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Research Article

Effect of Vitamin D3 on Colonization Genes Expression of Haemophilus influenzae Isolated from Children with Otitis Media Associated with Temporomandibular Joint Pain

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ABSTRACT

Background: Acute otitis media (AOM) is the middle ear inflammation caused by various microorganisms, including bacteria. Haemophilus influenzae is a common bacterium that causes this inflammatory condition. This study aimed to determine the role of cholecalciferol (D3) in the expression of colonization genes in H. influenzae isolated from children with otitis media, which is associated with temporomandibular joint pain.

Subjects and Methods: A total of 160 ear swabs and blood samples were collected from children with recurrent acute otitis media (AOM) for culture and sensitivity tests. The number of patients with pain in the TMJ was recorded, whether by pressure on the joint area or during mouth opening. Vitamin D3 was measured using an Enzyme-Linked Immunosorbent Assay. The minimum inhibitory and bactericidal concentrations of vitamin D3 were identified. Quantitative real-time PCR was used to evaluate the impact of this vitamin on the expression of pilA, hmw1, and hmw2.

Results: This study showed that H. influenzae caused 28.12 % of AOM, and this inflammation occurred in 30.6% of children aged 1-2 years. Interestingly, 14.38 % of AOM patients had TMJ pain, while 85.62 % did not. Vitamin D3 levels in AOM patients were lower than those in normal children. The current study demonstrated that the expression of colonization genes in H. influenzae, the most common bacterium causing AOM, is upregulated in the absence of vitamin D3. However, these genes' expressions are downregulated in the presence of this vitamin.

Conclusions: This study demonstrated that vitamin D3 inhibited the colonization gene expression of H. influenzae and altered the expression of these genes, suggesting therapeutic roles in infection prevention.

Introduction

There are two types of ear infections, which are acute suppurative otitis media (ASOM) and chronic otitis media (COM) [1,2]. Acute OM (AOM) usually affects children younger than 2 years old. It starts quickly and shows up as fever and pain in the ear of a child who is already sick in other ways; it is mainly caused by bacteria. If the eardrum perforates, which happens about 5% of the time but has been seen at higher rates, it might be associated with ear discharge [1-3]. In addition, in some cases, the infection from the middle ear can spread to the temporomandibular joint (TMJ), which is a small, complicated

joint in the body. The mandibular ramus joins the condylar process, an ellipsoid hard tissue (bony structure) with a thin neckline [4,5]. Several potential reasons for OA have been recognized. They are inflammatory, metabolic, and mechanical [6]. Pain is the most characteristic sign of TMJ OA [7]. Growth disruption of the craniofacial bone may develop from otitis media on the TMJ. The patient's clinical findings indicate that long-term follow-up is essential to track changes in craniofacial growth in persons with a history of recurrent otitis media [8]. Upper respiratory tract infections caused by bacteria are strongly associated with AOM, which are mainly caused by Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis [9,10]. Respiratory tract infections and invasive diseases like meningitis and septicemia, with underlying illnesses, are caused by nontypeable Haemophilus influenzae (NTHi) among children. Several adherence factors, such as hemagglutinating, pili, and colonization proteins, encourage upper respiratory tract colonization, a necessary condition for illness [11]. Whether particular adhesions contribute to the microorganism's diffusion inside the respiratory tract or to sterile regions is debatable. Over the last ten years, it has become clearer that bacteria have developed various strategies to control gene expression to survive and multiply in hosts during the many stages of infection [12]. Vitamin D3 receptors are found in many cells, suggesting that Vitamin D3 may play a role in the development of infection [1-3]. All immune system cells, especially active T and B lymphocytes, macrophages, and dendritic cells, have vitamin D3 receptors. Researchers found that chemotactic and phagocytic qualities increase in places where vitamin D3 is present. This means that these properties make them better at killing microbes, and now people are interested in how vitamin D3 affects the immune system [2,9,11]. In addition, the development and progression of degenerative joint illnesses, such as temporomandibular joint osteoarthritis, may be significantly influenced by vitamin D3 due to its direct and indirect effects on bones and joints [13]. It was shown that vitamin D and its derivatives may serve as promising molecular therapies for treating bacterial infections, particularly multidrug-resistant (MDR) strains. Vitamin D exhibits antibacterial activity against both Gram-positive and Gram-negative microorganisms. The potential anti-infective effects of antibiotic-free vitamin D treatment and/or adjuvant therapy, in combination with antibiotic compounds, are being explored for the treatment of infectious disorders such as Mycobacterium tuberculosis and H. pylori infections [14]. This study aimed to determine the role of cholecalciferol (D3) on the expression of colonization genes (pilA, hmw1, and hmw2) of H. influenzae isolated from children's otitis media, which is associated with temporomandibular joint pain.

