Section: Research Paper

FORENSIC ANALYSIS OF BACTERIAL PATHOGENS AND TOXINS, A REVIEW STUDY

Jahnavi Chivukula, Amit* Chauhan, Roseanne Mascarenhas, Nilofer Nasreen, Manobita Debbarma, Megha Rani Das, Shruti Thomas, Anto Akil I, Madhavan P, Anirudh Joshi K, Fr. Jobi Xavier.

Department of Life Sciences, CHRIST (Deemed to be University), Bengaluru, Karnataka, INDIA 560029.

Corresponding Author: Amit Chauhan

Email I'd: amit_chauhan777@yahoo.in

Contact Details: +91-9540067484

ABSTRACT

Identification and analysis of toxins present in biological samples has become essential to investigate any case including homicide, suicide, sexual assault cases, bio-crime, etc. Microbial growth phases, mechanism of synthesis, action of toxins can be studied to solve cases. Alongside, typical colonization of microbes in humans varies basedon geographical location which helps to determine the actual location of crime and disposal of cadaver if transported. Several techniques have been used to detect toxins/ pathogens at lowest possible concentrations/numbers. The most common methods include Gas Chromatography-Mass Spectroscopy (GC-MS), Fourier Transform- InfraRed Spectroscopy (FTIR), Sequence targeting of biomolecules, etc.

KEYWORDS: Pathogens**,** Forensic analysis, Toxins, Bacterial growth, crime, analytical techniques etc.

INTRODUCTION

Bacteria are omnipresent microorganisms with a versatile genome which can adapt to extreme conditions on both allies and foes to humans. The most primitive way of survival in bacteria is by forming an endospore. Anendospore is an escape mechanism wherein the live parts are enclosed in a thick covering which is resistant to extreme conditions. Bacteria have developed pathogenicity by inheriting virulent plasmids from ancestors, which can synthesize toxins and infect specific hosts. Few bacteria directly target the immune system and elicit a response. These organisms mutate frequently, posing a threatto the world, especially in terms of gaining resistance to antibacterial drugs. Bacterial toxinsare classified into two categories- Exotoxins and Endotoxins[1].

Endotoxins are the part of the outer membrane in Gram-negative bacteria called the Lipopolysaccharide (LPS) layer. These toxins are released only when the bacterial cell undergoes lysis and death, releasing the LPS in the Extracellular matrix (ECM) (*Bacterial Endotoxins/Pyrogens | FDA*). The endotoxins are involved in causing Gram Negative shock. Gram Negative shock is a condition where the LPS released into the ECM triggers arobust host immune response leading to the secretion of various chemokines and cytokines by patrolling immune cells. These chemical signals keep on multiplying and are secreted inlarger quantities recruiting active cells of the immune system, all this leads to elevated immune response and frustrated phagocytosis. This frustrated phagocytosis can lead to organdysfunction or system.

The other class of toxins help invade the host immune system by damaging hostcells. In addition to cell lysis, these toxins can also lead to autophagy, pyroptosis, or activation of the MAPK pathways, altering the host's immunological response to infection and altering both local and systemic inflammatory responses. Exotoxins are peptide or protein molecules synthesized by bacteria that are secreted into ECM[2]. Unlike Endotoxins, bacterial cell lysis is not required to release them. Toxins engage with specific eukaryotic target proteins in the cytosol to modify host proteins post-translationally, which frequently affects cellular signaling cascades and inflammatoryresponses.

The examination of biological samples for the presence of poisons and their quantity is within the range of a therapeutic dosage or above the threshold for harm can both be determined by the toxicology report. These findings can be utilized to conclude a substance's potential impact on a person's demise, disease, or physical or mental disability. To identify the specific source of the sample such as individually, if possible, methods, involved to identify the enforcer of an attack, microbial forensics combines the practices of epidemiology with the characterization of microbial and microbial-related evidence[3]. In this review, we will be dealing with various locations where bacteria can colonize and their forensic application.

CHARACTERISTIC MICROBIOME IN VARIOUS LOCATIONS ON HUMAN BODY

Skin Microbiome

Several body Sites offer a variety of micro-environments with varying UV radiation exposure, pH, temperature, moisture, sebum concentration and topography. Human skin is colonized by microorganisms after birth. Along with time, an increasingly complex ecosystem forms which is composed of indigenous and transient microorganisms. Sequencing of the skin microbiome showed that the human skin microbiota comprises around 113 phylotypes that belong to six bacterial divisions. The microbial members of this community are determined by several environmental and physiological parameters such as anatomic location, local humidity, the production of sebum and sweat, and the hormonal status and age of the host. Sweat glands acidify the skin, making conditions unfavorable for certain microorganisms. Free fatty acids and antimicrobial peptides are examples of antimicrobial compounds in sweat that inhibit microbial colonization. The hydrophobic, lipid-rich sebum that anoxic sebaceous glands secrete lubricates and protects hair and skin from microorganisms[4]. The relationship between the human skin and the colonizing microorganisms can have various features ranging from mutualistic and commensal to saprophytic and parasitic relationships. The consequences for skin health depend largely on the host status and to an extent also on the species composition and the strain identities of the skin microbiota.

Abundant resident bacterial genera on the surface layers of the human skin include *Staphylococcus, Propionibacterium, Micrococcus* and *Corynebacterium*. Sebaceous glands constitute a selective environment allowing growth of *P. acnes* and *Malassezia* species. Bacteria like *Staphylococcus* and *Corynebacterium spp.* that flourish in humid settings were abundant in the bends of the elbows and the feet.

