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Streptococcal infection in young pigs

I. An immunochemical study of the causative agent (PM streptococcus)

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Outbreaks of streptococcal infection in pigs 2–6 weeks after birth are fairly common both in England (Field, Buntain & Done, 1954) and in the Netherlands (de Moor, 1963). The infection takes the form of a bacteriaemia frequently involving the brain and joints. In the early stages it responds to treatment with antibiotics, but some animals recover spontaneously, while others, untreated, die in the early stages of the disease.

The general characteristics of the causative streptococcus have already been described by Field *et al.* (1954). It is a haemolytic diplococcus, non-pathogenic for mice, guinea-pigs and rabbits, but causing arthritis and meningitis on intravenous or subdural inoculation into 24-day-old pigs. Field and his co-workers were unable to assign the streptococcus to any of the established serological groups (A to O) and this was also the experience of de Moor, who examined strains isolated from the disease in Holland. Some of the strains isolated by Field and by de Moor appeared to be identical (de Moor, 1963).

It is the object of this report to describe the serological relationships of streptococci isolated from nineteen outbreaks of the disease in England, mostly in East Anglia. The streptococci, here designated PM streptococci, were isolated at autopsy from the heart blood, joints and brains of pigs dying from the infection. The PM streptococci belong to Lancefield's group D and to a single serological type within that group. Cocci serologically identical with these were found in ante-mortem throat and blood cultures from some of the affected animals as well as from throat cultures of apparently normal litter-mates. Serological matches for these streptococci were also found in throat and nose cultures from a small number of sows without manifest infection (Elliott, Alexander & Thomas, 1966).

Through the co-operation of Mr Field and Dr de Moor I have been able to compare their strains with the PM streptococci to be described in this report. I have found that Field's strain 428 and de Moor's strains designated group S are serologically identical with the PM streptococcus.

METHODS

Serological methods

Streptococcal cultures and extracts. The PM streptococci were grown either in Todd Hewitt broth containing 10 % of horse serum or in dialysate broth (Dole, 1946) made with Pfanstiehl peptone and containing 1 % glucose.

Saline extracts were made by resuspending in a small volume of 0·85 % NaCl the centrifuged cocci from broth cultures. The suspensions were incubated at 37° C. for 2 hr., after which the supernatant fluids were separated from the cocci by centrifugation.

Acid extracts were made either by heating the cocci to 100° C. for 10 min. in hydrochloric acid at pH 2 or by stirring the cocci in 5 % trichloracetic acid for 24 hr. at 4° C.

Streptococcal antisera were prepared in rabbits by the method previously described (Elliott, 1960).

Precipitin tests were carried out using the capillary technique (Swift, Wilson & Lancefield, 1943).

Analytical methods

Electrophoresis was carried out on Pevikon in 0·1 M veronal buffer pH 8·6 at 400 V. for 12–14 hr. at 4° C. The Pevikon block (45 cm. × 7 cm.) was then cut transversely into 1 cm. strips and each suspended in 1 ml. H_2O. The 'antigens' thus eluted were identified by precipitin tests with appropriate antisera.

Paper chromatography was used for the identification of monosaccharides after hydrolysis of carbohydrates in 4 N-HCl at 100° C. for 6 hr. in sealed ampoules. The following solvents were used: butanol, ethanol, water (4:1:1); butanol, pyridine, water (3:2:1·5); isopropyl ether, formic acid for phosphorus compounds (9:6). Spraying agents were aniline hydrogen phthalate and, for phosphorus compounds, amidol reagent followed by ultraviolet irradiation. Glucosamine and galactosamine were differentiated chromatographically after conversion to pentoses (Stoffyn & Jeanloz, 1954). Glucose was estimated by the glucose oxidase reaction (Glucostat), xylose and methylpentose by the cysteine–sulphuric acid methods (Dische, 1949; Dische & Shettles, 1948), and hexosamines by a modification of Elson & Morgan's procedure (1933).

GROUP IDENTIFICATION OF PM STREPTOCOCCI

Serological reactivity of extracts from PM streptococci

Saline and hydrochloric acid extracts made from PM streptococci as described under Methods were examined by precipitin tests with streptococcal group antisera kindly provided by Dr R. C. Lancefield. From the results shown in Table 1, it can be seen that extracts prepared from all strains precipitated with group D antisera; these reactions occurred regardless of the type specificity of the antisera and were interpreted as being group specific. Positive reactions also occurred with some, but not all, group E and group N antisera; these reactions were probably due to type- rather than group-specific antibodies and their significance is unknown. Some of the PM streptococcal extracts reacted with antisera to the C polysaccharide of pneumococcus. Preliminary study indicates that this reaction is probably due to a serological relationship between cell-wall components of the PM streptococci and pneumococci.