Subjects and Methods

This study included the collection of 160 ear swabs and blood samples from AOM patients in the otolaryngology department at Al-Khalis Hospital in Diyala. The patient's age was between one and 12 years old. The patients were distributed to 83 males and 77 females. The number of patients with pain in the TMJ was recorded, whether due to pressure on the joint area or during mouth opening. The blood samples were used to measure the levels of vitamin D3.

Patients with acute otitis media (AOM) who were diagnosed in the otolaryngology department at Al-Khalis Hospital, and the number of patients with pain in the temporomandibular joint (TMJ) were included during the study period (individuals aged 1-12 years).

while patients with other AOM diseases in the middle ear and patients who are under antibiotic therapy, which may affect the culture results. Additionally, patients aged 13 years or older were excluded.

Enzyme-linked Immunosorbent Assay for vitamin D3 measurement

The levels of vitamin D3 in the AOM patients were measured by ELISA using their blood samples, and a level less than 15 ng/mL was considered vitamin D insufficiency according to the company's instructions (Sigma Aldrich, USA).

Bacterial growth conditions

Ear swabs were cultured on MacConkey, Blood, and Chocolate agar medium, supplemented with Vitox (Oxoid Ltd., Basingstoke, Hampshire, UK) at 37°C in the presence of 5% CO2. Positive growth was assessed based on morphological characteristics, including shape, size, margin, consistency, and colour of colonies, as well as microscopic features and biochemical tests. Furthermore, the Vitek II system was used for detecting bacterial proliferation and conducting antibiotic sensitivity tests according to the manufacturer (bioMérieux, France) [15,16].

Minimum inhibitory (MIC) and bactericidal concentration (MBC)

H. influenzae colonies were collected from agar plates and cultured into Brain Heart Infusion broth. The inoculated broth was then incubated 24 hours at 37°C with 5% CO2. The bacterial culture was centrifuged at 1600g for 5 minutes, after which the supernatant was discarded. Bacterial cell suspensions (0.2 OD600) were prepared in Brain Heart Infusion (BHI) medium. The microdilution process was performed in a sterile 96-well plate with a total volume of 200 µL. Each concentration of vitamin D (6 mg/ml, 3 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml) was examined in a doubling dilution series using triple positive controls (BHI and H. influenzae alone). A plate reader was used to measure the optical density (OD600) of the solutions at baseline and over 24 hours at 37°C with 5% CO2. The negative control for each vitamin D concentration consisted of BHI supplemented with each vitamin D concentration alone, without the addition of bacteria. To determine the MBC value for each concentration, decimal dilutions were performed from the MIC solutions. Agar plates were inoculated with 50 µl and incubated overnight at 37°C with 5% CO2. Lastly, the colonies were counted. The MIC and MBC assays were conducted three times [17,18,19].

Antibiotic Sensitivity Test for H. influenzae

All bacterial isolates underwent antimicrobial susceptibility testing to identify resistance patterns to commonly used antibiotics, as recommended by the Clinical and Laboratory Standards Institute (CLSI). The Kirby-Bauer disc diffusion method was used as the principal susceptibility testing technique [19]. Briefly, the turbidity of the McFarland standard was adjusted to 0.5 to create bacterial suspensions, which were injected onto Mueller-Hinton agar plates. The inoculation plates were covered with antibiotic discs, which were then incubated for 18 to 24 hours at 35 to 37 °C. The groups of antibiotics that were tested in this study, which are most common antibiotics used to treat this bacterium included aminoglycosides (gentamicin, tobramycin, and streptomycin), carbapenems

(imipenem), extended spectrum cephalosporin (cefotaxime and ceftazidime), penicillins (penicillin and amoxicillin), macrolide (azithromycin and erythromycin), phenicols (chloramphenicol), tetracyclines (tetracycline), lincosamides (clindamycin), glycopeptide (vancomycin), and rifamycins (rifampicin) [20,21]. Based on the CLSI breakpoints, the results were interpreted as susceptible, intermediate, or resistant. The diameter of the inhibition zones surrounding the antibiotic discs was assessed.