S. epidermidis may act as an opportunistic pathogen when it breaches the skin surface and enters the bloodstream. It is a causative agent of hospital-acquired infections in immunocompromised patients, mostly associated insertion of medical devices where it forms biofilms. The detachment of bacterial cells from biofilms leads to bacteraemia. The lipophilic facultative anaerobe *P. acnes* can hydrolyse triglycerides present in sebum, one factor which may explain their successful colonisation of sebaceous units. *P. acnes* employs lipases to break down triglyceride lipids in sebum and proteases

to release the amino acid arginine from skin proteins, which releases free fatty acids and helps the bacteria cling to the skin. *Propionibacterium spp.* abundance was found to positively connect with cheek sebum levels from face samples. *P*[5]. *acnes* is suspected to be of major importance in the pathogenesis of acne and also in a number of other opportunistic infections.

The lipids of sebum and the stratum corneum are also utilized by auxotrophic *Malassezia* and *Corynebacterium spp*. *Corynebacterium spp*. utilize these lipid compounds to generate the Coryne mycolic acids that coat their cell surface. *Staphylococcus spp*. are halotolerant andutilize the urea that is present in sweat as a nitrogen source. Various *Staphylococcus spp*. canalso produce adherens that promote attachment to the skin and proteases that liberate nutrients from the stratum corneum.

Bacteria invading the blood

Once pathogens gain access to the circulatory system of the host, it is extremely difficult to restrain their growth and multiplication. They can enter various organs and tissues of the body, causing a systemic infection or a state of sepsis. Once the bacteria have colonized the bloodstream, there are some infectious agents that can cross the blood brain barrier, thus gaining entry into the brain. The CNS, consisting of the brain and the spinal cord, is protected by the skull bones and the meninges. The meninges (Dura mater, Arachnoid, and Pia mater) consist of specialized endothelial cells that form the blood-brain barrier. These cells have specific intercellular tight junctions, restricting the movement of molecules across them. The bacteriathat have the ability to cross the BBB have various mechanisms to get past this. The brain is an immune-privileged site, and so it relies on the innate immune system cells and cells circulating in the bloodstream to eliminate any foreign substance[6].

The bacteria may enter cerebral parenchymal cells via the perforated postcapillary venules, which can facilitate the adhesion of these bacteria with the cell by mimicking certain receptors on the surfaces of the cells, like the choline-binding protein (CbpA), which binds to the laminin receptor. Once these pathogens bind to the cerebral cells, they may either trigger intracellular signaling cascades that make the intercellular tight junctions lose their integrity, or degrade them via the secretion of some substances[7,8]. With the destruction of this barrier, the bacteria gain access to the brain and this canresult in disastrous consequences.

Detection of when bacteria have invaded the blood would be useful for medical or forensic purposes. In severe sepsis, the multiple organ dysfunction syndrome (MODS) is a frequent complication and the main reason for death. During sepsis, non- neuroendocrine parenchymal cells secrete large amounts of procalcitonin into the blood by a continuous constitutive pathway. However, the post-translational conversion to mature calcitonin hormone is not seen, indicating unusual reasons for its synthesis, especially sepsis.

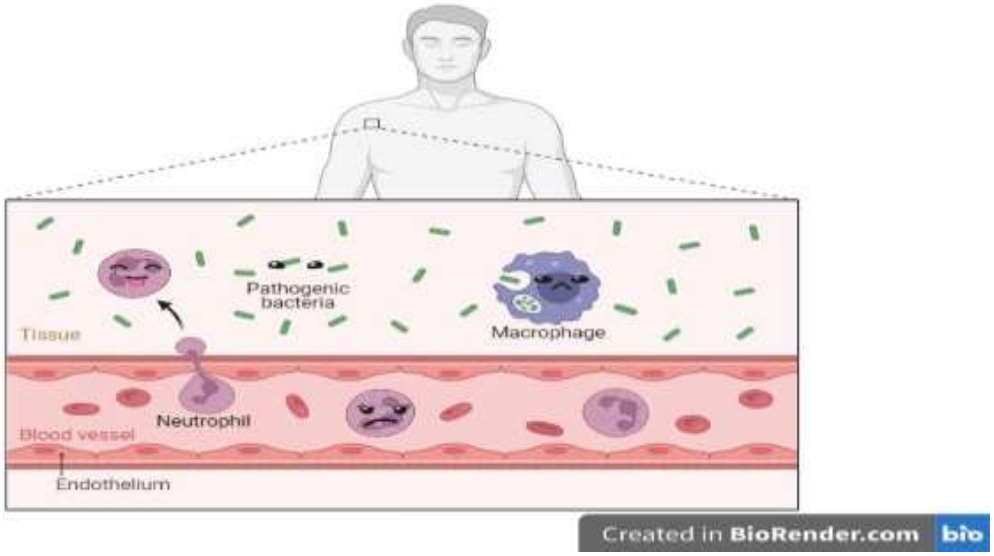


Fig. 1; Immune response towards bacterial invasion

Link between Gut and Female Reproductive Microbiome

It is advised that pregnant women avoid food of uncertain origin that may lead to digestive distress/food poisoning as it can harm the fetus as well. In forensic examination of sepsis during pregnancy or miscarriages, it would be useful to determine if the cause of fetal death was due to poorly prepared food, which could be traced by the time taken for bacteria to achieve colonization. Sequencing of human placenta microbiomes revealed that they resemble typically oral microbiota, particularly that of the tongue and tonsils, that may havespread through hematogenous dissemination during pregnancy, as opposed to vaginal or intestinal germs. Bacteria can invade the cells of the gastrointestinal tract, get transported through the blood and colonize placental or fetal tissues. For instance, chorioamnionitis and placental/fetal colonisation are linked to *Streptococcus spp.* and *F. nucleatum*, found in the amniotic fluid[9]. 64 Brazilian women undergoing elective caesarean sections had *Streptococcus mutans* (SM)- DNA detected in their cord blood (CB), maternal peripheral blood (PB), and maternal saliva (SA). The correlation between the detection of SM in PB and CB was favorable.