Gel diffusion tests

In gel diffusion tests using the Ouchterlony plate method, extracts from PM streptococci, from *Streptococcus faecalis* (cell-wall types 1, 4) and from *Strep. durans* (cell-wall type 26) when tested against group D antiserum (cell-wall type 1) formed precipitin lines that showed continuity and indicated probable identity of the antigenic determinants involved in the reactions. The same appearances were obtained when a group D reactive antiserum made against a porcine strain, A 227, was substituted for the *Strep. faecalis* antiserum. Strain A 227 was isolated from the throat of a 5-week-old piglet convalescent from an experimental infection produced 3 weeks previously with a PM streptococcus, strain PM 23. Culturally and serologically the two strains resembled one another except that PM 23 was encapsulated and pathogenic for piglets, whereas A 227 had no capsule and was not pathogenic (Elliott *et al.* 1966). It is possible that A 227 was an attenuated variant of PM 23 produced through residence in the throat of the convalescent, immune piglet.

Table 1. *Precipitin reactions of crude acid extracts from PM streptococci with streptococcal group antisera*

	Rabbit antisera to streptococci of stated group and type							
	Group D			Group E	Group N			Groups A, B, C, G, L, M, O
Antigen	Type 1	Type 3	Type 40	Strain K 129	Strain 6681	Strain R 7	Strain R 9	
Acid extracts from PM strep. (19 strains)	+	+	+	±	+	–	–	–

± Indicates positive reactions with three out of seven rabbit antisera.

Chemical analysis of the group D reactive component of PM streptococci

It has previously been shown that streptococci that compose Lancefield's group D are characterized by a serologically reactive polymer consisting of glucose and α-glycerophosphate, the so-called 'intracellular teichoic acid' (Elliott, 1962; Wicken, Elliott & Baddiley, 1963). In order to obtain additional evidence supporting the relationship of PM streptococci to group D, partially purified group extracts from four strains were analysed for the presence of glucosyl glycerophosphate. The four strains included two, PM 1 and PM 23, isolated from diseased piglets, a serologically identical strain, C 22 N, isolated from the nose of a normal sow and strain A 227 described in the preceding paragraph.

Extraction and purification of group substances

The streptococci were grown in 20 l. amounts of dialysate broth incubated for 18 hr. at 37° C. Because the yield of purified group substance from PM streptococci was small (from 1 to 5 mg./l. of culture), group reactive material extracted from

the cocci by a variety of methods was pooled and then partially purified before chemical analysis. Preliminary extraction in saline was carried out as described under Methods. The cocci were then disrupted by shaking with glass beads (Ballotini no. 13) in a Braun disintegrator; the resulting supernatant fluid was rich in group substance and could be separated from the disrupted cocci by high-speed centrifugation. Finally, the residual bacterial debris was extracted several times with trichloracetic acid as described under Methods.

To the pooled extracts was added ethanol (2 vol.) and the resulting precipitate, which contained the group reactive component, was re-dissolved in a small volume of water. Concentrated extracts thus prepared contained, in addition to the group D reactive substance, a large admixture of type-specific material probably derived from the bacterial capsule (see below) and from the bacterial cell walls. As shown in Table 2, separation of the type and group components was readily achieved by electrophoresis on Pevikon at pH 8·6. Under these conditions the group substance was negatively charged and moved to the positive end of the Pevikon block, whence it could be eluted free from contamination with type-specific material. The group reactive material eluted in water from the Pevikon was dialysed against water and finally frozen and dried. The lyophilized material was submitted to chemical analysis without further purification.

Table 2. *Precipitin reactions of electrophoretically separated components of extracts from PM streptococci*

	Rabbit antisera to group D streptococci				
	Group reactive			Type specific	
Antigen (extracts from PM. streptococci)	Type 1*	Type 3*	Type 10*	PM 1†	PM 23†
Fraction A (group specific)‡	+	+	+	−	−
Fraction B (type specific)§	−	−	−	+	+

* Group D cell-wall types.
† PM streptococci.
‡ Fast-moving component negatively charged at pH 8·6.
§ Slow-moving component negatively charged at pH 8·6.