RNA Extraction of H. influenzae

The RNA of H. influenzae isolates was extracted by culturing this bacterium in Brain Heart Infusion (BHI) broth supplemented with Haemophilus Test Medium Supplement under conditions of 5% CO2. RNA extraction was performed in the mid-log phase of bacterial culture using the RNeasy Mini Kit (Qiagen, Germany). And then 20 U of RNase-free DNase (QIAGEN S.p.A.) was added to the RNA for 20 minutes at 25°C on the RNeasy columns, according to the manufacturer's guidelines, to remove the contaminated DNA. The extracted RNA was visualised using 1.0% agarose gel electrophoresis [22].

Determination of Colonization Genes Expression in H. influenzae

The primers for gene expression were designed using the core-binding domain sections of hmw1A, hmw2A, pilA, and 16SrRNA using DNAMAN sequence analysis software (version 5.2; Lynnon Corp., Quebec, Canada) [23] (Table 1). The primers were used in a LightCycler 2.0 system (Roche, Mannheim, Germany) for quantitative real-time PCR (qRT-PCR) using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen Life Technologies Corp). The housekeeping gene (16SrRNA) was used, 1.0 µl One-step enzyme mix, 10.0 µl Syber green 2× (MgSO4 3 mM), 1.0 µl BSA 20×, and 0.25 µM of each primer were included in the qRT-PCR mixture (total volume 20 µl). The RNA was extracted using the RNeasy kit (Qiagen, Germany). The Quantiscript RT kit was used for cDNA production. 10 µL of SYBR Green I (Roche) and 0.5 µL of forward and reverse primers were used. Then, 5 µl of cDNA was mixed with them, and the volume was completed to 20 µl by the addition of nuclease-free water and put in a thermocycler (Roche, Switzerland).The reverse transcriptase steps were firstly, at 50°C for 2 minutes and then a denaturation step at 95°C for 2 minutes, there were 35 amplification cycles, each lasting five seconds at 95°C, ten seconds at 55°C, and ten seconds at 72°C. By amplifying an internal fragment of the 16SrRNA gene using ten-fold serial dilutions of known concentrations (100 ng/µl, 10 ng/µl, 1 ng/µl, 0.1 ng/µl, and 0.01 ng/µl) as templates of genomic DNA to produce quantitative standard curves [22]. For every gene from every H. influenzae isolate, three separate assays were done, each in triplicate.

Expression of H. influenzae Colonization Genes in the Presence of Vitamin D3 as established by the MIC assay, the MIC is 0.5 mg/ml. Therefore, the concentration 0.25 mg/ml of vitamin D3 was used to examine the impact of this vitamin on the expression of genes of H. influenzae, which was treated with this concentration of vitamin D3, and the no-treatment (control) group. Brain heart infusion broth (BHI) containing cholecalciferol at the above concentration was inoculated with H. influenzae. Following the manufacturer's recommendations, the cultures were cultured for 24 hours at 37°C and 5% CO2 before being pelleted at a weight of more than 10,000g. The RNA from the collected cells was extracted using the RNeasy kit (Qiagen, Germany). A spectrophotometer (DeNovix, USA) was then used to determine the amount of RNA, and the Quantiscript RT kit (Qiagen) was used to generate equimolar amounts of cDNA. SYBR Green I (Roche) was used in qPCR experiments, with 10 µL and 0.5 µL of each forward and reverse primer per well. Then, 5 µl of cDNA was added to them. Then the volume was completed to 20 µl by addition of nuclease-free water after being placed in a thermocycler (Roche, Switzerland), and the samples were subjected to denaturation (95°C) and activation (50°C). 40 amplification cycles at 60°C for 1 minute and 95°C for 3 seconds were achieved, and then, the cooling phase and melting curve were programmed. Because it is stable in various environmental settings, the housekeeping gene (16SrRNA) was used to normalize the gene expression data [24].

Table 1: The RT-PCR primers used to determine the gene expression.

