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MOLECULAR STUDY OF VIRULENCE GENES OF *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* ISOLATES FROM CEREBROSPINAL FLUID SHUNT-ASSOCIATED INFECTIONS IN PEDIATRICS BY PCR TECHNIQUE

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Abstract

Hydrocephalus is a serious disease that affects humans in different age groups and refers that collects excessive fluid accumulated in the brain, which may be congenital or acquired as a result of bacterial, infectious, parasitic or fungal infection. In the absence of many studies or scientific research to illustrate the cases of the other spinal cord fluid, we have been able to conduct this study, which included the conduct of several direct and chemical phenotypic tests and the 120 spinal cord samples (CSF) collected from the children The sleepers and arrivals to Azadi educational hospital and Kirkuk General Hospital in Kirkuk city, who were diagnosed by a neurosurgical specialist in the spinal cord for patients with hydrocephalus for the period from July 2017 until April 2018, the results showed that 46.1% of The samples were abnormal in terms of color, and the presence of sediment or blood in the sample as well as variations in the rate of natural values of the three variables of Glucose and Protein and the total number of white blood cells with a differential count of the quality of these cells. The study also included the isolation and diagnosis of bacterial species causing the CSF of the samples of the specimens by the introduction of the phenotypic tests and the Cultural Characteristics and Biochemical tests and the API in its diagnosis, *Staphylococcus aureus* and *Pseudomonaus aeruginosa* pathogen was blamable for a huge scale of infections in human because of their invention of secreted and other cell-surface related virulence factors that regulate by various genes. This study was carrying out to realize the frequency of Staphylococcus species in Kirkuk city using specific genes for that purpose. PCR technique was applied to amplify the *Staphylococcus aureus* virulence genes like sea, see, tst, and fnbA, and *Pseudomonaus aeruginosa* virulence genes like sea, see, tst, and fnbA and they determined PCR depending on the sizes of the PCR products amplicons. Out of 120 collected samples from CSF in children , only 44 isolated is appear bacterial growth and *Staphylococcus aureus* (22.73%) were revealed and *Pseudomonaus aeruginosa* (15.90%) According to phenotypic and biochemical tests. From the 4 examined genes that uses from *Staphylococcus aureus*, most abundant gene was *FnbA* 50% (5cases) followed by *sea* and *seb* 40% (4 cases). While *tst* (1 case) 10% gene reported as the least frequently detected gene. From the 4 examined genes that uses from *Pseudomonaus aeruginosa* , most abundant gene was *alg* 100% (7 case), *exoenzyme S, PlcH* 85.71% (6 cases) While *Exo A* 71.42% gene reported as the least frequently detected gene.

Key words: Cerebral ventriculitis, Child Hydrocephalous, Cerebrospinal Fluid Shunt, CSF Bacteria, Virulence factor of *Staph. aureus*, Virulence factor of *P. aeruginosa*