The cervicovaginal region is home to trillions of bacteria while the upper reproductive tract (uterine cavity) was formerly thought to be sterile. Bacterial ascent from the lower genital tract, admission through the mother's bloodstream, or active transport of microorganisms by immune cells from the gut or mouth cavity are a few ways that bacteria might reach the placenta. Preterm premature rupture of membranes, preterm delivery, respiratory distress, sepsis, and occasionally fetal/newborn death are all caused primarily by bacterial infection of the amniotic cavity and inflammation. Acute chorioamnionitis (AC) is a placental inflammation linked to miscarriage. AC is the infiltration of neutrophils into the amniotic membranes and placental chorionic plate, which is typically caused by an ascending infection. It can result in cerebral palsy, retinopathy of prematurity, and neonatal necrotizing enterocolitis (NEC). Group B *Streptococcus, Fusobacterium nucleatum, Peptostreptococcus, Escherichia coli, Bacteroides species, Ureaplasma urealyticum* and *Listeria*

monocytogenes are the primary bacteria that cause AC.

The rough structure of the gut is displayed in Fig 1. The rectum serves as both a source and a reservoir for the microorganisms that inhabit the cervicovaginal region. Bloating, diarrhoea, and discomfort in the abdomen are symptoms of small intestinal bacterial overgrowth (SIBO). The small intestinal mucosa andcrypts show inflammation and atrophy, the villi are blunted and gut permeability is increased by intraepithelial lymphocytes. SIBO is brought on by anaerobes such *Bacteroides*, *Lactobacillus*, and *Clostridium* as well as aerobes like *Streptococcus, E. coli*, *Staphylococcus*, and *Klebsiella*. Paracellular routes were used by bacteria to get from the cell to the basal media. Alpha-toxin released by *S. aureus* results in the development of transmembrane pores and the downregulation of tight junction proteins[10, 11]. The tight junction proteins Claudin and ZO1 were decreased, which lessened intestinal integrity and enhanced monolayer permeability. Cell-cell contacts were stabilised by an increase in bacterial ICAM-1. Increased apoptosis was induced by *Klebsiella* and *Streptococcus*. *K. pneumoniae* uses transcellular translocation to spread from the gut flora into other organs, causing systemic infections. By giving pyogenic liver abscess (PLA) *K. pneumoniae* strains a competitive edge over the gut microbiota, type VI secretion system T6SSs enables diffusion into distant organs. Bacterial-host cell interactions, pathogenicity, adhesion, and translocation across intestinal epithelial monolayers are all facilitated by the *K. pneumoniae* Sap (sensitivity to antimicrobial peptides) transporter. *K. pneumoniae* invaded and spread across Caco-2 monolayers when they were added to the apical side, which resulted in the release of the cytokines tumour necrosis factor TNF-α and Interleukin (IL)-6[12]. Patients with intestinal inflammation had aggregates of the oral bacteria *Fusobacterium nucleatum* living in their intestinal mucosa. It releases substances that affect the host, such as outer membrane vesicles (OMVs), close to the epithelium. IL-8 and TNF were secreted by colonic epithelial cells as a result of OMVs.

Colonization of bacteria in fetus

Humans have hemochorial placentation which promotes the growth and development of the fetus. The placenta has three layers when it is fully developed: the decidua of the mother's endometrium, the outer chorion surrounding the amniotic sac with villi and trophoblast, and the inner amnion, a single layer of ectodermal epithelium enclosing the embryo. The trophoblast cells that make up the placenta are found in the blastocyst's extraembryonic trophectoderm layer. Direct interaction between maternal blood and placental villi is made possible by the implantation of blastocyst and the foetal trophoblast cells' deep penetration into the mother's uterine blood vessels. The trophoblast multiplies and merges to form a transient invasive syncytiotrophoblast six to seven days Post Conception (PC)[13]. After ten days of conception, these cells become cytotrophoblasts (CTBs), which merge and differentiate into the invasive extra-villous cytotrophoblasts (EVTs) and the villous cytotrophoblasts (VTs). The embryo is encircled by an expanding syncytiotrophoblast[14].

Neonatal blood and amniotic fluid infection with organisms frequently populating the lower genital tract is the cause of early onset sepsis in neonates. About 25% of healthy women have the gram-positive commensal bacterium Group B *Streptococcus* (GBS) in their reproductive tract and digestive system. GBS are beta- hemolytic bacteria that commonly infect babies and cause cerebral palsy, persistent pulmonary illness, and delayed development. Pathogens can break the placental layers and enter the human amniotic fluid through the cervix when there is an excess of hemolysin. It crosses the decidua, chorion, amnion, and amniotic epithelium, and are mostly found in the choriodecidual region. Preterm labour showed greater hemolytic activity of GBS[15]. A stop codon

mutation in the kinase domain, a V343M substitution in CovS, a deletion in the promoter of covR/S, or one of several other mutations were present in GBS from amniotic fluid and chorioamnion from preterm labour.

Human amnion epithelial cells (hAEC) obtained from placentas were likewise invaded by 4% of the adhering GBS as a result of a loss in barrier resistance brought on by the pluripotent toxin beta-hemolysin/cytolysin. Increases in the cytokines IL-6, IL-8, IL-1β, CXCL1, and CCL20 as well as a 2.5-fold increase in the recruitment of NF-B into the nucleus were brought about by the hyper-hemolytic strain covR. Neonatal morbidity and preterm labour are brought on by IL-6, IL-8, and IL-1β. The pigment of GBS is granadaene, an ornithine rhamnolipid. At an effective concentration 50 (EC50) of 0.11 M, an 8-minute exposure to the pigment promotes hemolytic activity in erythrocytes and alters the membrane's morphology from disc-like to echinocytes and spheroechinocytes. Since the pure pigment is bound to the bacterial cell surface and is not secreted, it may cause hole formation in hAEC but not an inflammatory response. For the inflammatory response to be induced, host cells must be exposed to hemolysin that is linked with bacterial cell surfaces.