Chemical analysis

The methods employed in analysing the group reactive material from strains PM 1, PM 23, C 22 N and A 227 are described under Methods. Examination of acid hydrolysates by paper chromatography revealed the presence of glycerophosphate, glucose and xylose in all four strains. Table 3 shows the results obtained from quantitative chemical analysis of these partially purified group D extracts. In addition, analytical data derived from a previous study of group antigen from three known group D strains (D 76, C 1 and C 3) are given for comparison (Elliott, 1962).

It can be seen that the chemical data support the serological findings and confirm the presence of group D antigen in strains PM 1, PM 23, C 22 N and A 227. The presence of xylose is distinctive but does not appear to affect the serological

specificity of the antigen for, as already shown, gel diffusion precipitin tests indicated identity between group substances from all the strains listed in Table 3.

Table 3. *Carbohydrate content of group D substances isolated from streptococci of porcine and non-porcine origin*

Strain designation	Source	Glucose (%)	Xylose (%)	Glycero-phosphate
PM 1	Bacteriaemia in piglet	17·5	11·0	+
PM 23	Bacteriaemia in piglet	26·9	3·2	+
C 22N	Nose of normal sow	12·3	2·4	+
A 227	Throat of piglet convalescent from streptococcal bacteriaemia	26·2	2·2	+
D 76 (type 1)	Cheese	25·6	.	+
C 1 (type 4)	Cheese	30	.	+
C 3 (type 26)	Cheese	30	.	+

Strains D 76, C 1 and C 3 were obtained from Dr R. C. Lancefield.
Glycerophosphate was identified by paper chromatography but not determined quantitatively.

THE TYPE-SPECIFIC CAPSULAR COMPONENT OF PM STREPTOCOCCI

Formalized PM streptococcal vaccines elicit in rabbits the production of antibody specifically reactive with some component of these micro-organisms. Such antisera reacted strongly with saline or acid extracts prepared from PM streptococci isolated in all nineteen outbreaks investigated. They reacted minimally or not at all with extracts of other group D streptococci. By precipitation reactions with these antisera the type-specific component of the PM streptococci could be identified in unconcentrated filtrates of 18 hr. broth cultures. More concentrated preparations of the type-specific substance were obtained by saline extraction of the living cocci as described under Methods. The ease with which the type-specific substance could be extracted without apparent injury to the cocci suggested a superficial location, possibly in the form of a capsule. Further evidence for this was found in the 'swelling' reaction shown by the cocci in the presence of type-specific immune serum and by the characteristic precipitin haloes that surrounded colonies of PM cocci growing on nutrient agar incorporating such serum.

Characterization of the type-specific (capsular) component of PM streptococci

Extraction methods

The PM streptococci to be used as a source of type-specific substance were grown in 20 l. amounts of dialysate broth from which they were harvested after 18 hr. incubation at 37° C. Three methods were used to separate the type-specific substance. First, the soluble type-specific material was recovered from culture filtrates by precipitation with ammonium sulphate 0·8 saturation (fraction 1). Secondly, the cocci harvested from the cultures were allowed to autolyse in the presence of sodium deoxycholate (0·1 %, w/v) and the soluble type and group substances thus liberated separated from the residual bacterial debris by centrifugation (fraction 2). Finally, after autolysis, the residual bacterial debris was

extracted with 5% trichloracetic acid at 4° C. Several extractions, each of 24 hr. duration, were usually required; the extracts were pooled (fraction 3).

Purification procedures

From the three fractions much of the contaminating nucleic acid was removed by treatment first with ethanol (0·2 vol.) in the presence of 1% $CaCl_2$ (Anderson & McCarty, 1951) and then by digestion with deoxyribonuclease. Protein was removed by digestion with streptococcal proteinase followed by prolonged dialysis against distilled water. After these preliminary measures the fractions invariably contained a mixture of type- and group-specific substances. These were separable by electrophoresis on Pevikon at pH 8·6. In the case of the capsulated streptococci (strains PM 1 and PM 23) both group- and type-specific components migrated towards the anode, but the former moved more rapidly than did the latter and separation was usually achieved after electrophoresis for 12–14 hr. Immobile cell-wall material was excluded from the electrophoretically separated components of the capsulated strains, PM 1 and PM 23. In the case of the non-capsulated strain, A 227, the type-specific component was immobile on electrophoresis at pH 8·6. It seems probable that this component was derived from the streptococcal cell wall. The electrophoretically separated type-specific components were eluted from the Pevikon in water, dialysed free from buffer and finally frozen and dried before chemical analysis. The yield of type-specific was greater than that of group-specific substances and usually amounted to between 10 and 20 mg./l. of culture.