Primers	Sequence (5′–3′)	Size (bp)
16SrRNA-F	TCCTAAGAAGAGCTCAGAGAT	120
16SrRNA-R	TGATCCAACCGCAGGTTCC	
PilA-F	CTATATACACATAATTCCACATCAGCCTTA	125
PilA-R	CCACCATCGCAATTCCTTCTT	
hmw1-F	CCGGTGGTTTTGTGGAGACGTCG	133
hmw1-R	TGAAGTATTGCTGCGTCCTG	
hmw2-F	CCGGTGGTTTTGTGGAGACATCG	121
hmw2-R	GCGAAGGGGGTCTTCGGCTTCA	

The Statistical analysis was done by using Packages for the Social Sciences program (SPSS, 2019) to identify the differences among the groups and factors in this study [25]. The Chi-square test was used to determine the significant differences between percentages at 0.05 and 0.01 probability levels. Results were categorized as Significant (P≤0.05), Highly Significant (P≤0.01), and NS = non-significant.

Results

This study revealed a distribution of 83 males (51.87%) and 77 females (48.13%), with no significant differences found between the two groups. This study showed that 30.63 % of AOM patients were less than two years of age. However, the cases of AOM decreased in children older than six years old. It was found in this study that the AOM patients who had TMJ pain were 5.63% male compared to 8.75% female, and the AOM patients without TMJ pain were distributed as 46.25% male and 39.38% female. The number of patients who had pain in the TMJ, whether from pressure on the joint area or while opening the mouth, was recorded (Table 2), and the results of current study showed that 14.38 % of AOM patients had TMJ pain and 85.62 % of patients without TMJ pain, with a significant difference between them (P≤0.0001).

Table 2: Distribution of patients according to results of TMJ pain.

TMJ pain	No	Percentage (%)
Yes (+ve)	23	14.38
No (-ve)	137	85.62
Total	160	100%
Chi-Square: χ^2	---	81.225 ***
(P-value)		(0.0001)
**** (P≤0.0001)		

This study showed that the mean ± SEM of Vitamin D3 in AOM patients was 13.13 ± 0.457 ng/mL, which is considered vitamin D insufficiency in these patients (Figure 1).

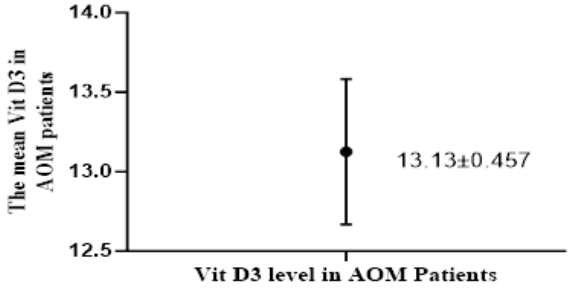

Figure 1: The mean of vitamin D3 in AOM patients

Table 3: Fold difference in expression of genes of H. influenza in the presence of vitamin D3 relative to their expression in the absence of vitamin D3 using qRT-PCR. '- 'indicates down-regulation of genes, ± represents the standard deviation for three measurements Gene taq.

Gene name	Gene Function	Fold difference in the absence of vitamin D3	Fold difference in the presence of vitamin D3
pilA	Colonization, biofilm formation	61.12 ± 0.03	-2.8 ± 0.05
hmw1	Adhesion, colonization	4.07 ± 0.08	-3.3 ± 0.02
hmw2	Adhesion, colonization	2.8 ± 0.45	-4.01 ± 0.12

This study identified the bacterial species that caused AOM in patients. It showed that 28.12% of AOM cases were caused by H. influenzae, followed by S. pneumoniae (15%), Pseudomonas aeruginosa (14.73%), and K. pneumoniae (12.5%). However, Serratia appeared to be one of the few bacteria that cause AOM, as its percentage is 3.13%.

The results showed that azithromycin, vancomycin, and cefotaxime were the most effective antibiotics for H. influenzae growth because this bacterium appeared sensitive against them at 84.4%, 84.4%, and 80%, respectively. However, this bacterium appeared to be 100% resistant to tobramycin, 84.4% resistant to gentamicin, and 73% and 62.2% resistant to tetracycline and erythromycin, respectively.

The results showed that the expressions of pilA, hmw1, and hmw2 appeared to be downregulated in the presence of vitamin D3. On the other hand, the expression of these genes appeared up-regulated in the absence of this vitamin (Table 3).