Introduction

Hydrocephalus is defined as the accumulation of cerebrospinal fluid in the pockets of the brain (ventricles in the brain) due to insufficient passage of cerebrospinal fluid from its point of production to the point of absorption in the circulatory system (Andreas *et al*., 2017), as is described as a disease caused by brain failure in Develop correctly during the prenatal period (Greenberg, 2016). This condition is characterized by a rapid increase in the head circumference or unusually large head size due to the excessive accumulation of cerebrospinal fluid in the brain and this condition causes the onset of symptoms of vomiting, drowsiness, irritability and deviation Downward eye to the symptoms of fatigue in general (Ortega *et al*., 2016), The treatment of hydrocephalus is also formed by the introduction of drainage(Cerebrospinal fluid Shunt)(CSF Shunt) to the peritoneal cavity, pleural cavity or to the atrium of the heart as this procedure is accompanied by many Potential problems of infection (Kestle *et al*., 2016), The Shunt Infection is mainly due to the colonization of the natural flora of the drainage device, whether the bacteria on the skin or the different potential of the body, where 90% of the infection occurs due to types of streptococcal The cluster and most of these infections occur within the first few months after surgery (Parker *et al*., 2015), where the infection caused a failure to convert, forcing the Shunt to be removed or replaced by the child's exhibition to hospitalization for a long time (Erik *et al*., 2018). The name means “water on the brain.” and Brain damage can occur as a result of the fluid buildup (Hlatky *et al*., 2003). This can lead to developmental, physical, and intellectual impairments, It requires treatment to prevent serious complications (Sarguna *et al*., 2006), Central Nervous System (CNS) Shunt surgery is the most common treatment and Shunt infection is one of the devastating complications associated with shunt placement, responsible for significant morbidity leading to shunt malfunction and chronic ill-health (Kaufman *et al*., 1997). There are considerable variations worldwide. Post-operative infection of cerebrospinal fluid (CSF) shunts have varied from a low of 2% to high of 27% (Winn *et al*., 2006; Wang *et al*., 2004). Shunt infection is defined as isolation of organism from shunt fluid, shunt tube, reservoir and/or blood culture along with the clinical signs and symptoms suggestive of shunt infection (Sarguna *et al*., 2006) Hydrocephalus mainly occurs in children and adults over 60. The National Institute of Neurological Disorders and Stroke (NINDS) estimates that 1 to 2 of every 1,000 babies are born with hydrocephalus (Winn *et al*., 2006). *Staphylococcus aureus* and *Staphylococcus epidermidis, Pseudomonas aeruginosa* and different genus of Enterobacteraceae are reported to the most common etiological agents responsible for shunt infection (Sarguna *et al*., 2006; Winn *et al*., 2006). Despite technological advances and administration of new antibiotics, infection continues to be one of the most frequent and serious complications of CSF shunts (Wang *et al*., 2006). Despite the importance of the issue due to the increase this disease in prevalence among newborns, there are no specialized or similar studies in the city of Kirkuk on this subject and there are no reports showing the cause of the increase in deaths among children with these diseases, and because of the importance of the disease and increase the rate of spread in Kirkuk and the absence of hospitals or specialized centers to treat or follow the cases of hydrocephalus compared to the developed countries, so the aim of the study was to isolate and diagnose positive and negative gram bacteria caused the spinal cord isolated from patients hydrocephalus and identify Percentages of each type in addition to the investigation of some Virulence Factor which may have a role in the increasing spread of the disease and the statement of its causes, also ensure the study of the phenotypic and physiological changes and conduct some chemical tests to estimate the concentration of Glucose and Protein on CSF samples of Newborn as well as the differential count of the quality of the purulent cells in case of infection.

Materials and Methods

Study included the collection of 120 samples of spinal cord fluid (CSF) in children patients which have head shunt injuries of hydrocephalous, of both sexes males and females, and from different age groups ranging from newborns up to the age of one years suspected of suffering from spinal fluid injury based on clinical symptoms and diagnosis of doctors Specialty in Kirkuk General Hospital and Azadi Teaching Hospital in the city center. Samples collected from CSF were collected under sterile conditions for the period from July 2016 to April 2017 .After the arrival of the spinal cord samples to the laboratory, each sample was divided into three sections in sterile test tubes for biochemical tests, bacteriological tests and differential cell count assays. Each CSF sample are cultured on brain heart infusion broth to activate for the purpose of obtaining secondary farms for the isolation of germs complex dietary requirements where the samples were plotted on the blood agar medium and MacConky agar and incubated dishes at 37 °C for 24 hours, and then taken one colony from each sample to perform the purification process by streak on Nutrient agar to isolate pure colonies. For subsequent tests (Visual tests)(in-kind) including Color, Appearance, Total pus cells count, Differential cell count, and chemical tests of CSF including Estimation of glucose and Estimation of protein ,For the exact diagnosis of bacterial species, many bacteriological and biochemical tests have been carried out to confirm the purity of bacterial isolates according to (Parija, 2014; Brown, 2007). The search include also the study of several Virulence Factors by using PCR technique that effected on CSF Shunt infection in children. Chromosomal nucleic acid was purified from *S. aureus* with Genomic DNA Kits for purification of genomic DNA kit (G-spin) according to the manufacturer instructions (biotechnology/Korea). No reference strains were available for the *S. aureus* genes (*sea, tst, seb, fnbA*) and *P. aeruginosa* genes *(alg, exoenzyme s, PlcH*, *exotoxin A*) a all primers used in this study was summarized in table1. A PCR was performed in a final volume of 20 µl containing 100 ng of DNA template, 1X of buffer, 0.3 µm of each primers, 200 µm of dNTP mix, 1.5 mM of $MgCl_2$ and 1U of Taq DNA polymerase (Pioneer, Korea). The PCR products were resolved on a 1.5% (w/v) agarose gel stained with Red safe staining souluion; DNA bands were visualized and photographed under an ultraviolet transilluminator.