Table 4. *Chemical analysis of type-specific substance (capsular 'antigen') from two serologically identical strains of PM streptococci and type-specific cell-wall antigen from a non-capsulated strain*

		Carbohydrates in type specific fractions (acid hydrolysate)		
Strain designation	Source of type-specific fraction	Glucose (%)	Hexosamine (%)*	Rhamnose (%)
PM 1 (capsulated)	Culture filtrate (fraction 1)	7·6	18·0	.
PM 1 (capsulated)	Autolysate + TCA† extract of residual debris (fractions 2 and 3)	10·8	23·0	.
PM 23 (capsulated)	TCA extract (fraction 3)	12·0	20·0	.
A 227 (non-capsulated)	TCA extract from cell walls	27·2	23·0	20·0

* Hexosamine = glucosamine + galactosamine.
† TCA denotes trichloracetic acid (5%).

Chemical analysis of type-specific substances

The carbohydrate components of the purified type-specific substances were identified by paper chromatography of acid hydrolysates and their concentrations determined as described under Methods. No attempt was made to identify protein constituents in the purified preparations. The results are shown in Table 4.

DISCUSSION

The streptococci here described as the cause of neonatal infection in pigs belong to a single serological type within Lancefield's group D. The type-specific component appears to be a capsular polysaccharide composed of glucose, glucosamine and galactosamine. The group reactive component is an 'intracellular' teichoic acid immunologically related to and probably identical with those characteristic of *Strep. faecalis* and *Strep. durans*. Chemically, it resembles them in containing glucose and glycerophosphate but differs from them in containing a small amount of xylose. This pentose does not appear to affect the serological specificity of the group substance, although examination of a larger number of antisera to the porcine streptococci might reveal a xylose specificity. In this connexion it will be recalled that some but not all rabbit antisera to group A streptococcal glycerophosphate contain antibody to the alanine component (McCarty, 1964). It has previously been shown that the group substances (glucosyl glycerophosphates) produced by different strains of group D streptococci differ in their glucose content (Wicken *et al.* 1963). The demonstration of xylose as a component of the porcine streptococcal group substance provides further evidence of the variable composition of the group D 'antigen'. Such variability is in keeping with the heterogeneous nature of group D, which includes streptococci diverse in habitat and physiological characteristics. As shown in Table 5 the porcine strains here

Table 5. *Comparison of PM streptococcus with other group D streptococci*

Characterization by	PM streptococci	*Strep. faecalis*	*Strep. bovis*
Group D 'antigen'	+ Glucose-xylose-glycerophosphate	+ Glucose-glycerophosphate	+ Not analysed
Capsule (serologically type-specific)	+	−	+
Slime production (dextran)*	−	−	+
Growth in 40% bile*	± Slow	+	+
Growth in penicillin (5 units per ml.)	−	+	Not tested
Heat resistance (60° C.)	−	+	Variable

* E. Barnes (personal communication).

described differ in many respects from *Strep. faecalis* and *Strep. bovis*. For this reason there would appear to be some justification for establishing within group D an additional subgroup with status equivalent to that of *Strep. faecalis* and *Strep. bovis* but to include streptococci with the characteristics of the PM strain. The PM streptococci appear to be identical with those isolated from piglet infections by Field and by de Moor, who designated his strains 'group S'. We share the experience of de Moor (1963) in that these micro-organisms have been isolated only from pigs so that the new subgroup within group D might appropriately be designated *Streptococcus suis* and the PM strains, Capsular Type 1.

SUMMARY

1. Streptococci causing neonatal infection in piglets have been identified serologically as belonging to group D.

2. Glycerophosphate, glucose and xylose have been identified as components of the group substance isolated from these micro-organisms.

3. The streptococci isolated from piglets with this disease in England appear to belong to a single serological type characterized by a capsular polysaccharide.

4. Glucose, glucosamine and galactosamine have been identified as components of the type-specific substance.

5. It is suggested that these streptococci should be designated Capsular Type 1 in *Streptococcus suis*, a new subgroup in group D.

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