Genital mycoplasmas *Ureaplasma parvum*, *U. urealyticum*, and *Mycoplasma hominis* are frequently spread through sexual contact. Detection of genital mycoplasmas, acute chorioamnionitis (61.5%), and foetal pneumonia (16.9%) during the second and third trimesters were associated with spontaneous abortions, according to a study of 130 placentaand foetal lung samples. High amounts of inflammatory cytokines in cord blood, as well as high levels of oral and urogenital commensal bacteria, were found in preterm patients with severe chorioamnionitis and funisitis. There were variations in the microbiota of the placental parenchyma between term and preterm placentas[16].

U. parvum, Fusobacterium nucleatum, Streptococcus agalactiae and *S. thermophilus* were abundant in pregnancies with severe chorioamnionitis. With these oral commensal bacteria, there were variations in the manufacture of secondary metabolites and in lipid metabolism. Inflammation in the placenta during chorioamnionitis can be brought on by declines in butyrate and riboflavin. The amniotic fluid's glucose is used up by a rise in glycerophopholipid metabolism, siderophore group nonribosomal peptides, and the pentose phosphate pathway[17]. Labor-inducing prostanoids and arachidonic acid are produced fromthe conversion of glycerophospholipids.

NAME OF ORGANISM	TOXIN PRODUCED	AREA OF COLONIZATION	MECHANISM OF EVADING IMMUNE SYSTEM	VACCINES AVAILABLE	REFERENCE
Streptococcus pneumoniae	Pneumolysin	Upper respiratory tract, sinuses, and nasal cavity	Polysaccharide capsule – restricts phagocytosis, masks TLRs on bacterial cell wall	PCV13	Le Guennec et. al., 2020; Rai et.al., 2016; Ring et. al., 1998; Gonzalez-Escartin et.al,2017
Neisseria meningitidis	Polymorphic toxins	Endothelial cells lining brain capillaries in the subarachnoid space, the parenchyma, and the choroid plexus	Tfp adhesion with CD147 and β2AR, the adhesin complex protein (ACP), autotransporter meningococcal serine protease A (MspA)	Available for serogroups A, C,W and Y	Le Guennec et. al., 2020; Jamet and Nassif, 2015; Mairey et. al., 2006; Pron et. al; 1997; Maissa et. al., 2017; Hung et.al, 2013; Turner et. al.,2006; Rosenstein et.al,2001
Streptococcus agalactiae (Group B *Streptococcus* - GBS)	Uncharacterized streptococcal pyrogenic exotoxins	Genitourinary and gastrointestinal tracts in asymptomatic individuals, Blood and CNS insymptomatic	Survive inside macrophages - orange carotenoid pigment with free-radical scavenging properties. bacterial lipotechoic acid (LTA), pilus protein PilA, serine-rich repeat proteins (Srr), streptococcal fibronectin-binding protein (Sfb) and alpha C protein	Polysaccharide conjugate vaccine (PCV)	Le Guennec et. al., 2020; Al Akhrass et. al., 2013; Edmond et. al., 2012; Liu et. al. 2004; Tazi et. al., 2012; Madhi et. al., 2017

Table 1; Common Microbes found on/in Humans

Toxin identification

The concept of proteome analysis is defined as the separation, identification and quantification of the entire protein complement expressed by a genome, a cell or a tissue. Early detection procedures for protein toxins rely on *in vivo* or *in vitro* methodologies. Later, immunological tests with enhanced mass spectrometric (MS) detection were created; these tests can produce results considerably more quickly and are easier to carry out thanbioassays.

Immunoassay

Immunoassays are assays which are bioanalytical methods for quantification of antigen using antibodies or vice versa. When an equal amount of antigen is present with an equal number of antibodies, it forms an antigen-antibody complex which is visible as insoluble precipitate. The quantification can be done by comparison with known concentration precipitations or by use of additional proteins such as enzymes, fluorescent tags, etc. When known concentration precipitations are observed, a standard graph is plot upon which the unknown concentration is found using the graph.

GEL DIFFUSION ASSAYS

Gel diffusion assays are conventional techniques which are followed even till now for few tests due to its high specificity. One of the most conventional and still famous gel diffusion is Ouchterlony double diffusion which can detect even 0.002 µg of *Staphylococcus* toxin. The process involves formation of 3 wells in a triangular manner and diffusion of positive control sera with test sera in 2 wells and an antiserum for the positive control in the third well. Formation of a curved line proves the presence of a particular toxin in the test sample while cross lines indicate absence of the toxin. This is a qualitative assay[18]. The quantitative assay is called as radial immunoassay in which one well is loaded with test sera along with known concentrations of positive control sera in other wells diffused with agar-antiserum complex providing different diameters according to concentration present, which can be used for plotting a standard graph and to know the concentration in test sera.

ELISA

The labelled immunoassay known as the enzyme-linked immunosorbent assay, or ELISA, is the industry standard for immunoassays. This immunological test, which may identify and measure molecules like antibodies, antigens, proteins, glycoproteins, and hormones, is exceedingly sensitive. Due to its versatility, speed, simplicity, specificity, sensitivity, affordability, and compatibility with actual samples, monoclonal antibody (mAb)-based detection techniques have been increasingly popular in recent years for the detection of toxins[19]. There are two types of ELISA, namely direct and indirect ELISA. In the direct method, an enzyme conjugated antibody which is called primary antibody directly binds to the specific toxin and when provided with substrate, the enzyme converts it into product which is usually a chromogenic in nature, indicating the presence of toxin, while no change in color indicates the absence of toxin. In the indirect method, an antibody specific to the primary antibody is conjugated with enzyme so that specificity will be higher than direct ELISA. Modification of these two types will further yield sandwich ELISA and competitive ELISA[20].