Discussion

Acute otitis media is more likely to happen during winter when upper respiratory tract infections are most common. This is because viral and bacterial respiratory tract illnesses raise the risk of AOM. The existing study exhibited that the percentage of TMJ pain in patients was 14.38%. This may be clarified by the fact that the side effects of otitis media that lead to septic arthritis involve the transmission of infection from the middle ear to the temporomandibular joint (TMJ) [26,27]. Direct or hematogenous spread through the synovial blood vessels can cause involvement of the TMJ. One of three routes (congenital cartilaginous canal dehiscence, dehiscent squamotympanic fissures, or unable to close Huschke's foramen) can spread from the ear to the TMJ [27,28]. Additionally, reports suggest that distinguishing between septic and reactive arthritis can be challenging, particularly in atypical cases, such as those involving H. influenzae infections. Currently, there are no guidelines addressing the potential for concurrent arthropathies, which complicates treatment strategies. While corticosteroids are typically prescribed for reactive arthritis, they may adversely affect septic arthritis. Early initiation of antibiotic therapy aimed to prevent sepsis and complications from septic arthritis. Continued suspicion of septic arthritis, even when symptoms suggest reactive arthritis, led to effective treatment outcomes. There is a pressing need for evidence-based guidelines to assist physicians in managing multiple arthropathies [29].

Microflora bacteria that live in the nose and throat often cause AOM illnesses. This study showed that H. influenzae and S. pneumoniae were the most common bacteria that cause AOM. Similarly, it was reported that S. pneumoniae, and H. influenzae are the most critical bacteria and cause 10 to 40 percent of AOM cases. If the right care isn't given, problems can happen that can be life-threatening [30]. Individual sensitivity is shown by the different infection rates in children from the same social background. The current study showed that all the AOM patients had vitamin D3 insufficiently as determined in the serum of patients using ELISA, which is the best serological method [31,32]. A vitamin D3 insufficiency was reported among the OM patients, which shows a link between OM and vitamin D3. Vitamin D targets have been present in the skin, stomach, liver, thymus, breast, parathyroid glands, and lymphocytes. These studies have shown that vitamin D does more than help the body utilize calcium [2, 9]. There is a strong link between vitamin D and both innate and acquired immunity. Microorganisms are killed by antimicrobial peptides (defensin, cathelicidin) and reactive oxygen products released by natural defence [33,34]. Calprotectin and S100 proteins, which are natural immune system factors that play a significant role, also increase when Vitamin D is active [33]. Cathelicidin is made when there is an infection

in the skin because it activates the Toll-like receptor (TLR) in keratinocytes. Vitamin D and the body's natural immune system are thought to work together to protect against germs in the surroundings. This affects the location of the infection [35]. It has been shown that when Vitamin D3 is present, the monocytes and macrophages' chemotactic and phagocytic abilities improve, as well as their ability to kill microbes. [36,37]. The results of this study showed that vitamin D3 plays a significant role in the expression of colonization genes in H. influenzae, and these results agreed with other studies that found that not getting enough vitamin D3 has been linked to a higher chance of sinusitis, upper and lower respiratory tract infections [24]. It was suggested that Vitamin D3 could raise these levels. Vitamin D3 has also been shown in some tests to be effective as an extra treatment for many illnesses [38,39,40]. Furthermore, it was reported that vitamin D3 decreases P. gingivalis growth and diminishes the expression of its virulent factor genes. This dual effect on P. gingivalis with the inflammatory response in host cells promises to develop an innovative and cost-effective therapeutic approach. Specifically, 1,25(OH)2D3 lowers the virulence of P. gingivalis by reducing the expression of genes responsible for virulence factors, including adhesins (fimA, hagA, and hagB) and proteinases (rgpA, rgpB, and kgp) [41]. In addition, this study is the first to determine the role of vitamin D in the colonization genes expression in a fastidious bacterium that causes the majority of AOM.

Conclusion

We investigated the role of cholecalciferol (D3) in the expression of colonization genes of H. influenza. The microbe isolated from children's otitis media. Vitamin D3 may improve the outlook for individuals with severe otitis media. There is a strong link between vitamin D3 deficiency and the long-term effects of otitis media. H. influenzae is the most common cause of AOM. This current study showed that the expression of colonization genes in H. influenzae is up regulated without vitamin D3. On the other hand, these genes' expressions are downregulated in the presence of this vitamin. In summary, Vitamin D3 plays a significant role in the expression of genes in this bacterium.

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Conflict of Interest

Authors declare no conflict of interest.

Data availability

Data are available upon reasonable request.

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