Table 1: Oligonucleotide primers used in this study

Primer	Oligonucleotide sequence , 5' to 3'	(bp)
exoenzymes	CGT CGT GTT CAA GCA GAT GGT GCT G CCG AAC CGC TTC ACC AGG C	444
Alg	CGT CTG CCG CGA GAT CGG CT' GAC CTC GAC GGT CTT GCG GA	313
PlcH	GCA CGT GGT CAT CCT GAT GC TCC GTA GGC GTC GAC GTA C	607
Exo	GAC AAC GCC CTC AGC ATC ACC AGC CGC TGG CCC ATTCGC TCC AGC GCT	397
Seb	TCG CAT CAA ACT GAC AAA CG AGG TAC TCT ATA AGT GCC TGC CT	475
Sea	TTG CGA AAA AAG TCT GAA TTG C ATT AAC CGA AGG TTC TGT AGA AGT A	200
Tsst	AGA TGA AGT AGT TGA TGT GTA TGG CAC ACT TTT AGA ATC AAC CG	451
FnbA	GAG CAG CAT TAT TCT TAG TTT GTG GCC CTT GTA CT	270

Results and Discussion

The pathogens that cause bacterial infection in infants with hydrocephalous and their antimicrobial susceptibilities have changed in this era of increasing antimicrobial resistant as shown in Table 1 that of the total 120 CSF samples taken from samples, 44 samples gave bacterial growth, 19 males with 43.18% and Females 25.82% .

Table 1: Number and Percentage of total infected and non-infected sample .

Percentage	Non Infected	Percentage	Infected	Total Number	Gender
66.67	38	43.18	19	57	Male
61.31	38	56.82	25	63	Female
63.33	76	36.67	44	120	Total

The appearance of the high percentage of negative isolates is due to several reasons, including the probability that the infection may be virulence or might be due to anaerobic germs that have not been isolated in this study or may be the injury caused by fungi or parasites. The results of our study do not correspond to (Alenezi *et al.*, 2018) which showed that 60.9% of the infected samples appeared in males while females recorded a percentage of 39.1%, as well as the study of researchers (Gwenn *et al.*, 2018), which established the rule of sampling in males by 70%, and this Matches what it said (Klein, 2005) that females are more likely to be infected with different infections. Samples of bacterial growth were diagnosed based on phenotypic, Culture Characteristic and Biochemical tests and the use of an API in the classification of bacterial species, where the results indicated that of the total of 44 bacterial isolates, 19 were 43.18% isolated from the Gram Positive bacteria while 25 isolation 56.82% are Gram Negative bacteria. The appearance of Gram positive and negative bacteria in CSF samples have the ability of germs to reach the Central Nervous System by crossing the blood and brain barrier to infect the meningococcal membranes surrounding the brain or through wounds caused by accidents and fractures that infect The skull or through the natural flora of the respiratory tract which becomes opportunistic due to the weakness of the immune system or may be transmitted from the pregnant mother to the fetus during the embryonic stages of development or by the transmission of germs that are part of the natural flora in the gut where it travels to Brain by the head Shunt to reach the brain and the inflammation occurs (Brown *et al.*, 2006; Winn *et al.*, 2006). The table 2 shows the *Staphylococcus aureus* bacteria was diagnosed at the highest isolation rate 22.73% of total isolates of gram positive bacteria while *P. aeruginosa* with the highest isolation rate of 15.90% of total isolates of gram negative bacteria

Table 2: Bacterial Isolation that isolated from CSF Stunt infection.