MASS SPECTROMETRY METHODS

It is used to determine the molecular weight of ionized particles such as atoms, molecules, and clusters by using differences in the ratios of their charges to their respective masses (mass/charge; m/z). These techniques work best in complicated matrices where they can be combined with different separation methods like one- and two-dimensional polyacrylamide gel electrophoresis and gel-free methods that are based on liquid chromatography. All of the peptides in the mixture are fragmented in data-dependent studies, and the MS/MS spectra of those fragments are compared to a relevant protein database. However, this kind of experiment provides no data regarding the quantity. For quantitative and affirmative cases, targeted (data independent) experiments are pertinent. Here, the approach merely searches and fragments the interest peptides that were previously picked. There are two guiding concepts for targeted (data independent) MS/MS investigations, each of which makes use of a distinct mass spectrometer. First, triple quadrupole (QqQ) mass spectrometers are used for selective reaction monitoring (SRM)[21]. In this experiment, a specific peptide is targeted by the first quadrupole's mass filter, which then passes it to the second quadrupole, which acts as a collision cell to break up the peptide. The third quadrupole then filters the specific fragment product reaction ion. The precise pair of m/z values connected to the chosen fragment and the precursor peptide and its selected fragment is referred to as transition which is greater than parent m/z.

Parallel reaction monitoring (PRM), the second technique, uses hybrid quadrupole-Orbitrap mass

spectrometers but operates on a similar concept to SRM. The targeted precursor ion is filtered and fragmented as previously said, but the Orbitrap mass spectrometer records the entire MS/MS spectrum[22]. As a result, tens to hundreds (and thousands in wide-screen investigations) of targeted proteins can be quantified in the same run.

The most popular technique for identifying bacteria is MALDI TOF MS. This method is particularly quick because it relies on the introduction of the sample rather than sample preparation ontoa MALDI plate of a bacterial colony. Intact or trypsin-digested ribosomal or intracellular protein and peptide profiles of whole bacterial cells produce a distinctive bacterial "fingerprint" that can be used to identify contaminating bacteria with accuracy. The detection of *Mycobacterium avium subspecies paratuberculosis* is an illustration of a successful application of MALDI TOF MS for the discriminating between bacterial subtypes (MAP). A pathogenic bacterium called MAP causes disease in ruminants like cattle paratuberculosis(PTB).

BIOSENSOR BASED METHOD

A biosensor is a device that detects chemicals in living organisms by measuring living organisms or biological molecules. Finding a molecular species to create a binding with the target pathogen for sensing is how recognition is accomplished. In order to improve measurement accuracy, various bioreceptors have been added to biosensors. Antigen/antibody, enzyme, nucleic acids/DNA, biological structures/cells, biomimetic, andbacteriophage are the most common types of bio receptors used. With the exception of a tiny group of catalytic ribonucleic molecules, all enzymes belong to the protein group. A complex molecule known as an antibody or antigen is made up of hundreds of individual amino acids that are organized in a carefully planned pattern. They have a specialized affinity for a certain structure that serves as a bioreceptor. The complementarityof adenine: thymine (A:T) and cytosine: guanosine (C:G) pairs inside the DNA provides the specificity of the biorecognition in nucleic acid/DNA based bioreceptor forms. An entire cell/microorganism of biorecognition, or a specific cellular component, is capable of creating a specific binding, which is used in cellular based bioreceptors[23]. An artificial receptor that has been created and developed to resemble a biological receptor is known as a biomimetic-based bioreceptor. Biomimetic-based bioreceptors are created through genetic engineering, plastic membrane fabrication, and molecular imprinting.

DIFFERENT TYPES OF BIOSENSING METHODS

To detect pathogens, various biosensing methods have been developed and have been widelyused for some time. Optical, electrochemical, and mass-based transduction are the most sensitive and accurate methods. A bioreceptor surface is required in biosensor applications for recognition purposes in order to specify different pathogens.

Biosensor based on optical method

This method is extremely effective and reliable for detecting pathogens and endotoxins. To develop optical biosensors, basic optics properties such as reflection, refraction, absorption, dispersion, and so on are used. Transducers such as optical fibre, Raman infrared spectroscopy, surface plasmon resonance, and others are used to develop optical biosensors. Laser light travels through the tapered optical fibre on the detection surface before being detected. The light is propagated through the fibre or waveguide and detects foodborne pathogens and various endotoxins. These methods were used to develop optical-based biosensors to detect pathogens such as *E. coli, Salmonella, Listeria*, and others have reported endotoxin detection using an optical-based biosensor[24].

Biosensor based on electrochemical method

The changed potential and current are observed when the sensing electrode interacts with the sample; this biosensor is known as an electrochemical biosensor. There are several types of electrochemical biosensors, including potential (potentiometric), current (amperometric), conductance (conductometric), and impedance (impedimetric). The amperometric-based biosensor has been used to detect *E. coli, Salmonella,* and other pathogens. Endotoxin and phthalate detection in water is accomplished using an impedimetric- based biosensor.

