there is a formidable research process to cover before it is declared safe for human consumption.

A NOTE ON ANTI-OXIDANT PROPERTIES OF CUD

Reactive oxygen species like super oxide, free radicals play a significant role in periodontal tissue destruction during periodontal diseases[30], when they are produced in excess by the neutrophils during oxidative burst process[31] CUD is under scrutiny for its anti-oxidant properties as well. Redistillate of cow's urine was shown to contain a large number of volatile fatty acids and also showed the presence of about 2.6 mmol of antioxidants, which might play a role in the prevention/protection of the free radicals mediated DNA damage[30]. Anti-oxidants like glutathione seem to be making major strides towards actually being of use in periodontal therapy[32], it stands to reason that CUD with a little more research on its antioxidant composition**,** could be heading in the same direction. Apart from exhibiting antibacterial activity the antioxidant property could act as an adjunct in treating periodontal diseases. But we still have a long way to go before a human clinical trial can firmly establish the efficacy of antioxidant activity of CUD.

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TABLES

Table 1

CUD	**100**	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.6**	**0.8**	**0.4**	**0.2**
Pg	S	S	S	S	S	S	S	S	S	S
Pi	S	S	S	S	S	S	S	S	S	R
Aa	R	R	R	R	R	R	R	R	R	R
CHX										
Pg	S	S	S	S	S	S	S	S	S	S
Pi	S	S	S	S	S	S	S	S	S	R
Aa	S	S	S	S	S	S	S	S	S	S

Table. 2

Aa	**100**	**50**	**25**	**12.5**
CUD	50 CFU	100 CFU	120 CFU	150 CFU
CHX	NG	NG	NG	NG
Pg	0.2	0.4	0.8	1.6
CUD	NG	NG	NG	NG
CHX	NG	NG	NG	NG

Pi	0.2	0.4	0.8	1.6
CUD	126 CFU	18 CFU	12 CFU	NG
CHX	NG	NG	NG	NG

Table. 3

	CUD 500 µl/ml					**CHX**
	75µl	50 µl	25 µl	10 µl	5 µl	500 µl/ml
Pg	11mm	-	-	-	-	16mm
Pi	10mm	-	-	-	-	18mm
Aa	-	-	-	-	-	25mm

Footnotes for tables

* Serial dilutions used : *10^{-1}=200 microliter (from initial tube 20 microliter drug+380 microliter BHI) + 200 microliter BHI*

† S-Sensitive, R-Resistant

‡ CFU = colony forming units, NG=no growth

Table 1. Minimum inhibitory concentration (MIC)

Table. 2 Minimum bactericidal concentration (MBC)

Table. 3 Agar diffusion

Legends of Figures (Images)

Fig.1 Subculturing plates Blood Agar for Pg Pi BHI agar for Aa

Fig 2 Inoculation for subculturing

Fig.3 Brain heart infusion broth

Fig 4 MIC Pg

Fig 5 MIC Pi

Fig 6 MIC Aa

Fig.7 Zone of inhibition Pg

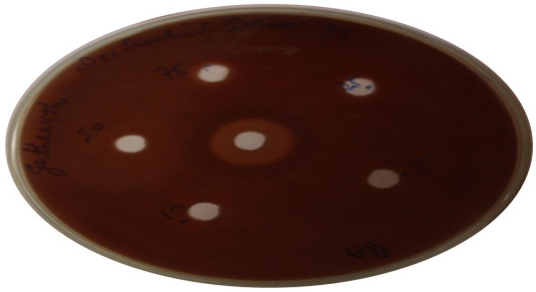
Fig 8 zone of inhibition Pi

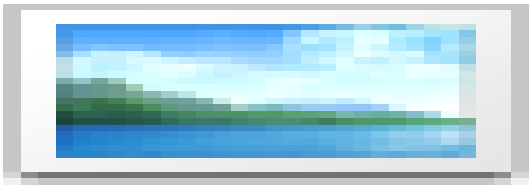
Fig. 9 zone of inhibition Aa

Fig.10 CFU Pg

Fig.11 CFU Pi

Fig 12 CFU Aa





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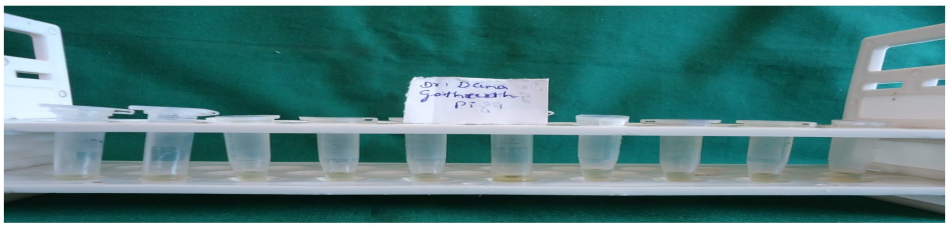