Percentage	Isolation Number	*Spp.*
22.73	10	*Staphylococcus aureus*
9,09	4	*Staphylococcus epidermidis*
14.29	6	*E. coli*
15.90	7	*Pseudomonas aeruginosa*
9.09	4	*Klebsiella pneumonia*
4.55	2	*Acinatobacter spp.*
11.36	5	*Streptococcus*
2.27	1	*Enterococcus cloacae*
11.36	5	*Brukholderia cepacia*
100%	42	Total

The results of the Ocular tests and tests of Biochemical

1- **Color and clarity :-** The results of sample CSF color and clarity Table(3) indicated that 63.33% were natural samples and 36.67% were abnormal samples in terms of color and clarity compared to the comparison tube containing distilled water, where the (Fisvhbuch & Dunning,2000) that it is possible to determine the clarity of the CSF samples through the examination Direct to the naked eye and the degree of clarity depends on the turbidity and the color.

Table 3: Type of CSF sample according color and clarity.

Percentage	Number	CSF Sample
63.33	76	Normal CSF
36.67	44	Abnormal CSF
100	120	Total

2- Total White Blood Cells Count : The results show Table (4) that 63.33% of the samples did not show purulent cells (no cell), while 10% of the CSF samples contained pus cells with fewer than 1000 cell/ml, 15% of the samples contained 1000 cell/ml cells representing the highest percentage of the sampled, while the number of pus cell exceeded more than 1000 cells/ml in 11.67% of the samples of the infected CSF. A study (Kestenbaum *et al.*, 2010) indicated that the determination of the total number of white blood cells in the CSF is a rudimentary indicator of the pathological condition of the central nervous system and concluded that the values of WBC in a Newborns and children can be used in the precise interpretation of the results.

Table 4 : Number and Percentage of total WBC Count.

Percentage	Number	Number of WBC
63.33	76	No Cell
10	12	Less than 1000 cell
15	18	1000 cell
11.67	14	More than 1000
100	120	Total

3- Deferential White Blood Cell : The cell type was determined whether the Neutrophil or lymphocytes cells, the results Table (5) indicated the rule of the cells by 56.82% in the sampled samples while the ratio of lymphocytes was 43.18%.

Table 5: Number and Percentage of total Deferential WBC Count.

Percentage	Number	Type of WBC cell
56.82	25	Neutrophil
43.18	19	Lymphocytes
100	44	Total

4. Glucose : The results show Table(6) that (35%) of the specimens showed hypoglycaemia in the cerebrospinal fluid below the normal rate while (59.17%) was within the normal rate of glucose in the spinal cord fluid, while the (5.83%) high values above the normal rate of chlorose in the spinal cord fluid, described the levels of Low CSF of 2.2 ml mol/L is a characteristic of bacterial meningitis or cerebrospinal fluid or tumors, while the increase is reflected in trauma, traumatic conditions or diabetes (Nigrovic *et al.*, 2012).

Table 6: Number and Percentage of Glucose concentration.

Percentage	Number	Glucose concentration
35	42	Less than Normal Rang
59.17	71	Normal Rang
5.83	7	More than Normal Rang
100	120	Total

5- Protein : The results Table(7) indicated that 39.17% of the CSF samples contained protein values that were higher than the normal level of protein in the spinal cord fluid, while 58.33% of the samples were containers of protein values within the natural range and 2.5% of the samples had protein values below the normal rate of protein concentration in the spinal fluid. Spinal cord, the ratio of natural protein to the spinal fluid in adults is 15-45 mg/dl, whereas in newborns it is 1500 mg/L due to the immaturity of the blood barrier in the brain while 180-580 mg/L for children aged 6 to 12 years (Sechusen *et al.*, 2003).