INCORPORATION OF AMINO ACID COMPOSITION AND FUNCTIONAL DOMAIN FOR IDENTIFICATION OF BACTERIAL TOXINS

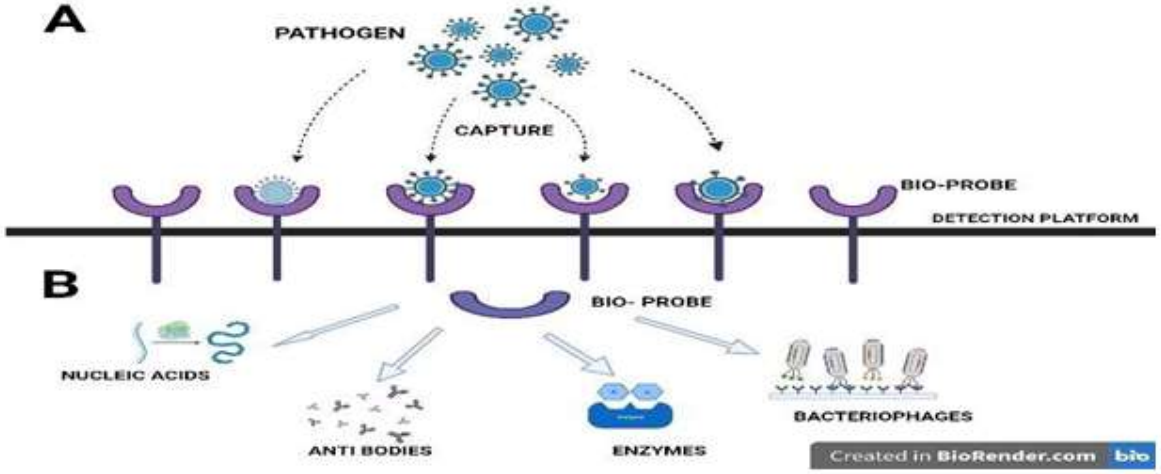
Bacterial toxins can also be determined by a computational method which is based on identifying amino acid sequences and functional domain information. SVM (support vector machine) model was designed using a 20-dimensional vector that consists of the composition scores for twenty amino acids. For the identification of functional domains an SVM model was designed using a 15-dimensional vector consisting of 40 distinguishable domains represented by a binary score: 1 if present and 0 otherwise[25].

NANO BIOSENSORS

Nano biosensors are used in the detection of several microbial toxins and pathogenic microorganisms by nanomaterial-based sensing platforms. Due to the growing awareness of the health dangers connected with the intake of seafood and water, research on the electrochemical detection of algal toxins has gained attraction in recent years. For the electrochemical detection of algal toxins, a number of nanomaterials, including nanoclusters (NCs), graphene, CNTs, and carbon nanofibers, have been combined with particular identification components. The development of electrochemical algal toxin biosensors has focused mostly on the MCs (MC-LR) generated by cyanobacteria. For the detection of algaltoxins, amperometric and impedimetric biosensors have typically been used.

Nearly all foods and feeds may include mycotoxins, which are secondary metabolites of fungi that can appear under a variety of environmental situations. These poisons can harm the kidneys and cause liver cancer when consumed in excess. Researchers had to develop a variety of biosensing methods for their detection due to the serious health dangers associated with these poisons. The performance of electrochemical biosensors for the sensitive detection of mycotoxins has improved as a result of the intriguing features of nanomaterials. Aflatoxin is the general term used to describe the mycotoxins, ochratoxins, fumonisins, zearalenone, and citrinin. Research on identifying bacterial pathogens in food matrices has grown significantly in recent years[26]. For the detection of foodborne pathogens and the toxins they produce, such as

Figure 4 Detection methods using Bio probe.



Forensic significance of Bacteria and their toxins

In addition to the capsule, *S. pneumoniae* can also manufacture pneumolysin (Ply), a hazardous intracellular protein with 471 amino acids and a mass of 53 kDa. It is created during the late log phase of growth and is a member of the family of cholesterol- binding cytolysins. This toxin can create oligomers, which can then create pores in epithelial membranes to cause pro-inflammatory signalling and activatethe complement-classical pathway.

Time lapse of toxin production and ingestion of pathogens can aid in determining Post- Mortem Index (PMI). For example, Thanatomicrobiome is composed of certain external microbes that occupy the internal organs or the different commensals whose relative levels vary greatly after death. The external microbial populations connected to a human cadaver are described by the necrobiome.

The polysaccharide intercellular adhesin is one staphylococcal carbohydrate that aids in the accumulation of bacterial cells (PIA). It is an amorphous exopolysaccharide formed during the active growth phase, as a crucial component ofbiofilm. It facilitates cell-cell adhesion and serves to protect the expanding colony from thehost immune system.

Toxic metabolites secreted by biological systems serve as biomarkers for determining post-mortem index (PMI). They can help in the estimation of time since death using an emerging technique, "metabolomic fingerprinting". When the cause of death remains unclear, analysisof toxic metabolites from post-mortal biological specimens can aid in understanding the pathogenesis of a few fatal disorders; "metabolomic autopsy" can be used to examine causeof death[27].

Use of biological organisms and their toxins in war is not a modern concept. Romans contaminated the water supplies of their adversaries with carcasses of decaying fauna. Tartar soldiers in the battle of Kaffa threw plague infected dead bodies at walls of their enemies in what is considered a bioweapon. A cult in 1993 dispersed spores of *Bacillus anthracis*; there were no infections as it was a vaccine strain. Bioterrorism involves both biological organisms (bacteria, fungi, virus and their toxins) as well as mechanical gadgets that serve to transport them. These are considered forensic evidence when examining biocrimes. Gene sequencing and identification of strains endemicto regions can be used to trace the source of the pathogenic bacteria[28]. They also aid in identifying the cause of death. Enterotoxins, such as one produced by *Staphylococcus aureus*, causes food poisoning. Their presence in the stomach contents of the victim is valuable in forensic investigations.