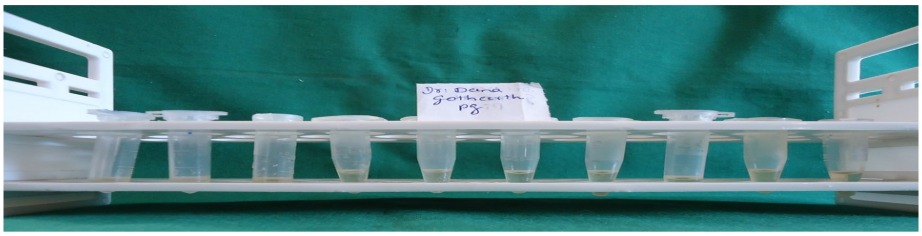

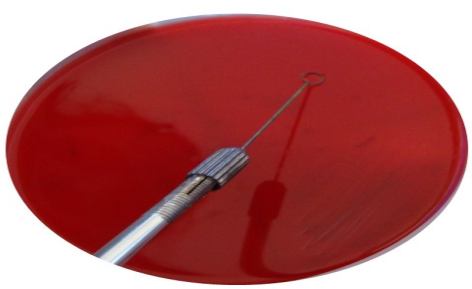

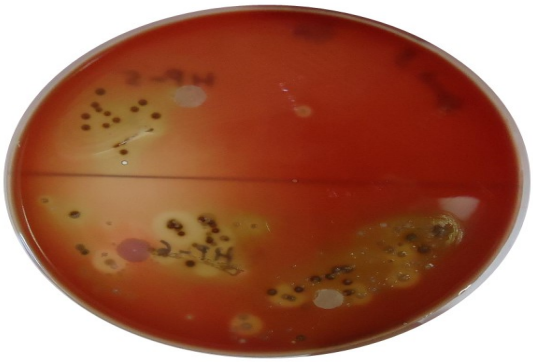

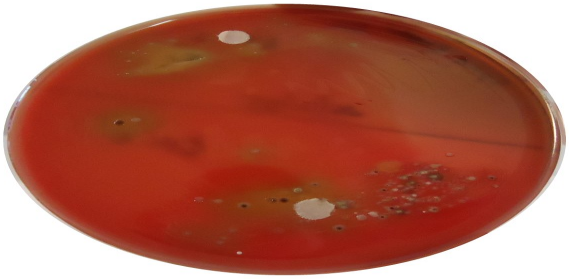

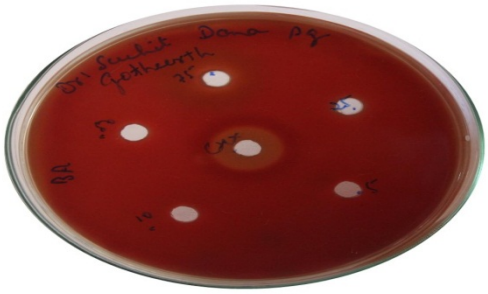

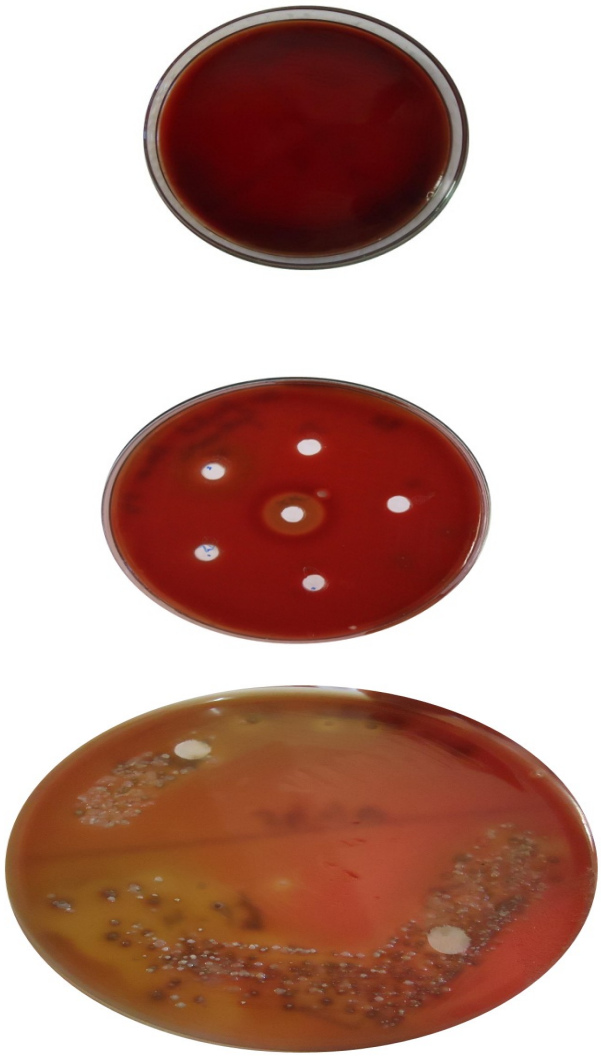

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