Table 7: Number and Percentage of Protein concentration.

Percentage	**Number**	**Protein concentration**
2.5	3	Less than Normal Rang
58.33	70	Normal Rang
39.17	47	More than Normal Rang
100	120	Total

Virulence Gene Result

Out of 120 collected samples from CSF, only 10 *Staphylococcus aureus* (22.73%) were revealed , Many genes like *sea*, *see*, *tst*, *seb*, and *fnbA* genes was determined by either multiplex or uniplex PCRs depending on the sizes of the PCR products amplicon. From the 4 examined genes, in (Table 8) most abundant gene was *fnbA* 50% (5 cases) followed by *sea* 40% (4 cases) and *seb* 40% (4 case) While *tst* gene 10% (1 case) reported as the least frequently detected gene.

Most of *S. aureus* strains have the ability to yield one or more toxins including *sea*, *see, eta* genes and these toxins characterize as highest source of food poisoning staphylococcal. 95% of these outbreaks were due to classical toxins such as *sea*, *see* and *eta* (Omoe *et al.*, 2002). Our results revealed that many genes were identified more frequently in MRSA isolates and these outcomes numbers were agreed with the earlier researches which indicated that *sea*, *see*, *tst*, and *fnbA* genes were detected more frequently in MRSA isolates (Hawrr Wahab *et al.*, 2014; Sila *et al.*, 2009; Hu *et al.*, 2008). Products of these genes with PCR explored in our research revealed classical polymorphism of the gene and it will allowed characterization genetic of these isolates (Hookey *et al.*, 1998). The deviation in frequency of these genes among many other researches could be explained according to diverse factors like geographical variance, which may be further affected by ecological origins of *S. aureus* strains, type of disease and clinical samples ,quantity and quality of collected samples and procedure used to detect these genes and to isolation and purification the bacteria. Only 7 *Pseudomonas aeruginosa* (15.90%) were revealed , From the 4 examined genes, in (Table 9) most abundant gene was *alg* 100% (7 case) and *exoenzymes* and *PluH* 85.71% (6 case), While *Exo A* gene 71.42% (5 case) reported as the least frequently detected gene.

Table 8: Demonstrates the results of the presence of virulence genes in *S. aureus*.

N.	**Isolation N.**	**DNA Result**	***Seb***	***Sea***	***Tsst***	***fnbA***
1	S2	+	-	-	-	-
2	S3	+	-	-	-	-
3	S4	+	+	+	-	+
4	S7	+	+	+	-	+
5	S10	+	-	+	-	+
6	S12	+	-	-	-	-
7	S 29	+	+	-	+	-
8	S 31	+	-	-	-	-
9	S 37	+	+	-	-	+
10	S 41	+	-	+	-	+

Table 9 : Demonstrates the results of the presence of virulence genes in *P. aeruginosa*.

N.	**Isolation N.**	**DNA Result**	***exoenzymes***	***Alg***	***PlcH***	***Exo A***
1	P 1	+	+	+	-	+
2	P 2	+	+	+	+	+
3	P 3	+	+	+	+	-
4	P13	+	+	+	+	+
5	P11	+	+	+	+	+
6	P 4	+	+	+	+	+
7	P22	+	-	+	+	-

Conclusion

CSF shunt infection plays a significant role in outcome of patients with hydrocephalus. Early detection and management of shunt infection with appropriate antibiotics along with prompt removal and replacement of the alternative drainage system has provided best results. Identification of risk factors and causative agents may also help prevent shunt infection. In addition, it is important that surveillance of shunt infection should be done with feedback of appropriate data to surgeons in an attempt to reduce shunt infection rate. In conclusion, the incidence of coding genes which cause infection which recorded in the present study specify that the control of these genes appears to be a characteristic feature of *S. aureus*. Furthermore, control of numerous virulence genes seems to be more communal than it's believed.

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