Disease outbreaks occur around the world. These involve forensic and epidemiological investigations. This is to ascertain if it was brought about by natural causes or if it involveddeliberate exposure with criminal intent.

Discussion

Even though only around 5% of bacterial species are harmful, they have historically been responsible for a disproportionately high number of illnesses and fatalities in humans. They have proven to be adaptive in various conditions and causing numerous diseases. Bacteria produce toxins which are capable of causing diseases especially in human beings, these types of bacteria are called Bacterial pathogens and the study of these bacterial pathogens and its effects on human body in forensic aspects is called Microbial forensics. Microbial forensicscombines epidemiology techniques with the identification of microbiological and microbiology-related data to help identify the identity of the perpetrator(s) by identifying theprecise source of the sample, as individually as possible.

Bacteria in humans can be found in various parts of the human body ranging from outermostcovering skin to innermost blood circulatory system, owing to their ability to adapt and survive in extreme conditions. Though many bacteria are considered healthy/ good types of bacteria (many don't have the ability to invade and infect the human body), some have proven to be opportunistic and capable of causing various diseases ranging from Pulmonary Tuberculosis to Gonorrhoea. Identifying these bacteria present in the human body in a forensic perspective can be a means of identifying and deducing the possible cause of death, lifestyle, locality and geographical area, possible location of death and time of death of the victim.

As microbial forensics has been gaining more and more importance and as there are developments in many technologies and procedures for identification of bacterial pathogens and its toxins. These identification methods range from identifying specific species of bacteria to itswhole genome level of sequencing to identify the specific type of bacteria and its toxins. Preliminary detections of the bacteria include the techniques and characteristics of conventional microbiology. These techniques include fatty acid analysis, serotyping, and microscopy in addition to culture, phage sensitivity, staining, and microscopy.

Technologies for detecting and identifying pathogens based on nucleic acids allow for the examination of both genetic and structural data. PCR allows specific fragments of genomic DNA to be isolated and their copy number amplified. Over time, enhancement of methods and technologies have created newer variations of PCR which help in accurate and faster detection. Mass spectrometry (MS) combined with PCR analysis revolutionized the ability to identifyrespiratory pathogens. MS is useful in identifying many non–nucleic acid chemical species that may provide clues to microbial identity, origins, and production processes. Proteins, peptides, lipids, carbohydrates, inorganic metals and organic metabolites may provide information about an organism's source environment, how it was produced, and the level ofsophistication of the preparation.

As science enhanced, many more techniques like ELISA, Southern Blotting, Western Blotting, Fluorescence assays etc., have been developed and have proved to be economical and less time consuming. Based on its accuracy, sensitivity and economic availability, there has been frequent use of these techniques in forensic toxicology and forensic microbiology for cases involving the bacterial pathogens and its toxins resulting in the death of victims. Genome sequencing coupled with various identification techniques has been proven to be accurate, sensitive andless time consuming and has been successful in giving various information about bacterial pathogens, its toxins and its effects on

humans which has applications in understanding the pathogens and its faster identification.

Future Perspectives

Currently, microbial forensics is in the developing stage. A global code of conduct and set of quality assurance standards for laboratories practicing microbiological forensics must be developed through time and with experience. By doing so, problems and data variability that could be contested in judicial procedures will be reduced. Prioritizing current efforts is necessary, particularly for viruses and poisons that are more likely to be exploited in bio crimes. It is necessary to create various standards for the validation of the approaches used to classify various threat agents that may be applied forensically to attribute criminal conduct. For a deeper understanding of microbial population genetics, careful evaluation ofregional microbial population dynamics is necessary.

The previous ten years have seen a revolution in research across a wide range of disciplines, from personalized medicine to forensics, thanks to advancements in MPS and related approaches. These days, complicated samples or entire genomes can be sequenced quickly and affordably. Therefore, in a polyphasic approach, these novel techniques can be utilized as an addition to established microbiological techniques. However, logical standards for a microbiological forensics information database have not yet been established. Any microbial forensics laboratory needs a knowledge base includingdatabases on genomes, microbiology, forensic techniques, associated materials and related evidence assays, bioinformatics, and standardized instruments if it is to be effective.

CONFLICT OF INTEREST: NA

SOURCE OF FUNDING: NA

ETHICAL CONSIDERATION: NA

REFERENCES

1. Aagaard, K. M. Author response to comment on “the placenta harbors a unique microbiome.” Science Translational Medicine: 2014: 6(254), 317.
2. Al Akhrass, F., Abdallah, L., Berger, S., Hanna, R., Reynolds, N., Thompson, S., Hallit, R.,& Schlievert, P. M. Streptococcus agalactiae toxic shock-like syndrome: Two case reports and review of the literature. Medicine, 2013: 92(1), 10–14.
3. Antony, K. M., Ma, J., Mitchell, K. B., Racusin, D. A., Versalovic, J., & Aagaard, K. The preterm placental microbiome varies in association with excess maternal gestational weight gain. American Journal of Obstetrics and Gynecology, 2015: 212(5), 653.e1-16.
4. Bonasoni, M. P., Palicelli, A., Dalla Dea, G., Comitini, G., Nardini, P., Vizzini, L., Russello,G., Bardaro, M., & Carretto, E. Klebsiella pneumoniae chorioamnionitis: An underrecognized cause of preterm premature rupture of membranes in the second trimester. Microorganisms, 2012; 9(1), 96.
5. Bourmaud, A., Gallien, S., & Domon, B. Parallel reaction monitoring using quadrupole-Orbitrap mass spectrometer: Principle and applications. Proteomics, 2016; 16(15–16), 2146–2159.
6. Byrd, A. L., Belkaid, Y., & Segre, J. A. The human skin microbiome. Nature Reviews. Microbiology, 2018; 16 (3), 143–155.
7. Coelho, M. A., Sampaio, J. P., & Gonçalves, P. Living and thriving on the skin: Malassezia

genomes tell the story. MBio, 2013; 4 (2), e00117-13.

8. Darwish, I. A. Immunoassay methods and their applications in pharmaceutical analysis: Basic methodology and recent advances. International Journal of Biomedical Science: IJBS, 2006; 2 (3), 217–235.
9. Edmond, K. M., Kortsalioudaki, C., Scott, S., Schrag, S. J., Zaidi, A. K. M., Cousens, S., & Heath, P. T. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet, 2012; 379 (9815), 547–556.
10. Fardini, Y., Chung, P., Dumm, R., Joshi, N., & Han, Y. W. Transmission of diverse oral bacteria to murine placenta: evidence for the oral microbiome as a potential source of intrauterine infection. Infection and Immunity, 2010; 78(4), 1789–1796.
11. Forbes, J. D. Clinically important toxins in bacterial infection: Utility of laboratory detection. Clinical Microbiology Newsletter, 2020; 42 (20), 163–170.
12. G Abril, A., G Villa, T., Barros-Velázquez, J., Cañas, B., Sánchez-Pérez, A., Calo-Mata, P.,& Carrera, M. Staphylococcus aureus exotoxins and their detection in the dairy industry and mastitis. Toxins, 2020; 12 (9), 537.
13. Gao, J., Tang, Y., Sun, X., Chen, Q., Peng, Y., Tsai, C. J.-Y., & Chen, Q. Downregulation of ribosomal contents and kinase activities is associated with the inhibitive effect on the growth of Group B Streptococcus induced by placental extracellular vesicles. Biology, 2021; 10 (7), 664.
14. Gonzalez, M. R., Bischofberger, M., Pernot, L., van der Goot, F. G., & Frêche, B. Bacterial pore-forming toxins: The (w)hole story? Cellular and Molecular Life Sciences 2007; 65(3), 493–507.
15. Graves, S. F., Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Sturdevant, D. E., Rasmussen, D. L., Kirpotina, L. N., Quinn, M. T., & DeLeo, F. R. Sublytic concentrations of Staphylococcus aureus Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. Journal of Leukocyte Biology, 2019; 92 (2), 361–374.
16. Gupta, R., Raza, N., Bhardwaj, S. K., Vikrant, K., Kim, K.-H., & Bhardwaj, N. Advances in nanomaterial-based electrochemical biosensors for the detection of microbial toxins, pathogenic bacteria in food matrices. Journal of Hazardous Materials, 2021; 401 (123379), 123379.
17. Hsieh, P.-F., Lu, Y.-R., Lin, T.-L., Lai, L.-Y., & Wang, J.-T. Klebsiella pneumoniae type VI secretion system contributes to bacterial competition, cell invasion, type-1 fimbriaeexpression, and in vivo colonization. The Journal of Infectious Diseases. 2019; 219 (4), 637–647.
18. Hsu, C.-R., Chang, I.-W., Hsieh, P.-F., Lin, T.-L., Liu, P.-Y., Huang, C.-H., Li, K.-T., & Wang, J.-T. A novel role for the Klebsiella pneumoniae Sap (sensitivity to antimicrobial peptides) transporter in intestinal cell interactions, innate immune responses, liver abscess, and virulence. The Journal of Infectious Diseases. 2019; 219 (8), 1294–1306.
19. Jamet, A., & Nassif, X. Characterization of the Maf family of polymorphic toxins inpathogenic

Neisseria species. Microbial Cell (Graz, Austria). 2015; 2 (3), 88–90.

20. Knöfler, M., Haider, S., Saleh, L., Pollheimer, J., Gamage, T. K. J. B., & James, J. Human placenta and trophoblast development: key molecular mechanisms and model systems. Cellular and Molecular Life Sciences: CMLS, 2019; 76 (18), 3479–3496.

21. Kwak, Y.-K., Vikström, E., Magnusson, K.-E., Vécsey-Semjén, B., Colque-Navarro, P., & Möllby, R. The Staphylococcus aureus alpha-toxin perturbs the barrier function in Caco-2 epithelial cell monolayers by altering junctional integrity. Infection and Immunity, 2012; 80 (5), 1670–1680.

22. Latino, M. A., Botta, G., Badino, C., Maria, D. D., Petrozziello, A., Sensini, A., & Leli, C. Association between genital mycoplasmas, acute chorioamnionitis and fetal pneumonia in spontaneous abortions. Journal of Perinatal Medicine. 2018; 46 (5), 503–508.

23. Los, F. C. O., Randis, T. M., Aroian, R. v., & Ratner, A. J. Role of Pore-Forming Toxins in Bacterial Infectious Diseases. Microbiology and Molecular Biology Reviews. 2013; 77 (2), 173–207.

24. MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., & MacCoss, M. J. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics (Oxford, England). 2010; 26 (7), 966–968.

25. Marriott, H. M., Mitchell, T. J., & Dockrell, D. H. Pneumolysin: a double-edged sword during the host-pathogen interaction. Current Molecular Medicine. 2008; 8 (6), 497–509.

26. Moser, G., Guettler, J., Forstner, D., & Gauster, M. (2019). Maternal platelets—friend or foeof the human placenta? International Journal of Molecular Sciences, 20(22), 5639.

27. Oliveira, M., & Amorim, A. Microbial forensics: new breakthroughs and future prospects. Applied Microbiology and Biotechnology. 2018; 102 (24), 10377–10391.

28. Parker, D., & Prince, A. Immunopathogenesis of Staphylococcus aureus pulmonary infection. Seminars in Immunopathology. 2012; 34 (2), 281–297.